Role of the Vps34p-interacting protein Ade5,7p in hyphal growth and virulence of Candida albicans

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The phosphatidylinositol (PtdIns) 3-kinase Vps34p of the human pathogenic yeast Candida albicans participates in virulence and in protein transport. In order to dissect these two functions, a search for proteins interacting with C. albicans Vps34p was performed using a yeast two-hybrid system. This study demonstrates the physical interaction between Vps34p and Ade5,7p, which is the bifunctional enzyme of the de novo purine nucleotide biosynthetic pathway. The interaction initially observed in a yeast two-hybrid system was confirmed in vitro with recombinant proteins. Given the complex formation between Ade5,7p and the virulence- regulating Vps34p, it was of interest to characterize the function of Ade5,7p in C. albicans. To this end, ade5,7 null mutants were generated. The resulting mutants were adenine deficient, and sensitive to the presence of metal ions. In addition, the ade5,7 null mutants were avirulent in a mouse model of systemic candidiasis, and showed reduced hyphal growth in an agar matrix under embedded conditions. In summary, Ade5,7p interacts with the multifunctional virulence regulator PtdIns 3-kinase Vps34p, and ade5,7 and vps34 null mutant strains show similar phenotypes regarding sensitivity to metal ions, hyphal growth and virulence.

INTRODUCTION

Candida albicans is an opportunistic human fungal pathogen that causes systemic infections in immunocompromised patients, and mucosal infections in healthy individuals (Odds, 1994). The virulence of C. albicans depends on several virulence factors, including the ability to switch between different morphogenetic forms, recognition and adhesion to host epithelial and endothelial cells, and also the secretion of proteinases and phospholipases (Cutler, 1991; Köhler & Fink, 1996). These virulence factors are affected by the phosphatidylinositol (PtdIns) 3-kinase Vps34p, which plays a role in protein transport from the Golgi to the vacuole, and in regulation of virulence factors, such as yeast-to-hyphae transition, adhesion to human cells, and secretion of proteinases. In addition, the C. albicans vps34 null mutant strain is avirulent in a mouse model of systemic candidiasis (Eck et al., 2000; Bruckmann et al., 2000). The multifunctional Vps34p protein interacts with the vacuolar ATPase subunit Vma7p (Eck et al., 2005), and the overlapping and related functions of these two proteins have been demonstrated by the related phenotypes of the vps34 and the vma7 null mutants. In this study, the Vps34p-interacting protein Ade5,7p was isolated and functionally characterized.

Ade5,7p is a bifunctional enzyme of the de novo purine nucleotide biosynthetic pathway in the yeast Saccharomyces cerevisiae. The de novo purine synthesis pathway involves 10 sequential enzymic steps that result in the generation of inosine monophosphate, followed by one of two paths to produce either adenosine monophosphate or guanosine monophosphate. In bacteria, enzymes involved in de novo purine synthesis are encoded by separate genes, while their eukaryotic counterparts are often encoded by single genes encoding multifunctional polypeptides (Henikoff, 1987; Zalkin & Dixon, 1992).

At least 13 proteins regulate the synthesis of adenine in S. cerevisiae (Jones & Fink, 1982). Some of these enzymes participate in specific steps of the pathway (encoded by Ade1, Ade2, Ade4, Ade5,7, Ade6, Ade8, Ade12 and Ade13), while others are required to produce additional substrates necessary to complete the pathway (encoded by

Abbreviations: AD, activation domain of Gal4p; AIRS, aminoimidazole ribotide synthetase; BD, binding domain of the transcription factor Gal4p; EBP, oestrogen-binding protein; FCS, fetal calf serum; GAAC, general amino acid control; GARS, glycinamide ribotide synthetase; GST, glutathione S-transferase; P, plasma membrane; PtdIns, phosphatidylinositol; V, vacuolar; VHHFLU, antibody against fluorescein.
ADE3 and AS35). Strains with mutations in two transcription factor genes, BASI and BAS2, show a partial adenine requirement for growth (Arndt et al., 1987). In the latter mutants, expression of the biosynthetic enzymes is low, indicating a positive regulatory role for the transcription factors (Daignan-Fornier & Fink, 1992). Another transcription factor, Gcn4p, which is positively regulated by Gcn1p, also stimulates the expression of ADE5,7 under conditions of purine limitation (Rolfe & Hinnenbusch, 1993).

In the yeast S. cerevisiae, Ade5,7p contains the activities for aminoimidazole ribotide synthetase (AIRS) and glycinamide ribotide synthetase (GARS), which are involved in steps two and five, respectively, of adenine biosynthesis. The deletion of ADE5,7 in S. cerevisiae leads to viable mutants that have an adenine requirement, and reduced fitness in rich medium (Giaeuer et al., 2002).

C. albicans contains an ADE5,7 homologous ORF (ORF 19,5061, IPF13495.2) that is 2409 bp long, and shows 62% identity to the corresponding S. cerevisiae gene (Arnaud et al., 2005; D’Enfert et al., 2005). This ORF shows high homology to sequences in S. cerevisiae that encode GARS and AIRS enzymic activity. Apparently, C. albicans Ade5,7p expression is regulated in a Gcn4p-independent fashion, in contrast to the S. cerevisiae orthologue (Yin et al., 2004).

Increased expression of the Candida ADE5,7 gene has been observed in genome-wide expression profiling as a response to the antifungal agent 5-flucytosine (Liu et al., 2005).

The deletion of C. albicans ADE2 (the gene encoding phosphoribosylaminoimidazole carboxylase) leads to reduced virulence, showing that de novo purine biosynthesis is involved in Candida pathogenicity (Donovan et al., 2001).

Given the physical interaction between the Ade5,7p, which is the bifunctional enzyme of the de novo purine nucleotide biosynthetic pathway, and the virulence-specific Vps34p, we were interested in characterizing the function of Ade5,7p in C. albicans. C. albicans Ade5,7p was cloned and recombinantly expressed, and the interaction of the recombinant Ade5,7p with Vps34p was shown. In addition, ade5,7 null mutants were generated, and these mutant strains were assayed for adenine requirement, detoxification, hyphal development and virulence.

**METHODS**

**Strains and growth conditions.** The C. albicans strains used in this study are listed in Table 1. Yeast strains were grown in YPD medium [2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract, YNB 0.7% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose] or Sabouraud medium [2% (w/v) glucose, 1% (w/v) peptone (casein)] at 30°C. Growth was monitored by counting cell numbers in a haemocytometer. Hyphal growth was induced on solid medium: cells were grown overnight in YPD at 30°C, washed with 0.9 M NaCl, diluted, and spread either on Spider plates [1% (w/v) nutrient broth, 0.2% (w/v) K2HPO4, 1.35% (w/v) agar, 1% (w/v) mannitol] (Lee et al., 1975), or on YPD plates supplemented with 15% (w/v) fetal calf serum (FCS), at a density of 20–100 cells per plate. Plates were incubated at 37°C for at least 7 days. Hyphal growth in liquid medium was induced by 10-fold dilution of late-exponential-phase cultures grown at 30°C, either into fresh YPD supplemented with 10% (w/v) FCS, or into Spider medium, at 37°C. Sensitivities of the mutants to various metal ions were assayed on YPD plates supplemented with the appropriate salts. The ade5,7 mutant strains were selected on CSM (complete supplement mixture) medium (Qbiogene).

**Two-hybrid screen.** The S. cerevisiae strain Y190 was transformed with the bait plasmid pGBK734, followed by transformation with the pGAD C. albicans cDNA library (Eck et al., 2005). A lacZ filter assay (Kranz et al., 2001) was performed, and positive clones were isolated by plasmid rescue. All other methods were performed according to standard techniques. The nucleotide sequence of the positive clone was determined, and compared with that of the Stanford C. albicans sequencing project (http://candida.bri.nrc.ca/candida/index.cfm).

**Expression of recombinant C. albicans Ade5,7p, ligand blot and pull-down assay.** Ade5,7p was recombinantly expressed as a GST (glutathione S-transferase) fusion protein (Ade5,7GST) in Escherichia coli. ADE5,7 cDNA was amplified using primer A (introducing a BamHI site) and primer B (introducing a HindIII site) (Table 2). The PCR product was cleaved with BamHI and HindIII, and cloned into the pGEXK vector, resulting in plasmid pGEX-Ade5,7. Subsequent transfection of E. coli, induction of protein expression, and purification, were carried out according to the manufacturer’s protocols. In addition, Vps34p was recombinantly expressed as a His6-tagged protein (Vps34His), and C. albicans EBP (estrogen-binding protein) was expressed as a GST-tagged protein (EBP-GST) (Eck et al., 2000; Madami et al., 1994). The fluorescent antibody VHFLUH was expressed as a His6-tagged protein (VHFLUHIS). For ligand overlay assays, approximately 0.5 µg recombinant protein was separated by SDS-PAGE (8%), and blotted onto a Protran transfer membrane (Schleicher & Schuell) by using standard protocols and a previously described method (Eck et al., 2005). The membrane was incubated overnight with recombinant GST-tagged Ade5,7p or EBP-GST (8 µg ml⁻¹), and then for 2 h with anti-mouse GST antiserum (1 : 4000; Amersham Biosciences Europe). After incubation with a secondary monoclonal peroxidase-labelled anti-mouse IgG antibody (1 : 4000), the blot was developed by using the ECL detection system. Pull-down assays were done according to a previously described method (Eck et al., 2005). Approximately 2.5 µg Vps34His protein was added to a Ni-NTA superflow agarose column (Qiagen). After washing, a 2.0 µg quantity of Ade5,7GST, EBP-GST or GSTp was added. The eluate was separated by SDS-PAGE. After blotting, the filter was incubated with mouse GST antiserum (1 : 3000), and developed by ECL.

**Deletion of the ADE5,7 gene by PCR-based gene targeting.** Primers used in this study are listed in Table 2. The method was performed according to Gola et al. (2003). Marker genes were amplified by primers S1 [bp –94 (upstream) to +7 of ADE5,7] and S2 (bp –101 to –1, downstream of ADE5,7). The proper deletion of the complete ADE5,7 ORF (2409 bp, 802 aa; the Stanford C. albicans sequencing project Assembly 19, ORF19.5061 on Contig10218; http://candida.bri.nrc.ca/candida/index.cfm) was confirmed by Southern analysis, according to standard protocols. The probe was amplified by PCR using primers AS5 (bp –1318 to –1294, upstream of ADE5,7) and AS4 (bp –8 to –31, upstream of ADE5,7).

**Reintegration of ADE5,7.** To construct the template for homologous reintegration of the ADE5,7 gene, the primers G1 (bp –577 to –538, upstream of ADE5,7) and G4 (bp –498 to –518, downstream of ADE5,7) (Table 2; chromosomal sequences are underlined, and
To construct PCR products of 2410 bp (HIS1 and Arg2 (bp 157 to 176, downstream of HIS1)), confirmed by PCR and Southern analysis. The PCR product (2.2 kb), His2 (bp 764 to 789, upstream of HIS1), Arg1 (bp 992 to 971, upstream of ARG4), and Arg2 (bp 932 to 911, downstream of ARG4) were used. The PCR product (2.2 kb) was restricted with BamHI, and integrated into pUC19 (pUCADE). The primers Arg1 and Arg2 were used. Proper integration of the HIS1 and ARG4 genes was tested by Southern analysis.

**Construction of prototrophic mutant strains.** To construct the prototrophic heterozygous mutant strain AU(A), the primers His1 (bp 597 to 758, upstream of HIS1), His2 (bp 597 to 758, upstream of HIS1), Arg1 (bp 992 to 971, upstream of ARG4), and Arg2 (bp 932 to 911, downstream of ARG4) were used. The PCR products of 2410 bp (HIS1) and 3338 bp (ARG4) were used to integrate the HIS1 and ARG4 genes into the original sites by homologous recombination in a two-step procedure. To construct prototrophic ade5,7 null mutant strains AU(A) and AUH(A), the primers Arg1 and Arg2 were used. Proper integration of the HIS1 and ARG4 genes was tested by Southern analysis.

### Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference/source</th>
</tr>
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<tbody>
<tr>
<td>E. coli XL1-Blue</td>
<td>supE44 hisD17 recA1 endA1 gyrA46 thi relA1 lac F’ [proAB+ lacF8 AM15 Tn10 (ter’)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>C. albicans SC5314</td>
<td>Wild-type</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>C. albicans BWP17</td>
<td>AURA3: imm434::AURA3 : imm434; HIS1:: hisG/ AURA1: hisG; Arg4:: hisG/Arg4:: hisG; ADE5:: ura3; HIS1: hisG; Arg5,6:: hisG-URA3::hisG</td>
<td>Wilson et al. (1999)</td>
</tr>
<tr>
<td>C. albicans CNC44</td>
<td>AURA3: imm434::AURA3 : imm434; AURA1: hisG/ AURA1: hisG; Arg5,6:: hisG-URA3::hisG</td>
<td>Negredo et al. (1997)</td>
</tr>
<tr>
<td>C. albicans AU</td>
<td>AURA3: imm434::AURA3 : imm434; HIS1:: hisG/ AURA1: hisG; Arg4:: hisG/Arg4:: hisG; ADE5:: ura3; HIS1/AD5,7</td>
<td>This work</td>
</tr>
<tr>
<td>C. albicans AH</td>
<td>AURA3: imm434::AURA3 : imm434; HIS1:: hisG/ AURA1: hisG; Arg4:: hisG/Arg4:: hisG; ADE5:: ura3; HIS1/AD5,7</td>
<td>This work</td>
</tr>
<tr>
<td>C. albicans AUH</td>
<td>AURA3: imm434::AURA3 : imm434; HIS1:: hisG/ AURA1: hisG; Arg4:: hisG/Arg4:: hisG; ADE5:: ura3; HIS1/AD5,7</td>
<td>This work</td>
</tr>
<tr>
<td>C. albicans AUR</td>
<td>AURA3: imm434::AURA3 : imm434; HIS1:: hisG/ AURA1: hisG; Arg4:: hisG/Arg4:: hisG; ADE5:: ura3; HIS1/AD5,7</td>
<td>This work</td>
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<tr>
<td>C. albicans AU(HA)</td>
<td>AURA3: imm434::AURA3 : imm434; HIS1:: hisG/ AURA1: hisG; Arg4:: hisG/Arg4:: hisG; ADE5:: ura3; HIS1/AD5,7</td>
<td>This work</td>
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<tr>
<td>C. albicans AUH(A)</td>
<td>AURA3: imm434::AURA3 : imm434; HIS1:: hisG/ AURA1: hisG; Arg4:: hisG/Arg4:: hisG; ADE5:: ura3; HIS1/AD5,7</td>
<td>This work</td>
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<tr>
<td>C. albicans AHU(A)</td>
<td>AURA3: imm434::AURA3 : imm434; HIS1:: hisG/ AURA1: hisG; Arg4:: hisG/Arg4:: hisG; ADE5:: ura3; HIS1/AD5,7</td>
<td>This work</td>
</tr>
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</table>

BamHI restriction sites are double underlined) were used to amplified a 3.5 kb fragment that contained the complete ade5,7 gene. The PCR product was digested with BamHI, and integrated into pUC19 (pUCADE). The primers A1 (bp 597 to 758, upstream of ARG4) and A2 (bp 157 to 176, downstream of ARG4) were used to amplify the selection marker gene ARG4. The PCR product (2.2 kb) was restricted with PmaCl, and integrated 210 bp downstream of ADE5,7 into the plasmid pUCADE. After digestion with BamHI, a 5.7 kb DNA fragment, containing the ADE5,7 gene with upstream and downstream sequences for homologous recombination, as well as the ARG4 gene as selectable marker, was used to integrate the ADE5,7 gene into the original locus. Reintegrates were selected on plates that did not contain arginine. Proper integration of ADE5,7 was confirmed by PCR and Southern analysis.

**Hyphal induction within agar matrix.** For filamentous growth within an agar matrix, *C. albicans* strains were grown overnight in YPD, and diluted to 5 × 10⁶ cells ml⁻¹. After growth for 4 h at 30 °C, the culture was diluted to 10⁷ cells ml⁻¹. A 100 μl volume of the diluted cells was mixed with YPS agar [1 % (w/v) yeast extract, 2 % (w/v) peptone (blood), 2 % (w/v) sucrose, and 1 % (w/v) agar-agar], and poured into plates. After 48, 96, 144, 192 and 240 h incubation at 23 °C, colonies were examined microscopically, and the percentage of filamentous colonies was plotted as a function of time.

**Virulence studies.** Six-week-old male outbred NMRI mice (Harlan-Winkelmann) were housed five per cage, and checked daily. The various strains of *C. albicans* were grown in Sabouraud glucose broth at 30 °C until late-exponential phase. Cells were washed three times, and resuspended in 0.9 % NaCl. A 200 μl suspension, containing between 5 × 10⁶ and 5 × 10⁷ cells (for quantification of kidney colonization after 3 days), or 5 × 10⁸ cells (for quantification of kidney colonization after 21 days), was used to infect immunocompetent mice by intravenous injection into the lateral tail vein. Survival
was monitored for 19–21 days. For comparison of survival curves, the log-rank test was used (Peto et al., 1977). A P value < 0.05 was considered to be significant.

To quantify kidney colonization of *C. albicans*, mice were sacrificed 3 days (infected with $5 \times 10^5$ cells) and 21 days (infected with $5 \times 10^4$ cells) after injection, and kidneys were homogenized in 3 ml physiological NaCl buffer. Serially diluted suspensions were then plated on YPD agar. After 4 days growth at 30°C, numbers of *C. albicans* colonies were counted. The studies using embryonated hen eggs as an alternative virulence model were done according the method described for *Pseudomonas aeruginosa* and *C. albicans* (Härtl et al., 1995, 1997).

**RESULTS**

*C. albicans* Vps34p interacts with Ade5,7p, which is the bifunctional enzyme of the *de novo* purine nucleotide biosynthetic pathway

Vps34p-interacting Candida proteins were identified using the yeast two-hybrid system. The bait protein Vps34p was expressed in *S. cerevisiae* Y190/pGBKT34, which was transformed with a *C. albicans* cDNA library inserted into the prey vector. A positive clone containing a 1.2 kb insert was selected by lacZ filter assay (data not shown). The sequence of the insert was determined. It contains an ORF of 1005 bp that starts 41 bp downstream of the BamHI restriction site and is in-frame with the GAL4 activation domain. The corresponding protein is 334 aa, and shows approximately 65% identity to the C-terminal end of Ade5,7p of *S. cerevisiae*. In addition, the sequence is identical to the *ADE5,7* ORF of the Stanford *C. albicans* sequence (Assembly 19, ORF19.5061 on Contig10219, 802 aa). This ORF shows a 62% similarity to the corresponding *S. cerevisiae* gene. We conclude that the identified clone represents the Candida homologue of *S. cerevisiae* *ADE5,7*.

The interaction of the truncated Ade5,7p and Vps34p was confirmed by retransformation and subsequent lacZ filter assays, and expression analysis of the HIS1 reporter gene, in different *S. cerevisiae* two-hybrid strains that contained different promoter constructs of the reporter genes.

First, the interaction was confirmed by the retransformation of *ADE5,7* into *S. cerevisiae* Y190/pGBKT34. All isolated colonies were blue, showing the expression of the lacZ gene that is induced by the interaction of BD (binding domain of the transcription factor Gal4p)-Vps34p and AD (activation domain of Gal4p)-Ade5,7p. In contrast, the control that lacked AD-Ade5,7p remained white, and this indicated that BD-Vps34p was not able to induce the transcription of lacZ reporter without AD-Ade5,7p (data not shown).

Second, we tested the expression of the HIS reporter genes in *S. cerevisiae* Y190 and HF7c. The interaction of Vps34p with Ade5,7p induced expression of these reporter genes, and resulted in growth on histidine-deficient solid medium (Fig. 1). *S. cerevisiae* strains lacking one of the two interacting proteins were unable to grow on plates without histidine, and this indicated that BD-Vps34p was not able to induce the transcription of lacZ reporter without AD-Ade5,7p (data not shown).

**Biochemical characterization of the Vps34p–Ade5,7p interaction**

The complete protein Ade5,7p was recombinantly expressed as a GST fusion protein, and Vps34p was fused with a His6 tag. Both recombinant proteins were isolated...
by affinity chromatography. Recombinant Ade5,7\textsubscript{GST} with a mobility of 125 kDa, reacted with GST antiserum (Fig. 2A, lane 2), and the 115 kDa Vps34\textsubscript{HIS} reacted with His antiserum, but not with GST antiserum (Fig. 2A, lanes 1 and 3). Direct physical interaction of the recombinant proteins was shown using a ligand blot and a pull-down approach.

For the ligand overlay, the recombinant denatured proteins Ade5,7\textsubscript{GST} and Vps34\textsubscript{HIS} were blotted onto a membrane, and incubated with Ade5,7\textsubscript{GST} (Fig. 2B, lanes 1 and 2). The denaturation reduced the chance of interaction. Nevertheless, the binding of Ade5,7\textsubscript{GST} to immobilized Vps34\textsubscript{HIS} was detected by anti-mouse GST antiserum (Fig. 2B, lane 2). This interaction was specific, as the EBP\textsubscript{GST} control protein did not bind to the immobilized Vps34\textsubscript{HIS} protein (Fig. 2B, lane 4), and the VHHFLU\textsubscript{HIS} control protein did not bind to the immobilized Ade5,7\textsubscript{GST} protein (Fig. 2B, lane 5).

For the pull-down assay, the Vps34\textsubscript{HIS} protein was immobilized to a nickel matrix, and Ade5,7\textsubscript{GST} protein was applied. In the eluate fraction, Ade5,7\textsubscript{GST} was identified (Fig. 2C, lane 1), thus demonstrating binding of Ade5,7\textsubscript{p} to Vps34\textsubscript{p}. When Ade5,7\textsubscript{GST} was applied to a nickel matrix in the absence of Vps34\textsubscript{HIS} no binding was detected (Fig. 2C, lane 2). Nonspecific binding of GST\textsubscript{p} or other proteins was excluded by analysing the application of GST\textsubscript{p} protein alone, and the recombinant EBP\textsubscript{GST} protein, on the column with Vps34\textsubscript{HIS}. The eluate fraction did not contain any control proteins, thus excluding the possibility of nonspecific binding (Fig. 2C, lanes 3 and 4).

**Disruption of the ADE5,7 gene in C. albicans**

The ADE5,7 null mutant strains of *C. albicans* BWP17 were generated to characterize the role of the putative purine nucleotide biosynthesis enzyme Ade5,7\textsubscript{p} in *C. albicans*. As a first step, two heterozygous mutants were generated that contained either the *URA3* (AU) or the *HIS1* (AH) gene as a selectable marker integrated at the *ADE5*\textsubscript{7} loci. As the absence of an *URA3* allele makes the interpretation of mutant phenotypes more difficult (Lay et al., 1998; Cheng et al., 2003), the *URA3* gene containing heterozygous mutant strain AU was used to assay the phenotypes.

In the heterozygous strains AU and AH, the second ADE5,7 allele was replaced by the *HIS1* gene, resulting in AUH, and by the *URA3* gene, resulting in AHU (Fig. 3A). Proper deletion of the ADE5,7 genes in all strains was confirmed by Southern analysis (Fig. 3C), and by PCR (data not shown). Chromosomal DNA of the *C. albicans* strains SC5314 (wild-type), CNC44 (Arg\textsuperscript{−}), BWP17 (parental strain) and the various mutant strains was isolated, and restricted with *Nde*I. The proper 8.8 kb wild-type fragment in *C. albicans* SC5314, CNC44 and BWP17 was identified by Southern hybridization (Fig. 3C, lanes 1, 2 and 3). The replacement of one ADE5,7 allele by the *URA3* gene (AU; Fig. 3C, lane 4) or the *HIS1* gene (AH; Fig. 3C, lane 5) resulted in an additional fragment of 2.2 or 7.9 kb, respectively. The replacement of the second ADE5,7 allele by the *HIS1* (Fig. 3C, lane 6) or *URA3* gene (Fig. 3C, lane 7) led to two ade5,7 null mutants (AUH and AHU, respectively). The loss of the 8.8 kb fragment was consistent with the replacement of the second allele (Fig. 3C).

The identical phenotypes of two independently constructed ade5,7 null mutants showed that ADE5,7 was indeed the cause of the changed phenotypes. To confirm this, an ADE5,7 reintegrate strain, AUR, was generated by replacing the *HIS1* gene in the null mutant strain AUH with the ADE5,7 gene and the *ARG4* gene (Fig. 3B). The 5.1 kb band shows the proper integration of ADE5,7 fused with *ARG4* (Fig. 3C, lane 8). The correct deletion of ADE5,7, and the reintegration of ADE5,7 and *ARG4*, were also confirmed by PCR (data not shown).

**Role of Ade5,7\textsubscript{p} in adenine biosynthesis and metal-ion homeostasis**

Ade5,7\textsubscript{p} is essential for the biosynthesis of adenine. The ade5,7 null mutant strains AUH and AHU did not grow on plates that did not contain adenine (Fig. 4A).

Vps34\textsubscript{p} is involved in the sequestration of toxic ions via a H\textsuperscript{+} gradient, in cooperation with the vacuolar ATPase (Kitanovic et al., 2005; Eck et al., 2005). Therefore, the effect of the Vps34\textsubscript{p}-interacting protein Ade5,7\textsubscript{p} on metal-ion homeostasis was assayed. We tested whether and how metal ions influenced the growth of the *C. albicans* ade5,7 null mutant strains AUH and AHU. The *C. albicans* wild-type strain and the ade5,7 mutant strains showed equal growth on plates supplemented with Co\textsuperscript{2+}. The same
results were found for plates supplemented with Mn$^{2+}$, Ni$^{2+}$ or Zn$^{2+}$ (data not shown). However, on plates supplemented with Cu$^{2+}$ and Ag$^{2+}$, the ade5,7 null mutants showed increased sensitivity towards high ion concentrations, thus demonstrating a moderate role of Ade5,7p in ion detoxification (Fig. 4B–D).

The growth of the ade5,7 mutants was inhibited by AgNO$_3$ at a concentration of 750 μM, CuSO$_4$ at 15 mM, and CuCl$_2$ at 4 mM. The vps34 null mutant strain CAV3 showed similar sensitivity to AgNO$_3$, CuSO$_4$ and CuCl$_2$ (Fig. 4B–D).

**C. albicans ade5,7 null mutant shows repressed filamentous growth under low-temperature embedded conditions**

Multiple pathways have been shown to regulate the induction of hyphal growth in *C. albicans*; these include the MAP kinase, the cAMP, the pH-regulated, and the embedded/microaerophilic pathways (Köhler & Fink, 1996; Brown *et al.*, 1999; Davis *et al.*, 2000; Ernst, 2000; Giusani *et al.*, 2002). The *C. albicans* ade5,7 null mutant strains showed the same characteristics as the wild-type strains when hyphal growth was induced via the MAP kinase pathway, the cAMP pathway, and the pH-regulated pathway (data not shown). Consequently, an additional yeast-to-hyphae transition, which is induced by embedded/microaerophilic conditions, was analysed. The hyphal growth in an agarose matrix at low temperature (23 °C) was tested. After 144 h, the AUH and AHU ade5,7 null mutant strains showed 10% and 20% filamentous colonies, respectively, while the percentage of filamentous colonies was 100% for the wild-type strains SC5314 and CNC44. In addition, the heterozygous mutant AU showed approximately 30% filamentous colonies, and the AUR revertant strain showed 13% filamentous colonies (Fig. 5A). After 10 days, approximately 80% of the colonies of the null mutant strain exhibited hyphal growth, whereas the wild-type strains, the heterozygous mutant strain AU, and the revertant strain, showed 90–100% filamentous colonies.

In addition, the colonies differed in mycelial phenotype after 6 days (144 h). The wild-type strains SC5314 and...
CNC44 exhibited intensive mycelial growth of the colonies. The heterozygous mutant AU and the revertant AUR colonies showed slightly reduced filamentous growth, and the colonies of the null mutant strains AHU and AUH showed few mycelial structures (Fig. 5B).

**Ade5,7p is required for virulence of *C. albicans***

The ade5,7 null mutants showed affected hyphal development under microaerophilic/embedded conditions. Consequently, the virulence of the mutant strains was tested in a mouse model of systemic candidiasis. All ade5,7 null mutants were avirulent in this animal model, and resulted in a significantly higher survival of infected mice compared with the wild-type strain and the Arg" strain CNC44. All mice infected with $5 \times 10^6$ mutated AUH, AHU, AUH(A) and AHU(A) *C. albicans* cells survived during the complete course of the experiment (Fig. 6A, B) and looked healthy. The heterozygous mutant strains AU and AU(HA), and the Ade5,7 revertant strain AUR, showed slightly, non-significant, reduced virulence, and 70–80% of the infected mice survived. However, most of these animals (75–90%) lost weight, rotated, or showed a crooked neck. All mice infected with $5 \times 10^6$ wild-type cells SC5314 died after 6 days.

Contrasting results were observed in the alternative virulence model of embryonated hen eggs. Most embryos (80–90%) of the eggs infected with $10^6$ null mutant cells died after 2 days. The wild-type strain SC5314, the heterozygote strain, and the revertant strain, showed similar significantly higher virulence. Only embryos of control eggs inoculated with NaCl survived (Fig. 6C).

Systemic candidiasis is often associated with colonization of internal organs, such as kidneys, lung or liver. It is known that in animal models of disseminated candidiasis, *C. albicans* exhibits a high predilection for the kidneys, which leads to late fatalities in the course of the infection.
Therefore, kidney colonization of mice infected with 5 × 10^5 cells of the strains SC5314, AU, AUH, AHU and AUR was examined 3 days post-infection (n=2). Kidneys of mice bearing strain SC5314 exhibited a high fungal burden of between 4.1 × 10^4 and 7.9 × 10^4 c.f.u. (g kidney tissue)^{-1}, whereas kidneys of mice infected with the null mutants AUH and AHU showed reduced c.f.u. [0.0–6.0 × 10^2 and 2.0 × 10^3 (g kidney tissue)^{-1}, respectively]. The heterozygote mutant and the revertant strains showed slightly higher c.f.u. than the null mutant strains [between 0.4 × 10^3 and 5.1 × 10^3, and 2.0 × 10^2 and 3.0 × 10^3 (g kidney tissue)^{-1}, respectively].

In addition, survivors of the virulence test were checked for kidney colonization. Examination of kidney colonization was assayed after 21 days for mice infected with 5 × 10^4 cells. Kidneys of mice bearing strain SC5314 exhibited a high fungal burden of between 0.9 × 10^6 and 1.0 × 10^6 c.f.u. (g kidney tissue)^{-1}, whereas kidneys of mice infected with the null mutant strains AUH and AHU showed a slightly reduced number of c.f.u. [between 1.4 × 10^5 and 8.2 × 10^5, and 1.0 × 10^4 and 1.1 × 10^5 (g kidney tissue)^{-1}, respectively]. The kidneys of mice infected with the heterozygous strain and the revertant strain exhibited nearly identical numbers of c.f.u. to the wild-type strain SC5314 [between 0.9 × 10^6 and 2.9 × 10^6, and 1.2 × 10^6 and 4.9 × 10^6 (g kidney tissue)^{-1}, respectively].

Fig. 4. ade5,7 null mutants show adenine auxotrophy, and increased sensitivities to metal ions. (A) C. albicans ade5,7 null mutant strains AUH and AHU show reduced growth on solid medium lacking adenine. (B–D) ade5,7 null mutant strains AUH and AHU, and the vps34 null mutant strain CAV3, show increased sensitivity to (B) CuCl₂, CuSO₄ and (C) AgNO₃ on solid medium, and (D) in liquid medium after growth for 48 h. The C. albicans wild-type strains SC5314 and CNC44, the heterozygous mutant strain AU and the ade5,7 revertant strain AUR grew equally well on solid medium lacking adenine. The same results were shown on solid and in liquid medium supplemented with AgNO₃ and on solid medium supplemented with CuCl₂ and CuSO₄, respectively.

Fig. 5. Ade5,7p is essential for hyphal development under microaerophilic/embedded conditions. After different incubation times at 23 °C, colonies were examined microscopically, and the percentage of filamentous colonies was plotted as a function of time. (A) The ade5,7 null mutant strains AUH and AHU showed a reduced number of filamentous colonies. (B) The colonies differed in mycelial phenotype after 6 days incubation.
These results clearly show reduced numbers of c.f.u. after 3 days in the kidneys of mice bearing the null mutant strains, and the heterozygous and revertant strains, whereas after 21 days, only the null mutant strains showed a slightly reduced number of c.f.u.

**Construction and characterization of the prototrophic mutant strains**

The auxotrophies of the mutant strains may influence the phenotypes. Therefore, we characterized *C. albicans* CNC44 Arg auxotroph in addition to SC5314 to exclude that the Arg auxotrophy of the null mutants influences the phenotypes. In addition, prototrophic heterozygous and homozygous mutant strains were constructed to rule out the potential of synthetic defects (Ade; Arg) in the ade5,7 null mutants. A prototrophic revertant strain was not constructed because the identical phenotypes of two independently constructed ade5,7 null mutants had already shown that ADE5,7 causes the changed phenotypes. The prototrophic mutants were characterized in an analogous way to the auxotrophic mutant strains. Insignificant differences were observed between the auxotrophic and prototropic strains. The reintegration of the HIS1 and ARG4 genes into the mutant strains did not change the phenotypes (data not shown) or the avirulence (Fig. 6A, B) significantly.

**DISCUSSION**

In this study, we demonstrate that Ade5,7p, the bifunctional enzyme of the de novo purine nucleotide biosynthetic pathway of the yeast *C. albicans*, interacts with the PtdIns 3-kinase Vps34p, and is involved in adenine synthesis, metal-ion detoxification, hyphal growth and virulence.

The physical interaction of Ade5,7p with Vps34p indicates a link between vacuolar transport and de novo nucleotide synthesis. This can be understood by the fact that both processes are regulated during periods of nutrient limitation. The recycling of cytoplasmic material in the vacuole results in the supply of material for the salvage pathway of purine synthesis, which is more active under nutrient limitation, and less active during de novo synthesis. The affected de novo synthesis may send a signal to start transport into the vacuole, possibly by physical interaction between Vps34p and Ade5,7p. Direct physical interaction between vacuolar transport proteins and enzymes of the de novo nucleotide synthesis has also been shown in *S. cerevisiae*. A physical interaction has been found between Ade5,7p and Apg12p, a protein of starvation-induced autophagocytosis, which is responsible for transport of cytoplasmic proteins into the vacuole (Ho et al., 2002).

Amino acids such as glutamine, glycine and aspartate are involved in de novo purine synthesis. Furthermore, general amino acid control (GAAC) is linked to purine synthesis, since the transcription factor Gcn4p, which regulates the transcription of more than 30 genes of GAAC, is required for maximal expression of one or several genes encoding purine biosynthesis proteins under conditions of purine...
limitation. Gcn4p stimulates the expression of ADE5,7 (Rolfes & Hinnebusch, 1993), and, in addition, ADE5,7 expression is significantly induced by the GAAC system in a GCN4-dependent fashion in S. cerevisiae (Yin et al., 2004). Therefore, we hypothesize that Vps34p controls amino acid metabolism, in addition to regulating purine synthesis. This is supported by the finding of a physical interaction between a protein of the GAAC, i.e. the C. albicans Gcn4p-activator protein Gcn1p, and Vps34p in the two-hybrid system (unpublished data). In C. albicans, Gcn4p acts as a global regulator of both metabolic and morphogenetic response (e.g. hyphal growth) to amino acid starvation (Tripathi et al., 2002). Such a co-regulation of purine synthesis and hyphal growth under low-temperature embedded conditions was shown here for Ade5,7p.

C. albicans ade5,7 null mutants were generated, and showed a defective growth on adenine-deficient medium, thus demonstrating a decisive role of Ade5,7p in the de novo purine biosynthesis pathway in C. albicans. The addition of extracellular adenine compensated this growth defect, and thus the salvage pathway is not affected. This effect is in agreement with data obtained for S. cerevisiae showing that Ade5,7p affects purine nucleotide biosynthesis (Giaever et al., 2002). The S. cerevisiae ade5,7 null mutant is viable, but exhibits a reduced fitness in rich medium (YPD). In addition, deletion of only one ADE5,7 allele in a diploid strain leads to reduced fitness in YPD medium (Deutschbauer et al., 2005). In contrast, the C. albicans ade5,7 null mutant strain was viable and showed normal growth in YPD medium. Therefore, the characterization of the ade5,7 null mutant strains is not biased by growth phenomena.

The vps34 and ade5,7 null mutant phenotypes could be caused indirectly by the absence of the corresponding genes. Nevertheless, in some cases, the genes could be involved directly in special cellular processes.

Cu$^{2+}$ resistance is most likely to be a prerequisite for the survival of C. albicans in the digestive tract of the host, since the Cu$^{2+}$ concentrations of about 10 $\mu$M in the stomach and duodenum are relatively high (Underwood, 1977). The ade5,7 null mutants showed an increased sensitivity toward high concentrations of Cu$^{2+}$ and Ag$^{2+}$, which in S. cerevisiae are detoxified by the ATPase Ctr1p located at the plasma membrane (Riggle & Kumamoto, 2000). The influence of targeting the plasma membrane (P)-ATPase by the vacuolar (V)-ATPase has been shown in S. cerevisiae (Perzov et al., 2000). We hypothesize that a defective Vps34p interacting with V-ATPase affects the distribution of P-ATPase in the ade5,7 null mutants, and thus leads to decreased Cu$^{2+}$ and Ag$^{2+}$ resistance (Eck et al., 2005; Perzov et al., 2000). The Ade5,7p protein possibly influences the P-ATPase, perhaps by interaction with the Vps34p-V-ATPase complex. In contrast to the vps34 null mutant (Poltermann et al., 2005), the ade5,7 null mutants did not show sensitivity to Co$^{2+}$, Mn$^{2+}$, Ni$^{2+}$ or Zn$^{2+}$. In general, the ade5,7 mutants showed an increased sensitivity towards metal ions that are detoxified by P-ATPase, whereas the vps34 mutant showed sensitivity to metal ions that are detoxified by both V- and P-ATPase. In summary, both Ade5,7p and Vps34p play a role in metal-ion detoxification, as this is done by plasma membrane ATPase.

Hyphal growth in a matrix is regulated by the transcription factors Efg1p and Czf1p. These two transcription factors act antagonistically, depending on the physical environment (Brown et al., 1999). Efg1p promotes filamentous growth at 37 °C in liquid media, and represses hyphal development during growth within a matrix at low temperature, whereas Czf1p antagonizes the repression mediated by Efg1p and induces hyphal growth at low temperatures when cells are embedded within agar matrix (Giusani et al., 2002). Previously, we have reported that the C. albicans vps34 null mutant shows hyperfilamentation in an agar matrix (Kitanovic et al., 2005). In this study, we observed that the Vps34p-interacting protein Ade5,7p shows the opposite phenotype. Thus, we hypothesize that Ade5,7p and Vps34p may act antagonistically, and in a similar way to the transcription factors Efg1p and Czf1p. Vps34p regulates hyphal growth induced by the cAMP and MAP kinase pathways, and reduces hyphal differentiation in a matrix (Bruckmann et al., 2000; Kitanovic et al., 2005). However, the Ade5,7p protein activated hyphal growth in a matrix, and, thus, the Ade5,7p–Vps34p complex may affect the balance of hyphal growth under various conditions.

The ade5,7 null mutants showed avirulence in the mouse model, and this can be attributed to reduced kidney colonization. In the model of embryonated hen eggs, the ade5,7 null mutants showed high virulence. This may be attributed to different environmental conditions, e.g. the availability of adenine in the eggs, whereas in blood the concentration of adenine is too low for survival of the ade5,7 null mutant strains. The reduced kidney colonization is especially significant after 3 days of infection. This indicates that the low adenine concentration in blood prevents colonization at the beginning of the pathogenesis. After reaching the kidneys, the null mutant can grow almost equally as fast as the wild-type, and this results in a reduced difference between null mutant and wild-type strain regarding the kidney colonization at the end of virulence test. In addition, the reduced virulence of an ade2 null mutant in an immunosuppressed murine model of systemic candidiasis has confirmed the requirement of adenine de novo biosynthesis for pathogenesis of C. albicans (Donovan et al., 2001).

Deletion or change of locus of the URA3 gene may complicate the characterization of null mutants (Lay et al., 1998; Cheng et al., 2003). In this study, the URA3 gene was integrated at the same locus in the heterozygous and null mutant strains. The related phenotypes of the heterozygous mutant strain and of the wild-type strain did not indicate a URA3-position effect in the ade5,7 null mutant strains.
Furthermore, the mutant phenotypes did not revert after addition of uridine to the medium (data not shown), and this confirms that the null mutant phenotypes do not result from a URA3-position effect.

In this study, we have shown that Ade5,7p, which is the C. albicans bifunctional enzyme of the de novo purine synthesis pathway, interacts with the vacuolar transport protein PtdIns 3-kinase Vps34p. The characterization of ade5,7 null mutants indicates that this interaction may influence cellular functions that are connected to detoxification, hyphal growth in a matrix, and virulence. In addition, the adenine auxotrophy of the ade5,7 null mutant strains seems to contribute to avirulence in the mouse model of systemic candidiasis.

ACKNOWLEDGEMENTS

We thank Federico Navarro-Garcia (Dept Microbiologia II, Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain) and Aaron P. Mitchell (Department of Microbiology, Columbia University, New York, NY, USA) for providing C. albicans strains CNC44 and BWIP17. We thank U. Stöckel and B. Weber for technical assistance.

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Edited by: D. Sanglard