Generation and functional in vivo characterization of a lipid kinase defective phosphatidylinositol 3-kinase Vps34p of Candida albicans

Juliane Günther, Monika Nguyen, Albert Härtl, Waldemar Künkel, Peter F. Zipfel and Raimund Eck

1Hans-Knoell-Institute for Natural Products Research, Department of Infection Biology, Beutenbergstrasse 11, D-07745 Jena, Germany
2University of Applied Sciences, Tatzendpromenade 1b, D-07745 Jena, Germany

Correspondence
Raimund Eck
reck@pmail.hki-jena.de

The phosphatidylinositol (PI) 3-kinase Vps34p of Candida albicans has lipid kinase and autophosphorylation activity and is involved in virulence and vesicular protein transport. In order to characterize the roles of lipid kinase activity, a chimeric Vps34 protein was created which lacks lipid kinase but retains autophosphorylation activity. To this end, six amino acids within the putative lipid-binding site of Vps34p were replaced by the homologous region of the PI 3-kinase-like C. albicans Tor protein. The resulting chimeric Vps34T protein was recombinantly expressed in Escherichia coli and shown to lack lipid kinase activity. The corresponding chimeric VPS34TOR gene was inserted into the genome of C. albicans, and this lipid-kinase-defective strain had a distinctive phenotype compared to those of the wild-type strain SC5314 and the vps34 null mutant. The lipid-kinase-defective strain was non-virulent, and showed altered hyphal growth, reduced adherence, as well as defective vacuole morphology and endosomal vesicle transport. These results demonstrate an important role for the lipid kinase activity of Vps34p in virulence and vesicular protein transport. On the other hand, the lipid-kinase-defective strain and the vps34 null mutant differ in their temperature- and osmotic-stress response. This indicates a possible role for activities different from the lipid kinase function of Vps34p.

INTRODUCTION

Candida albicans is the major systemic fungal pathogen in humans (Odds, 1994). This polymorphic yeast is capable of causing life-threatening infections in immunocompromised patients as well as a variety of mucosal infections in healthy individuals. Recent experimental data suggest that the virulence of C. albicans is dependent on several properties, including the ability of the yeast to switch between different morphogenetic forms, host epithelial and endothelial cell recognition and adhesion, as well as the ability to secrete proteinases and phospholipases (Cutler, 1991; Köhler & Fink, 1996). A number of virulence factors of C. albicans have been characterized; however, the mechanisms that enable this opportunistic fungus to become pathogenic have not yet been unraveled.

In C. albicans, the phosphatidylinositol (PI) 3-kinase Vps34p is involved in virulence. The C. albicans vps34 null mutant is unable to form hyphae on various solid media, shows a significantly delayed yeast to hyphae transition in liquid media, is hypersensitive to high temperature and hyperosmotic stress and exhibits reduced adherence to human cells. In addition, the vps34 null mutant displays enlarged and electron-transparent vacuoles and is avirulent in a mouse model of systemic candidiasis (Eck et al., 2000; Bruckmann et al., 2000; 2001).

PI 3-kinases control a wide variety of cellular processes in eukaryotic cells, including mitogenesis, protection from apoptosis, growth factor receptor down-regulation, stimulation of glucose uptake, endocytosis, actin cytoskeleton rearrangement and intracellular protein/membrane trafficking (DeCamilli et al., 1996; Toker & Cantley, 1997). PI 3-kinases are subdivided into three classes, based on sequence similarities, substrate specificity and regulatory properties (Vanhaesebroeck et al., 1997). Class I PI 3-kinases represent dual-specificity enzymes, and display both lipid kinase and protein kinase activity. The generation of a lipid-kinase-defective human PI 3-kinase γ with a defective putative PI-binding domain was used to show the transphosphorylation of the PI 3-kinase adapter protein p101 and the MAP protein kinase MEK-1 in vitro (Stoyanov et al., 1995; Bondeva et al., 1998; Bondev et al., 1999).

The class III PI 3-kinase Vps34p (Vps: vacuolar protein sorting) represents the only PI 3-kinase activity in the yeast
Saccharomyces cerevisiae (Schu et al., 1993; Vanhaesebroeck et al., 1997). ScVps34p regulates intracellular protein trafficking to the vacuole, retrograde endosome-to-Golgi transport, autophagocytosis and vacuole acidification (Herman & Emr, 1990; Zhou et al., 1995; Burda et al., 2002; Kihara et al., 2001; Munn & Riezman, 1994). Two distinct Vps34p-containing protein complexes were identified in S. cerevisiae. (Kihara et al., 2001). ScVps34p shows lipid kinase and autophosphorylation activity, and the protein is predominantly phosphorylated on serine residues (Stack & Emr, 1994). ScVps34 proteins containing mutated residues in the putative lipid kinase domain (amino acid positions 731, 735, 736 and 749) are defective in both autophosphorylation and lipid kinase function (Stack & Emr, 1994; Schu et al., 1993).

In this study, we generated a Vps34T mutant protein of C. albicans with specifically inactivated lipid kinase activity. A Candida mutant strain containing the lipid-kinase-defective gene was generated. The phenotype of this strain indicated that the lipid kinase activity is essential for the virulence, dimorphism, adhesion, vesicular protein transport and vacuole morphology of C. albicans. Additional activities of Vps34p, which are mainly involved in the stress response, were shown in vivo.

METHODS

Strains and growth conditions. The C. albicans strains and plasmids used are listed in Table 1. Strains were grown in YPD medium (2% glucose, 2% peptone, 1% yeast extract), Sabouraud medium (2% glucose, 1% peptone and casein) and SD medium [0.7% yeast nitrogen base without amino acids (Difco), 2% glucose] at 30°C. SD medium was supplemented with 20 μg uridine ml⁻¹ for ura⁻ strains. Cell numbers were determined using a haemocytometer. Hyphal growth was induced in liquid culture by diluting late-exponential-phase cultures grown at 30°C tenfold in fresh YPD medium supplemented with 15% fetal calf serum (FCS) at 37°C. On solid medium, hyphal growth was induced by nutrient limitation. Cells were grown overnight in YPD at 30°C, washed, diluted and spread on Spider plates. Between 20 and 100 cells per plate were incubated at 37°C for 14 days. Sensitivities of the mutant strains to KCl and NaCl were assayed on YPD plates. Escherichia coli XL-1 Blue, supE44 hisD41 recA1 endA1 gyrA46 thi relA1 lac I[promAB β lac F'proAB + lacM15 Tn10K Tet]) (Stratagene), was used for cloning, whereas E. coli M15 (Qiagen) was used for protein expression.

Construction of expression plasmid pVTPEX and integration plasmid pKEU1. To insert a KpnI restriction site in the lipid-binding domain of VPS34, a 380 bp C-terminal fragment of VPS34 was amplified using the following primers (coding sequences are underlined): 5′-CGGCCGTGACATATATTTATTTTTGCTCTGTTCATT-3′ and 5′-GCGGCGAAGGGTACGATGCGAGAAGGTTGACGACACTATTGAC-3′ (downstream). The XmnI and PsI restriction sites generated by the primers were used to clone the fragment into the Vps34p-6His-tag expression plasmid pVPEX1 (Eck et al., 2000), yielding pVPEX2. Then, the VPS34 coding sequence between the XmnI and KpnI restriction sites was replaced by synthetic double-stranded DNA consisting of the oligonucleotides 5′-GGCCGTGCAATATTACCTGCTGGAGTTGATACCCAGAGGAGGGTAC-3′ and 5′-GCTGAGTCTGAAGCTGCCTTCAG-3′ (CaTOR coding sequences – underlined – are found in Stanford’s Candida albicans sequencing project Assembly 19, ORF19-1903 on Contig135; URL: http://sequence-www.stanford.edu/group/candida/index.html) to generate the chimeric VPS34T gene containing the TOR PI head-group interaction site (Fig. 1). For the integration of the chimeric VPS34T gene in the C. albicans genome, an integration vector was constructed. To this end, the C-terminus of the VPS34 gene of pVTPEX was amplified using the following primers (pVTPEX sequences are underlined): 5′-GGCCGTGCAATATTACCTGCTGGAGTTGATACCCAGAGGAGGGTAC-3′ and 5′-GCTGAGTCTGAAGCTGCCTTCAG-3′ (downstream). The 450 bp fragment was cloned into VPS34 reintegration plasmid pKEU1 (Bruckmann et al., 2000) using XmnI and PnuCI restriction sites generated by the primers, yielding the VPS34TOR gene.

Expression of recombinant chimeric CaVps34T protein. Induction of high-level protein expression, purification of the 6His-tagged protein and Western blots with His-tag antibodies were carried out according to the standard protocols of the manufacturer (Qiagen).

Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. albicans strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC5314</td>
<td>Wild-type</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CAV3</td>
<td>Δvps34::hisGlΔvps34::hisG-URA3-hisG, Δura3::imm434/Δura3::imm434</td>
<td>Bruckmann et al. (2000)</td>
</tr>
<tr>
<td>CAV4</td>
<td>Δvps34::hisGlΔvps34::hisG, Δura3::imm434/Δura3::imm434</td>
<td>Bruckmann et al. (2000)</td>
</tr>
<tr>
<td>CAV5</td>
<td>Δvps34::hisG VPS34::URA3, Δura3::imm434/Δura3::imm434</td>
<td>Bruckmann et al. (2000)</td>
</tr>
<tr>
<td>CAV9</td>
<td>Δvps34::hisG VPS34TOR::URA3, Δura3::imm434/Δura3::imm434</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pKE2</td>
<td>pUC18 containing a 4-9 kb C. albicans genomic fragment bearing VPS34</td>
<td>Bruckmann et al. (2000)</td>
</tr>
<tr>
<td>pKEU1</td>
<td>As pKE2, but containing URA3</td>
<td>Bruckmann et al. (2000)</td>
</tr>
<tr>
<td>pKEU1T</td>
<td>As pKEU1, but containing chimeric VPS34TOR</td>
<td>This work</td>
</tr>
<tr>
<td>pVPEX1</td>
<td>pQE9 containing 3-1 kb fragment with complete VPS34</td>
<td>Eck et al. (2000)</td>
</tr>
<tr>
<td>pVPEX2</td>
<td>As pVPEX1, but with KpnI restriction site</td>
<td>This work</td>
</tr>
<tr>
<td>pVTEX</td>
<td>As pVPEX2, but with chimera VPS34T</td>
<td>This work</td>
</tr>
</tbody>
</table>


Enzyme assays. PI 3-kinase assays were carried out essentially as described by Schu et al. (1993). Approximately 0.15 μg of recombinant protein was assayed in 50 μl reactions containing 20 mM HEPES, pH 7.5, 10 mM MgCl₂, 0.2 mg sonicated phosphatidylinositol ml⁻¹, 60 μM ATP and 0.1 μCi [γ-³²P]ATP ml⁻¹. The reactions were incubated at 30 °C for 15 min, then terminated by the addition of 1 M HCl and lipids were extracted with 160 μl chloroform/methanol (1:1). The organic phase was dried, samples were resuspended in chlorofrom and spotted onto Silica gel 60 TLC plates (Merck), and the plates were developed in a borate buffer system (Walsh et al., 1991). Labelled phosphoinositides were analysed using a phosphoshamager.

Autophosphorylation assays were carried out as described by Czupalla et al. (2003). Recombinant protein (200 ng) was assayed in 50 μl reactions containing 0.1 % BSA, 1 mM EGTA, 0.2 mM EDTA, 7 mM MgCl₂, 10 mM MnCl₂, 100 mM NaCl, 40 mM HEPES, pH 7.4, 1 mM DTT, 1 mM β-glycerophosphate, 25 μM ATP and 0.1 μCi [γ-³²P]ATP ml⁻¹. The reaction was stopped after incubation at 30 °C for 30 min by adding 17 μl 4× sample buffer according to Laemmli (1970). The proteins were separated on SDS-polyacrylamide gels, dried and autoradiographic signals were analysed using a phosphoshamager.

Integration of the chimeric VPS34TOR gene in C. albicans. The chimeric VPS34TOR gene was introduced into the vps34 null mutant strain CAV4 (ura⁻) using the 6.0-kb BbvI/HindIII insert of pKEUT (Fig. 2). This insert contains the chimeric VPS34TOR gene, and includes upstream and downstream sequences to allow homologous recombination, as well as the URA3 gene as a selectable marker. Transformations were performed by electroporation, as described by DeBacker et al. (1999). Chromosomal DNA isolated from selected clones was digested with HindIII/KpnI and analysed by Southern hybridization with an [γ-³²P]dCTP-labeled EcoRI/ HindIII 4.9-kb insert of plasmid pKE2 (Bruckmann et al., 2000).

Fluorescent labelling and microscopy. Labelling with FM4-64 [N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylethylamino)-pyridinium dibromide] (Molecular Probes) was performed as described by Vida & Emr (1995). Candida cells were grown at 30 °C to an OD₅₆₀ of 0.8 to 1.6. Cells were harvested and resuspended at OD₅₆₀ 20–40 in YPD medium. The dye FM4-64 (16 nM) was added, and the cells were incubated for 15 min at 30 °C, followed by incubation in YPD without dye at OD₅₆₀ 10–20 for 1 h. For microscopic analysis, cells were placed on slides covered with a thin 1 % agarose film. Samples were observed for fluorescence using a filter of 546 nm excitation and 575–640 nm emission (Zeiss).

Adherence assay. The ability of C. albicans strains SC5314, CAV3 and CAV9 to adhere to human buccal epithelial cells (HBEC) in vitro were examined by a visual assay (Bailey et al., 1995). The Candida strains were grown, washed and counted according to the method of Bailey et al. (1995). HBEC from a male volunteer were washed with 1× PBS (Invitrogen) and counted. A total of 10⁵ Candida cells were incubated with 10⁶ HBEC in 1× PBS at 37 °C for 2 h. The cells were then transferred to microscope slides. Adherence was expressed as adherence of HBEC with adhering Candida cells.

Virulence studies. Male outbred NMRI mice (Harlan-Winkelmann), 6 weeks old, were housed five per cage and checked daily. C. albicans cells were grown in Sabouraud dextrose broth at 28 °C until late-exponential phase. Cells were washed three times and resuspended in 0.9 % NaCl. Portions (200 μl) of suspensions containing 5×10⁶, 5×10⁷ and 5×10⁸ cells were used to infect immunocompetent mice by intravenous injection into the lateral tail vein. Survival was monitored for 21 days. To quantify kidney colonization by C. albicans, mice were sacrificed 3 days and 21 days after injection, and kidneys were homogenized in 3 ml physiological NaCl buffer. Serially diluted suspensions were plated on YPD agar. After 3 days growth at 28 °C, numbers of Candida colonies were counted. Homogenized kidney material was also fixed with 10 % formaldehyde and stained with 25 μg Calcofluor White ml⁻¹ to detect C. albicans cells.

RESULTS

Chimeric Vps34T protein lacks lipid kinase activity but retains autophosphorylation activity.

In order to distinguish effects regulated by lipid kinase from additional activities, such as autophosphorylation, of C. albicans Vps34p, a chimeric Vps34T protein containing a mutated phosphatidylinositol (PI) binding domain was generated. To this end, six amino acids of the putative PI-binding site of Vps34p were replaced by the homologous...
region of Torp of \textit{C. albicans}, a related protein without lipid kinase activity (Fig. 1a). The recombinant chimeric Vps34T protein was expressed in \textit{E. coli}, isolated by affinity chromatography and detected by Western blot (Fig. 1b).

It was necessary to determine the lipid kinase activity of chimeric Vps34Tp, and therefore the formation of PI 3-phosphate (PI 3-P) by Vps34Tp was tested. Phosphorylated lipids were separated by TLC and monitored using a phosphoimager. The chimeric Vps34T protein did not form PI 3-P \textit{in vitro}, in contrast to wild-type Vps34p (Fig. 1c).

The autophosphorylation activity of chimeric Vps34Tp was tested by assaying the incorporation of radioactive phosphate. Both Vps34Tp and the wild-type Vps34 protein incorporate $^{32}$P, and thus both proteins are able to autophosphorylate \textit{in vitro} (Fig. 1b). These results prove that the exchange of six amino acids within the putative PI-binding domain of Vps34p specifically inactivates the lipid kinase, but preserves the autophosphorylation activity.

\textbf{Generating a lipid–kinase-defective Vps34p-containing \textit{C. albicans} mutant strain}

In order to investigate the role of the lipid kinase activity of Vps34p and that of the other activities, such as autophosphorylation, separately \textit{in vivo}, a \textit{C. albicans} mutant strain containing the chimeric VPS34TOR gene was generated. To this end, a 6.0 kb \textit{BbvI/HindIII} fragment of pKEU1T containing the chimeric VPS34TOR gene lacking a functional lipid kinase domain was generated. This fragment was transformed into \textit{C. albicans} Vps34p lipid-kinase-defective strain CAV9. Southern analysis of the chromosome DNA showed proper integration of the VPS34TOR gene into the chromosomal DNA of strain CAV4. Lane 1, \textit{C. albicans} wild-type strain SC5314; lane 2, vps34 null mutant strain CAV4; lane 3, VPS34 heterozygous revertant strain CAV5; lane 4, CAV9 containing the lipid-kinase-defective VPS34TOR gene. The blot was hybridized with an $[\alpha-^{32}\text{P}]\text{dCTP}$-labelled EcoRI/HindIII 4.9 kb fragment of plasmid pKE2.

\textbf{The lipid kinase activity of Vps34p plays a role in the virulence of \textit{C. albicans}}

The growth of the lipid-kinase-defective mutant strain CAV9 was examined, as the growth rate is important for the virulence of \textit{C. albicans}. Both the CAV9 and CAV3 strains containing the VPS34TOR gene lacking a functional lipid kinase domain were examined. The growth of both strains was compared to that of the wild-type strain SC5314. The results showed that the growth rate of CAV9 was significantly lower than that of SC5314, indicating that the lipid kinase activity of Vps34p plays a role in the virulence of \textit{C. albicans}.
mutant strains showed identical delayed growth. However, the growth rates of the mutants were similar to the wild-type strain SC5314. The delayed growth of the mutants may partly contribute to their avirulence. However, reduced growth is not always connected to avirulence (Augsten et al., 2002).

In order to identify whether the lipid kinase activity of Vps34p is involved in virulence or not, we tested the lipid-kinase-defective strain CAV9 in a mouse model for systemic candidiasis. We found that mutant strain CAV9 was non-virulent in mice. All mice infected with the mutant strain survived for three weeks: even animals infected with a high number of Candida cells ($5 \times 10^6$). An identical survival rate has been shown for the $vps34$ null mutant strain CAV3 (Bruckmann et al., 2000). In contrast, all mice infected with the same cell number of the wild-type strain SC5314 died after 2 days, and 20% of the mice survived for three weeks after infection with $5 \times 10^5$ cells. Nearly the same mortality rate has been observed for mice infected with the VPS34 heterozygous revertant strain CAV5 (Bruckmann et al., 2000). The avirulence of both the lipid-kinase-defective Vps34p-containing mutant strain CAV9 and the $vps34$ null mutant strain CAV3 shows the central role of the lipid kinase in virulence (Fig. 3).

Systemic candidiasis is often associated with colonization of internal organs, such as the kidneys, lung and 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). We have examined kidney colonization of mice infected with $5 \times 10^5$ cells of strains SC5314, CAV3 and CAV9 three days post infection ($n=3$). Kidneys of mice bearing strain SC5314 exhibited a fungal burden of $1.78-3.23 \times 10^4$ c.f.u. per gram kidney tissue, whereas kidneys of mice infected with CAV3 or CAV9 showed $7.1 \times 10^3-3.1 \times 10^4$ c.f.u. and $3 \times 10^4-1.9 \times 10^5$ c.f.u., respectively. After 21 days, survivors of the virulence test were checked for kidney colonization. We examined kidney colonization of mice infected with $5 \times 10^4$ cells of strain SC5314 ($n=2$) and $5 \times 10^5$ of strains CAV3 and CAV9 ($n=3$). Kidneys of mice bearing strain SC5314 exhibited a fungal burden of $1.0-1.3 \times 10^6$ c.f.u. per gram kidney tissue, whereas kidneys of mice infected with CAV3 and CAV9 showed $1.1 \times 10^3-1.5 \times 10^4$ c.f.u. and $8.1 \times 10^3-2.2 \times 10^4$ c.f.u., respectively. In the kidneys of mice infected with the mutant strains, only pseudohyphae were detected. These results clearly showed reduced c.f.u. in the kidneys of mice bearing the mutant strains CAV9 and CAV3, which is in agreement with the non-virulence of these strains in the virulence test.

The low c.f.u. of kidneys infected with the $vps34$ null mutant and the Vps34p lipid-kinase-defective mutant may indicate a lower ability of the mutants to adhere to endothelial cells in vivo, resulting in rapid clearance from the blood. Thus, the abilities of SC5314, CAV3 and CAV9 to adhere to HBE in vitro were determined in a visual assay (Bailey et al., 1995).

![Fig. 3. Pathogenicity of C. albicans Vps34p lipid-kinase-defective strain CAV9. C. albicans Vps34p lipid-kinase-defective strain CAV9 and wild-type strain SC5314 were tested in a mouse model of systemic candidiasis. Survival of mice infected with $10^4$ (□), $10^5$ (■) and $10^6$ (●) cells was monitored for 21 days ($n=10$, except $5 \times 10^5$, where $n=15$).](image)

The proportion of HBE with adhering wild-type Candida cells was 85% ($n=3$). The proportion of HBE with CAV3 or CAV9 cells on the surface was lower, at 45 and 50%, respectively.

**Lipid kinase activity of CaVps34p is required for hyphal growth**

The switch between yeast and hyphae is considered important to the virulence of C. albicans. The Vps34p lipid-kinase-defective strain CAV9 was examined in liquid medium and on solid medium under different hyphae-inducing conditions in order to test the influence of Vps34p lipid kinase activity on hyphal growth.

First, hyphal growth was induced in liquid YPD medium supplemented with serum. Under these conditions, the mutant strain CAV9 showed delayed hyphal formation. Moreover, approximately 80% of the hyphae were pseudohyphae. The delayed hyphal formation was more pronounced in the $vps34$ null mutant strain CAV3 (Fig. 4a). Identical results were obtained in liquid Spider medium (data not shown).

Second, hyphal formation was examined on solid Spider...
medium containing mannitol as carbon source. The lipid-kinase-defective strain CAV9 did not show hyphal development. The wild-type strain SC5314 formed normal mycelial colonies, which are indicative of filamentous growth. Nearly the same phenotype was observed for colonies of the VPS34 revertant strain CAV5 (Bruckmann et al., 2000). In general, the mutant strain CAV3 also showed a defect in hyphal growth. However, the mutant strains showed differences in colony morphology. The mutant strain CAV9 formed smooth colonies and CAV3 mutant colonies exhibited a rough surface (Fig. 4b). The same differences were observed on solid YPD plates supplemented with serum (data not shown). Microscopic examination showed an identical yeast form morphology of CAV9 and CAV3 cells (data not shown).

The differences in hyphal growth observed between the lipid-kinase-defective strain CAV9 and the wild-type strain indicate that the lipid kinase activity of Vps34p regulates the hyphal formation of C. albicans.

**Lipid kinase activity of Vps34p is involved in transport of prevacuolar endocytic compartments to the vacuole and vacuole morphology**

In the yeasts S. cerevisiae and C. albicans, Vps34p is involved in the transport of prevacuolar vesicles to the vacuole during the overlapping late steps of endocytosis and the CPY (carboxypeptidase Y) protein transport pathway from the late Golgi to the vacuole (Wurmser & Emr, 1998; Bruckmann et al., 2001). This function may be analysed by following the distribution of the fluorescent lipophilic dye FM4-64. In yeast, this dye allows endocytic uptake and vesicle-mediated transport to the vacuole to be monitored (Vida & Emr, 1995). In order to investigate the influence of the lipid kinase activity of Vps34p in this process, the endocytic transport of the dye was analysed in the mutant strain CAV9, containing the lipid-kinase-defective Vps34T protein, and was compared to the distribution in the C. albicans wild-type strain and the vps34 null mutant strain CAV3 by fluorescence microscopy (Fig. 5). The mutant strain CAV9 showed weak fluorescent staining of the vacuole membrane, but prevacuolar endocytic compartments in the cytoplasm were stained. In the wild-type strain SC5314, the dye had reached the vacuole, and a typical ring staining pattern was observed. This difference indicates that the lack of lipid kinase activity in the mutant strain CAV9 results in defects in the transport/fusion of prevacuolar endocytic vesicles to the vacuole. Thus, the lipid kinase activity of Vps34p seems necessary for a late step in endocytic protein transport to the vacuole.

In addition, enlarged vacuoles were observed in the Vps34p lipid-kinase-defective strain CAV9. In contrast, the wild-type strain showed normal vacuoles. This difference indicates that the lipid kinase activity of Vps34p is involved in maintaining normal vacuole morphology. This was also shown by the identical enlarged vacuoles in both mutant strains (Fig. 5).

**Activities of Vps34p different from the lipid kinase function are involved in high-temperature and osmotic stress response**

The C. albicans vps34 null mutant strain CAV3 is more sensitive to high temperature and hyperosmotic stress than the wild-type strain SC5314 (Bruckmann et al., 2000). Therefore, the contribution of the lipid kinase activity of Vps34p to high-temperature and osmotic stress responses was determined. The growth of the C. albicans Vps34p lipid-kinase-defective strain CAV9, the wild-type strain SC5314, the VPS34 revertant strain CAV5 and the vps34 null mutant strain CAV3 was assayed on YPD plates at 30, 38 and 40°C. The osmotic sensitivity was examined on solid YPD medium containing NaCl or KCl at 1 M or 5 M. The mutant strain CAV9 showed a reduced stress resistance compared to the wild-type strain and the revertant strain CAV5. However, the vps34 null mutant strain CAV3 was clearly more sensitive to high-temperature and osmotic stress than
DISCUSSION

*C. albicans* is one of the major human pathogenic fungi. The phosphatidylinositol (PI) 3-kinase Vps34p was characterized as important for virulence in a mouse model for systemic candidiasis. Therefore, we were interested to assay which activity of Vps34p is necessary for virulence.

In general, manipulations of the lipid-binding domain of the PI 3-kinase Vps34 interfere with both lipid kinase and protein kinase activity (Wymann et al., 1996; Stack & Emr, 1994). In human cells, a lipid-kinase-defective PI 3-kinase c with remaining protein kinase function was constructed, but little is known about the specific role of each of the two enzymic functions *in vivo* (Bondeva et al., 1998).

Here, we describe the exchange of six amino acids from the putative PI-interacting domain of Vps34p with the homologous region of the PI 3-kinase-like Tor protein, resulting in the chimeric Vps34T protein. Probably, the exchange of the interaction domain blocks binding of the substrate PI to Vps34Tp, while autophosphorylation is not influenced. This is surprising, because the change of asparagine, localized eight amino acids upstream from the first amino acid of the exchanged region, causes the loss of autophosphorylation activity in Vps34p of *S. cerevisiae* (Stack & Emr, 1994).

The chimeric Vps34T protein lacking lipid kinase activity but retaining autophosphorylation activity allows the separate study of the role of the lipid kinase activity of the PI 3-kinase Vps34p. A *C. albicans* lipid-kinase-defective strain, CAV9, was generated, which has the chimeric Vps34TOR gene integrated. This gene encodes a protein which specifically lacks lipid kinase activity. Otherwise, it is identical to the wild-type Vps34p. Thus, the Vps34p lipid-kinase-defective strain CAV9 and the VPS34 revertant strain CAV5, which shows a phenotype nearly identical to that of the wild-type strain SC5314, differ in only six amino acids in the VPS34 gene; this causes the lack of Vps34Tp lipid kinase activity in the mutant strain CAV9, although the autophosphorylation activity is maintained. The differences in the phenotypes observed here between the mutant strain CAV9 and the CAV5 or wild-type strain indicate an important role for the lipid kinase activity of Vps34p, as most likely this activity was specifically eliminated.

The chimeric Vps34T protein lacking lipid kinase activity but retaining autophosphorylation activity allows the separate study of the role of the lipid kinase activity of the PI 3-kinase Vps34p. A *C. albicans* lipid-kinase-defective strain, CAV9, was generated, which has the chimeric Vps34TOR gene integrated. This gene encodes a protein which specifically lacks lipid kinase activity. Otherwise, it is identical to the wild-type Vps34p. Thus, the Vps34p lipid-kinase-defective strain CAV9 and the VPS34 revertant strain CAV5, which shows a phenotype nearly identical to that of the wild-type strain SC5314, differ in only six amino acids in the VPS34 gene; this causes the lack of Vps34Tp lipid kinase activity in the mutant strain CAV9, although the autophosphorylation activity is maintained. The differences in the phenotypes observed here between the mutant strain CAV9 and the CAV5 or wild-type strain indicate an important role for the lipid kinase activity of Vps34p, as most likely this activity was specifically eliminated. Therefore, the phenotypic differences suggest that lipid kinase activity plays a role in virulence, hyphal growth and adhesion, as well as in vacuole morphology and vesicular protein transport to the vacuoles. This conclusion is confirmed by several defective features of the vps34 null mutant strain CAV3 which are identical to the phenotypes observed for the mutant strain CAV9. These phenotypes are likely connected to the lipid kinase activity of Vps34p, because both strains lack this activity.

However, minor differences exist between the Vps34p lipid-kinase-defective strain CAV9 and the vps34 null mutant strain CAV3 regarding hyphal formation. The delay of hyphal development of the mutant strains CAV9 and CAV3 may be connected to delayed growth. However, the delay of hyphal growth is lower for CAV9 than for CAV3. In addition, although both mutant strains do not form hyphae on hyphae-inducing solid agar, their colony morphology differs clearly. As both CAV9 and CAV3 are avirulent in a mouse model of systemic candidiasis, the differences in hyphal growth and colony morphology do not seem to be important for virulence.

The different phenotypes observed between the Vps34p lipid-kinase-defective strain CAV9 and the vps34 null mutant strain CAV3 are indicative of additional activities for the Vps34p protein *in vivo* because the lipid kinase activity...
was absent in both CAV9 and CAV3. These phenotypes are connected to stress response.

The generation of Vps34Tp, which lacks lipid kinase but retains autophosphorylation activity, allowed the assay of the role of lipid kinase activity in vivo. Moreover, the functions of lipid-kinase-independent activities in the stress response were shown. These results indicate that the lipid kinase activity of Vps34p plays a role in virulence, and will help to identify the proper signalling pathways required for virulence.

ACKNOWLEDGEMENTS

The authors thank B. Frais, U. Stöckel and B. Weber for technical assistance in the virulence test. This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES


Fig. 6. Stress sensitivity of C. albicans Vps34p lipid-kinase-defective strain CAV9. The growth of lipid-kinase-defective strain CAV9 was compared with that of the wild-type strain SC5314, the VPS34 heterozygous revertant strain CAV5, as well as vps34 null mutant strain CAV3, on YPD plates at 30, 38 and 40 °C and on plates incubated at 30 °C containing NaCl or KCl (1·2 M or 1·5 M).


