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

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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, glycylamide ribotide synthetase; GST, glutathione S-transferase; P, plasma membrane; PtdIns, phosphatidylinositol; V, vacuolar; VHHFLU, antibody against fluorescein.

ADE3 and *ASP5*). Strains with mutations in two transcription factor genes, *BAS1* 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 (Rolfes & Hinnebusch, 1993).

In the yeast *S. cerevisiae*, Ade5,7p contains the activities for aminoimidazole ribotide synthetase (AIRS) and glycine-amide 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 (Giaever *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 antimycotic substance 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 pGBKT34, 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 (Ade5,7_{GST}) in *Escherichia coli*. *ADE5,7* cDNA was amplified using primer A (introducing a *Bam*HI site) and primer B (introducing a *Hind*III site) (Table 2). The PCR product was cleaved with *Bam*HI and *Hind*III, and cloned into the pGEX3T vector, resulting in plasmid pGEX7-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 (Vps34_{HIS}), and *C. albicans* EBP (oestrogen-binding protein) was expressed as a GST-tagged protein (EBP_{GST}) (Eck *et al.*, 2000; Madani *et al.*, 1994). The fluorescein antibody VHHFIU was expressed as a His6-tagged protein (VHHFIU_{HIS}). For ligand overlay assays, approximately 0.5 µg recombinant protein was separated by SDS-PAGE (8%), and blotted on 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 Vps34_{HIS} protein was added to a Ni-NTA superflow agarose column (Qiagen). After washing, a 2.0 µg quantity of Ade5,7_{GST}, 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 -558, upstream of *ADE5,7*) and G4 (bp -498 to -518, downstream of *ADE5,7*) (Table 2; chromosomal sequences are underlined, and

Table 1. Strains used in this study

Strain	Genotype	Reference/source
<i>E. coli</i> XL1-Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F'[proAB⁺ lacI^q ΔM15 Tn10(ter')]</i>	Stratagene
<i>C. albicans</i> SC5314	Wild-type	Fonzi & Irwin (1993)
<i>C. albicans</i> BWP17	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg4 :: hisG/Δarg4 :: hisG</i>	Wilson <i>et al.</i> (1999)
<i>C. albicans</i> CNC44	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg5,6 :: hisG-URA3-hisG/Δarg5,6 :: HIS1</i>	Negredo <i>et al.</i> (1997)
<i>C. albicans</i> AU	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg4 :: hisG/Δarg4 :: hisG; Δade5,7 :: URA3/ADE5,7</i>	This work
<i>C. albicans</i> AH	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg4 :: hisG/Δarg4 :: hisG; Δade5,7 :: HIS1/ADE5,7</i>	This work
<i>C. albicans</i> AUH	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg4 :: hisG/Δarg4 :: hisG; Δade5,7 :: URA3/Δade5,7 :: HIS1</i>	This work
<i>C. albicans</i> AHU	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg4 :: hisG/Δarg4 :: hisG; Δade5,7 :: HIS1/Δade5,7 :: URA3</i>	This work
<i>C. albicans</i> AUR	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg4 :: hisG/Δarg4 :: hisG; Δade5,7 :: URA3/Δade5,7 :: ADE5,7/ARG4</i>	This work
<i>C. albicans</i> AU(HA)	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg4 :: hisG/Δarg4 :: hisG; Δade5,7 :: URA3/ADE5,7; Δarg4 :: ARG4; Δhis1 :: HIS1</i>	This work
<i>C. albicans</i> AUH(A)	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg4 :: hisG/Δarg4 :: hisG; Δade5,7 :: URA3/Δade5,7 :: HIS1; Δarg4 :: ARG4</i>	This work
<i>C. albicans</i> AHU(A)	<i>Δura3 :: imm434/Δura3 :: imm434; Δhis1 :: hisG/Δhis1 :: hisG; Δarg4 :: hisG/Δarg4 :: hisG; Δade5,7 :: HIS1/Δade5,7 :: URA3; Δarg4 :: ARG4</i>	This work

*Bam*HI restriction sites are double underlined) were used to amplify a 3.5 kb fragment that contained the complete *ADE5,7* gene. The PCR product was digested with *Bam*HI, and integrated into pUC19 (pUCADE). The primers A1 (bp –597 to –578, 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 *Pma*CI, and integrated 210 bp downstream of *ADE5,7* into the plasmid pUCADE. After digestion with *Bam*HI, 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.

Construction of prototrophic mutant strains. To construct the prototrophic heterozygous mutant strain AU(HA), the primers His1 (bp –764 to –742, upstream of *HIS1*), His2 (bp –749 to –727, downstream 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 AHU(A) and AUH(A), the primers Arg1 and Arg2 were used. Proper integration of the *HIS1* and *ARG4* genes was tested by 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 5×10^6 cells ml^{–1}. After growth for 4 h at 30 °C, the culture was diluted to 10^3 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 5×10^6 and 5×10^5 cells (for quantification of kidney colonization after 3 days), or 5×10^4 cells (for quantification of kidney colonization after 21 days), was used to infect immunocompetent mice by intravenous injection into the lateral tail vein. Survival

Table 2. Primers used in this study

Primer	Sequence (5'→3')*
A	<u>CGGGATCCGAAAAACGTAAATTGGTGGCAGC</u>
B	<u>CCCAAGCTTATGTGGAACTCCAAATTGACTA</u>
S1	<u>CATCGATTAGATTCAAAATCTTAGAACTTCTCCAAAACCTTATTCATT</u> <u>TGAGATTTTATTGAATATATTCCTCCCAACACAATTCAGCTTAACTATGTCATGAAGCTTCGTACGCTGCAGGTC</u>
S2	<u>ATATAATAATTTAAACATACAAAAGCAAGCAGTAAACAACAACAACAACAACAACCTCAATCGTCTAGT-</u> <u>TGTAATATCAACATTCTTATTTTCTTCTGATATCATCGATGAATTCGAG</u>
AS4	<u>GCTGAATTGTGTTGGGGAATATAT</u>
AS5	<u>AGAATGTTAAAACAGGGGACATCT</u>
G1	<u>ATCCGCGGATCCAAGGGTTGTGACACCTAATG</u>
G4	<u>ATGCGCGGATCCACTCAAAGCCGAGATAGACCT</u>
A1	<u>ATCGATCGCACGTGGGAATCACTTTTGGATGAA</u>
A2	<u>ATCGATCGCACGTGCATATAGACGTGTTGTGTGT</u>
Arg1	<u>GAAACAATGAATCGATGCTTTC</u>
Arg2	<u>CCTTGGTATTAGGTGATTTTGC</u>
His1	<u>CTCGTGCCGTGTTGAATGTTTGC</u>
His2	<u>CAGAACGCTCCGCACAAAAATC</u>

*Chromosomal sequences are underlined; restriction sites are double underlined.

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 5×10^5 cells) and 21 days (infected with 5×10^4 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 to 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 *Bam*HI 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 confirmed that the interaction between BD-Vps34p and AD-Ade5,7p induced the transcription of the histidine reporter genes (Fig. 1).

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

Fig. 1. *C. albicans* Vps34p interacts with Ade5,7p. The expression of the *HIS* reporter gene in *S. cerevisiae* Y190 and HF7c, caused by the interaction of Vps34p with Ade5,7p, results in growth on His-deficient solid medium (30 °C, 24 h). The proteins alone do not induce the expression of the *HIS* reporter gene. 3-AT, 3-amino-1,2,4-triazol.

by affinity chromatography. Recombinant Ade5,7_{GST}, with a mobility of 125 kDa, reacted with GST antiserum (Fig. 2A, lane 2), and the 115 kDa Vps34_{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_{GST} and Vps34_{HIS} were blotted onto a membrane, and incubated with Ade5,7_{GST} (Fig. 2B, lanes 1 and 2). The denaturation reduced the chance of interaction. Nevertheless, the binding of Ade5,7_{GST} to immobilized Vps34_{HIS} was detected by anti-mouse GST antiserum (Fig. 2B, lane 2). This interaction was specific, as the EBP_{GST} control protein did not bind to the immobilized Vps34_{HIS} protein (Fig. 2B, lane 4), and the VHHFLU_{HIS} control protein did not bind to the immobilized Ade5,7_{GST} protein (Fig. 2B, lane 5).

For the pull-down assay, the Vps34_{HIS} protein was immobilized to a nickel matrix, and Ade5,7_{GST} protein was applied. In the eluate fraction, Ade5,7_{GST} was identified (Fig. 2C, lane 1), thus demonstrating binding of Ade5,7p to Vps34p. When Ade5,7_{GST} was applied to a nickel matrix in the absence of Vps34_{HIS}, no binding was detected (Fig. 2C, lane 2). Nonspecific binding of GSTp or other proteins was excluded by analysing the application of GSTp protein alone, and the recombinant EBP_{GST} protein, on the column with Vps34_{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 Ade5,7p 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 *ADE5,7* 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⁻), BWP17 (parental strain) and the various mutant strains was isolated, and restricted with *NdeI*. 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 Ade5,7p in adenine biosynthesis and metal-ion homeostasis

Ade5,7p 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).

Vps34p is involved in the sequestration of toxic ions via a H⁺ gradient, in cooperation with the vacuolar ATPase (Kitanovic *et al.*, 2005; Eck *et al.*, 2005). Therefore, the effect of the Vps34p-interacting protein Ade5,7p 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 Co²⁺. The same

Fig. 2. *C. albicans* Vps34p interacts *in vitro* with Ade5,7p. (A) Recombinant Ade5,7_{GST} (125.0 kDa, lane 2), Vps34_{HIS} (120.0 kDa, lane 3), EBP_{GST} (81 kDa, lane 4), and VHHFLU_{HIS} (10 kDa). Vps34_{HIS} does not show cross-reactivity with anti-GST (lane 1). Ade5,7_{GST} and the control protein EBP_{GST} react with anti-GST (lanes 2 and 4). Vps34_{HIS} and the control protein VHHFLU_{HIS} react with anti-His (lanes 3 and 5). (B) Ligand blot. Membranes loaded with Ade5,7_{GST} and Vps34_{HIS}, EBP_{GST} and Vps34_{HIS}, or Ade5,7_{GST} and VHHFLU_{HIS} were incubated with indicated proteins (Ade5,7_{GST}, EBP_{GST} and VHHFLU_{HIS}), followed by incubation with anti-GST or anti-His. The asterisk marks possible degradation products of Vps34_{HIS}. (C) His pull-down. Ni-NTA beads loaded with Vps34_{HIS} (lanes 1, 3 and 4) and unloaded (lane 2) were incubated with Ade5,7_{GST} (lanes 1 and 2), EBP_{GST} (lane 3), and GSTp alone (lane 4), under native conditions. The eluted proteins were separated by SDS-PAGE, blotted, and detected by anti-GST. The correct sizes of recombinant proteins were checked by protein markers (not shown).

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 AgNO₃ at a concentration of 750 µM, CuSO₄ at 15 mM, and CuCl₂ at 4 mM. The *vps34* null mutant strain CAV3 showed similar sensitivity to AgNO₃, CuSO₄ and CuCl₂ (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

results were found for plates supplemented with Mn²⁺, Ni²⁺ or Zn²⁺ (data not shown). However, on plates supplemented with Cu²⁺ and Ag²⁺, the *ade5,7* null mutants showed increased sensitivity towards high ion

CNC44 exhibited intensive mycelial growth of the colonies. The heterozygous mutant AU and the revertant AUR colonies showed slightly reduced filamentous growth, and

Fig. 3. Disruption of the *ADE5,7* gene in *C. albicans*. The strategies used (A) to replace the chromosomal *ADE5,7* genes by *URA3* and *HIS1* genes, and (B) to reintegrate the *ADE5,7* gene, resulting in *C. albicans* revertant strain AUR, are shown. Thick black arrows indicate the *ADE5,7* gene; S1, S2, G1, G4, AS4, AS5, A1 and A2 are PCR primers (Table 2), pFA-*URA3* and pFA-*HIS3* are plasmids used for PCR with S1 and S2; *NdeI* is the restriction enzyme used for Southern analysis; *PmaCI* is the restriction enzyme used for cloning of *ARG4*; *BamHI* is the restriction enzyme used for cloning of *ADE5,7* (A, B). (C) Southern analysis of *NdeI*-digested chromosomal DNA from the following *C. albicans* strains: wild-type strain SC5314 (lane 1); Arg⁻ strain CNC44 (lane 2); parental strain BWP17 (lane 3); AU mutant strain (heterozygous *ADE5,7/ade5,7* mutant strain with integrated *URA3* gene, lane 4); AH mutant strain (heterozygous *ADE5,7/ade5,7* mutant strain with integrated *HIS1* gene, lane 5); AUH mutant strain (homozygous *ade5,7* null mutant strain, lane 6); AHU mutant strain (homozygous *ade5,7* null mutant strain, lane 7); AUR revertant strain (heterozygous *ADE5,7-ARG4/ade5,7* revertant strain, lane 8). The blot was hybridized with non-radioactive labelled PCR product, which was obtained with primers AS4 and AS5.

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 5×10^6 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 5×10^6 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 10^8 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

(Odds, 1994). 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. [$0.0\text{--}6.0 \times 10^2$ 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 3.0×10^2 (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 1.1×10^6 (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 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⁻ 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 prototrophic 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

Fig. 6. Ade5,7p is involved in the pathogenicity of *C. albicans*. (A, B) *C. albicans* wild-type strain SC5314, the heterozygous mutant strains AU and AU(HA), *ade5,7* null mutant strains AUH, AHU, AUH(A) and AHU(A), and *ADE5,7* revertant strain AUR were tested in a mouse model of systemic candidiasis. Survival of mice infected with 5×10^6 cells was monitored for (A) 21 days and (B) 19 days ($n=10$). (C) SC5314, AU, AUH, AHU and AUR were tested in an alternative virulence model of embryonated hen eggs. Survival of embryonated hen eggs infected with 1×10^8 *Candida* cells was monitored for 8 days ($n=10$). A *P* value of <0.05 was considered to be significant (Peto *et al.*, 1977).

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 μM 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.

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