The vesicle transport protein Vac1p is required for virulence of *Candida albicans*

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The putative vesicle transport protein Vac1p of the human pathogenic yeast *Candida albicans* plays an important role in virulence. To determine the cellular functions of Vac1p, a null mutant was generated by sequential disruption of both alleles. The *vac1* null mutant strain showed defective endosomal vesicle transport, demonstrating a role of Vac1p in protein transport to the vacuole. Vac1p also contributes to resistance to metal ions, as the null mutant strain was hypersensitive to Cu^{2+} , Zn^{2+} and Ni^{2+} . In addition, the loss of Vac1p affected several virulence factors of *C. albicans*. In particular, the *vac1* null mutant strain showed defective hyphal growth, even when hyphal formation was induced via different pathways. Furthermore, Vac1p affects chlamydospore formation, adherence to human vaginal epithelial cells, and the secretion of aspartyl proteinases (Saps). Avirulence in a mouse model of systemic infection of the *vac1* null mutant strongly suggests that Vac1p of *C. albicans* is essential for pathogenicity. In summary, the Vac1p protein is required for several cellular pathways, in particular those that control virulence and pathogenicity. Consequently, Vac1p is a novel and interesting target for antifungal drugs.

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INTRODUCTION

The dimorphic yeast *Candida albicans* causes life-threatening infections, particularly in immunocompromised patients, as well as a variety of mucosal infections in healthy individuals (Odds, 1994). Present evidence suggests that several factors control virulence of *C. albicans*, including the ability to switch between different morphogenetic forms, adherence to host epithelial and endothelial cells, and secretion of proteinases and phospholipases (Cutler, 1991; Köhler & Fink, 1996; Lo *et al.*, 1997; Odds, 1994). So far, a number of virulence factors of *C. albicans* has been characterized. However, the mechanism that enables this opportunistic fungus to become pathogenic has not been unravelled yet. Several classical virulence factors have been identified so far; however, it seems relevant to analyse virulence-determining signalling pathways as well. These define the molecular steps during switching from the non-pathogenic commensal form to the life-threatening pathogenic form. Several signalling pathways have been

linked to virulence, including hyphal induction pathways (Ernst, 2000a, b), and the vacuolar transport pathways (Bruckmann *et al.*, 2000, 2001; Eck *et al.*, 2000; Palmer *et al.*, 2003).

The phosphatidylinositol (PI) 3-kinase Vps34p of *C. albicans* is a key enzyme of the vacuolar protein transport, and is required for virulence. A *vps34* null mutant strain shows several defects related to virulence, including avirulence in a mouse model of systemic candidiasis, inability to form hyphae on different solid media, delayed yeast-to-hyphae transition in liquid media, hyperfilamentation under microaerophilic/embedded conditions, hypersensitivity to high temperature and hyperosmotic stress, and reduced adherence to human cells (Bruckmann *et al.*, 2000; Eck *et al.*, 2000). In addition, the *vps34* null mutant displays enlarged and electron-transparent vacuoles (Bruckmann *et al.*, 2001). The Vps34p protein of *C. albicans* interacts with Vma7p, a component of the *Candida* V-ATPase complex (Eck *et al.*, 2005). *vma7* null mutants are also avirulent (Poltermann *et al.*, 2005). Moreover, the secretion of aspartyl proteinases (Saps) and the resistance to toxic metal ions and fungistatic compounds are decreased in the *vps34* null mutant strain (Kitanovic *et al.*, 2005). The Vps34p

Abbreviations: CPY, carboxypeptidase Y; 5-FOA, 5-fluoroorotic acid; PI, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; Sap, secreted aspartyl proteinase.

protein exhibits lipid kinase and autophosphorylation activity. The characterization of a specific Vps34p lipid kinase defective mutant strain showed that the lipid kinase activity is essential for the role of Vps34p in pathogenicity (Günther *et al.*, 2005). Thus, lipid substrates of Vps34p, such as phosphatidylinositol 3-phosphate [PtdIns(3)P], are candidates to connect Vps34p signalling and virulence. Therefore, we speculated that the PtdIns(3)P-binding proteins Fab1p and Vac1p play a role in virulence.

PtdIns(3)P-binding proteins were identified in the yeast *Saccharomyces cerevisiae* and in mammalian cells. They contain a conserved FYFE finger domain named after the first four identified FYFE finger proteins Fab1p, YOTB, Vps27p and EEA1 (human homologous protein of Vac1p) (Burd & Emr, 1998; Stenmark & Aasland, 1999). In *S. cerevisiae* the binding protein Fab1p functions downstream of Vps34p as a PI(3) 5-kinase, which regulates vacuolar membrane turnover through the production of PdtIns(3,5)P₂ (Gary *et al.*, 1998). The PtdIns(3)P-binding protein Vac1p functions in Golgi-to-endosome vesicular protein transport (Weisman & Wicker, 1992). Because the lipid kinase function of Vps34p is essential for virulence of *C. albicans*, and the PdtIns(3)P-binding protein Fab1p is not involved in virulence, we focused on the functions of another PdtIns(3)P-binding protein, namely Vac1p (Augsten *et al.*, 2002; Günther *et al.*, 2005).

In *S. cerevisiae* Vac1p is a peripheral membrane protein, which belongs to the class D *vps* group, and plays a role in vesicle docking at the late endosome (multivesicular body). Mutations in the FYFE domain of the Vac1p protein result in mis-sorting of vacuolar proteins and temperature-sensitive growth (Burd *et al.*, 1997). Vac1p is an essential component of the endosomal SNARE (SNAP receptor) complex, which is responsible for the interaction and fusion of Golgi-derived vesicles with the late endosome (Burd *et al.*, 1997; Webb *et al.*, 1997). Furthermore, Vac1p links the Vps34p-signalling pathway to the fusion of transport vesicles with the endosome (Burd & Emr, 1998). This step is regulated by direct interaction with Rab GTPase signalling

protein Vps21p; the fusion is mediated by Pep12p and Vps45p (Horazdovsky *et al.*, 1994; Peterson *et al.*, 1999; Peterson & Emr, 2001; Tall *et al.*, 1999). In addition, the class C *vps* proteins Vps18p and Vps11p interact with Vac1p (Peterson & Emr, 2001; Srivastava *et al.*, 2000).

In this paper we show that Vac1p of *C. albicans* contributes to virulence, and that a *vac1* null mutant is avirulent. In addition, Vac1p affects several important virulence parameters, thus demonstrating that in *C. albicans* this PdtIns(3)P-binding protein acts as a central component of the virulence-determining Vps34p pathway.

METHODS

Strains and growth conditions. The *C. albicans* strains used in this study are listed in Table 1. Strains were grown in YPD medium [2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract], YP-sucrose medium [2% (w/v) sucrose, 2% (w/v) peptone, 1% (w/v) yeast extract], SD medium [0.7% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose, 1 M sorbitol], YNB medium (SD without 1 M sorbitol) or Sabouraud medium [2% (w/v) glucose, 1% (w/v) peptone (casein)] at 28 °C. SD and YNB were supplemented with 20 µg uridine ml⁻¹ for the growth of Ura⁻ strains. Uridine auxotrophs were selected on YNB plates containing uridine and 1 mg 5-fluoroorotic acid (5-FOA) ml⁻¹ (Sigma). Growth was monitored by measuring optical density at 600 nm in a SpectraMax 190 spectrophotometer. To induce hyphal growth on solid medium, cells were grown overnight in YPD at 30 °C, then 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 to 100 cells per plate. Plates were incubated at 37 °C for at least 7 days. Hyphal growth in liquid media was induced by diluting late exponential-phase cultures grown at 30 °C tenfold 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. *Escherichia coli* XL-1 Blue {*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F'*[*proAB⁺ lacI^f* Δ*M15 Tn10(tet^r)*]} (Stratagene) was used for cloning.

Construction of plasmids pV0, pV1 and pUCVU. The 5' region of *VAC1* was amplified by PCR using chromosomal DNA of *C.*

Table 1. Strains and plasmids

Strain or plasmid	Genotype or description	Reference/source
<i>C. albicans</i>		
SC5314	Wild-type	Fonzi & Irwin (1993)
CAI-4	Δ <i>ura3::imm434/Δura3::imm434</i> , isogenic to SC5314	Fonzi & Irwin (1993)
CV1	Δ <i>vac1::hisG-URA3-hisG/VAC1Δura3::imm434/Δura3::imm434</i>	This work
CV2	Δ <i>vac1::hisG/VAC1Δura3::imm434/Δura3::imm434</i>	This work
CV3	Δ <i>vac1::hisG/Δvac1::hisG-URA3 hisGΔura3::imm434/Δura3::imm434</i>	This work
CV4	Δ <i>vac1::hisG/Δvac1::hisGΔura3::imm434/Δura3::imm434</i>	This work
CV5	Δ <i>vac1::hisG/VAC1::URA3Δura3::imm434/Δura3::imm434</i>	This work
Plasmids		
pUC19	<i>Escherichia coli</i> cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pMB7	<i>C. albicans</i> gene disruption vector	Fonzi & Irwin (1993)

albicans SC5314 as a template and primers 1 and 2. Primer 1: 5'-GCGAGCTCGACGATATAACGGTTGTGTTG-3' (the underlined sequence is complementary to the genomic sequence, positions -144 to -124, upstream of *VAC1*); primer 2: 5'-GCGGTACCTGTCGTGTCACAAAGGGTGC-3' (positions +166 to +146). The resulting 325 bp PCR product was *SacI/KpnI*-digested and cloned into the disruption vector pMB7, yielding pV0 (Fonzi & Irwin, 1993). Primers 3 and 4 were used to amplify the 3' region. Primer 3: 5'-ACGCGTCGACCAAAGCGAGACGGTTTGATG-3' (positions +1232 to +1252); primer 4: 5'-GCCTGCAGCAAGCAGAACAAGTTATGACAGC-3' (positions -178 to -156; downstream of *VAC1*) yielded a 311 bp product, which was digested with *SalI* and *PstI* and

cloned into plasmid pV0, resulting in pV1 (Fig. 1a). For gene disruption, plasmid pV1 was cut with *SacI* and *PstI*. The digested plasmid pV1 was transformed to *C. albicans*. For homologous reintegration of the *VAC1* gene, *VAC1* was amplified by PCR with primer 5 [5'-GCGAGCTCTGAGAGTAATCCAAAGCAGTTAC-3' (-695 to -672, upstream of *VAC1*)] and primer 6 [5'-GCGGTAC-CACCCATGCGTGTCCAACAAC-3' (-905 to -884, downstream of *VAC1*)]. The 2961 bp PCR product was restricted with *SacI/KpnI* and cloned into pUC19 (Yanisch-Perron *et al.*, 1985), resulting in pUCV. The *URA3* gene was amplified with primer 7 [5'-ACGCCCCAAGGAATTGATTGGATGGTATA-3' (-411 to -393, upstream of *URA3*)] and primer 8 [5'-ACGCCCTTGGACCACCT-TTGATTGTAAATA-3' (-120 to -101, downstream of *URA3*)]. The 1364 bp PCR product was restricted with *StyI* and cloned into the *StyI* site (positions -244 to -249, upstream of *VAC1*) of pUCV, resulting in pUCVU. After digestion of pUCVU with *SacI* and *KpnI* a 4.3 kb insert was isolated that harbours the *VAC1* gene, upstream as well as downstream regions for homologous recombination, and the *URA3* gene as selectable marker.

Transformation of *C. albicans* and selection of *Ura*⁻ auxotrophs. The methods were carried out according to the procedures described previously (Boeke *et al.*, 1984; Eck *et al.*, 1997).

Disruption and reintegration of *VAC1*. To disrupt the *VAC1* gene, the previously described *hisG-URA3-hisG* cassette was used in a multistep procedure (Fonzi & Irwin, 1993). A 4.6 kb *SacI-PstI* fragment of pV1 containing the *URA*-blaster flanked by short sequences from the upstream and downstream sequences of *VAC1* and portions of the promoter and terminator, respectively, was used to transform *C. albicans* *Ura*⁻ strain CAI-4. To confirm proper deletion of the *VAC1* ORF (1338 bp, 445 aa, Stanford's *Candida albicans* sequencing project Assembly 19, ORF19.5662 on Contig231; <http://sequence-www.stanford.edu/group/candida/index.html>), the resulting *Ura*⁺ transformants were examined by Southern analysis using *HindIII/PstI*-digested chromosomal DNA and a labelled 300 bp *SacI-KpnI* fragment of pV1 as probe. Southern hybridization was also applied to evaluate segregants and transformants of the later disruption steps, according to standard protocols. In the first step one *VAC1* allele was replaced by the *hisG-URA3-hisG* cassette (CV1). Strain CV1 was plated on 5-FOA-containing medium for

Fig. 1. Disruption and reintegration of *VAC1* gene in *C. albicans*. (a) Restriction map of the plasmid pV1, illustrating the strategy for disruption of *VAC1*. The 5' and 3' regions of *VAC1* were obtained by PCR, and cloned in front or behind of the *hisG-URA3-hisG* cassette (4.1 kb, thick open boxes) in the disruption vector pMB-7. The *VAC1* gene of *C. albicans* CAI-4 or CV2 was disrupted by integration/recombination of the *SacI-PstI* fragment of pV1 into the chromosome. Vertical black lines show the location of restriction sites in (a) and (b). (b) Restriction map of the plasmid pUCVU illustrating the strategy for reintegration of *VAC1*. A 1.3 kb *HindIII* fragment containing the *URA3* gene (thick open box) was cloned behind *VAC1*. A *SacI-KpnI* fragment containing *VAC1* and *URA3* integrates into original locus of *VAC1* in CV4. (c) Southern analysis of *HindIII/PstI*-digested chromosomal DNA from the following *C. albicans* strains: parental wild-type strain CAI-4 (lane 1), heterozygous mutant strain CV1 (*VAC1/vac1::hisG-URA3-hisG*) (lane 2), heterozygous mutant strain CV2 (*VAC1/vac1::hisG*) (lane 3), null mutant strain CV3 (*vac1::hisG-URA3-hisG/vac1::hisG*) (lane 4), null mutant strain CV4 (*vac1::hisG/vac1::hisG*) (lane 5), and revertant strain CV5 (*vac1::hisG/VAC1-URA3*) (lane 6). The blot was hybridized with non-radioactive labelled *SacI-KpnI* insert of plasmid pV1 (a).

isolation of Ura⁻ segregants (CV2). A second transformation with the same disruption construct led to the isolation of a *vac1* null mutant strain (CV3). Again, Ura⁻ segregants were selected (CV4).

VAC1 was reintroduced into the null mutant strain CV4 by transformation with the 4.3 kb *SacI/KpnI* insert of pUCVU. Chromosomal DNA from selected clones was digested with *HindIII/PstI* and analysed by Southern blotting and hybridization (CV5).

Fluorescent labelling with FM4-64 and microscopy. Yeast cells were stained with the dye FM4-64 as described previously (Günther *et al.*, 2005; Vida & Emr, 1995). For microscopy, cells were placed on slides that were covered with agarose. Transport of FM4-64 was visualized microscopically using a BP510-550 excitation filter (U-MWG2), BA590 beam splitter and DM570 emission filter (Olympus BX51TF).

Hyphal induction within agar matrix. For filamentous growth within an agar matrix the various *C. albicans* strains were grown overnight in YPD, diluted to a concentration of 5×10^6 cells ml⁻¹, and cultivated for 4 h at 30 °C. Then 100 µl of the diluted cells (10^3 cells ml⁻¹) was mixed in YP-sucrose agar (YP-sucrose medium supplemented with 2% agar), plated, and incubated for 120 h at 22 °C. Colonies were examined microscopically, and the percentage of filamentous colonies was determined.

Hyphal induction by growth at neutral pH. *C. albicans* cells (100 µl of an overnight culture) were added to 20 ml acidic Soll's medium (Swoboda *et al.*, 1994), pH 4.5, and incubated for 12 h at 30 °C. Then 20 ml neutral Soll's medium, pH 6.5, was supplemented with 500 µl from the acidic culture. The hyphal growing cells were counted using a microscope.

Chlamydospore formation. Rice agar (2%, w/v, agar; 40 g rice per litre H₂O) was melted and after a short centrifugation the agar was plated onto a slide. *C. albicans* cells from an overnight culture were streaked onto this agar and covered with a coverslip. The slides were incubated in the dark at 25 °C in a damp environment. Chlamydospore formation was evaluated microscopically after 96 h. For growth in liquid medium the rice agar was diluted 1:1 (v/v) with water.

Adherence assays. *C. albicans* cells obtained from an overnight YPD culture at 30 °C were diluted into 20 ml fresh YPD medium (pH 6.0) to a concentration of 5×10^5 cells ml⁻¹. Following incubation for 16 h at 30 °C, the cells were washed and diluted in YPD medium, pH 4.5, to a concentration of 2×10^7 cells ml⁻¹. Human vaginal SK-LMS-1 cells (leiomyosarcoma) were cultivated in microtest plates in RPMI medium (Serva) to a concentration of 10^4 cells per well. The tissue culture cells were washed with PBS (140 mM NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2), and then 4×10^6 *C. albicans* cells were added to each well. After cultivation at 37 °C for 2 h, *C. albicans* cells were stained with Calcofluor white (12.5 µg ml⁻¹; Sigma) for 30 min, and non-adherent *C. albicans* cells were removed by washing with PBS. Finally, the fraction of adherent fluorescent yeast cells was determined in a fluorescence reader, using 360 nm for excitation and 460 nm for emission (FluoroScan, Labsystems). The wild-type *C. albicans* strain SC5314 was set to 100%. For statistical analysis Student's *t*-test was used. A *P* value ≤ 0.025 was considered significant.

Sap ELISA assay. This assay was carried out essentially as described previously (Kitanovic *et al.*, 2005). The primary mouse antibody (FX 7-10, which preferentially detects Sap2p but also other Saps) was kindly provided by Dr Borg-von Zeppelin, Göttingen, Germany.

Virulence studies. Male outbred NMRI mice (Harlan-Winkelmann), 6 weeks old, were housed at five per cage and

checked daily. The various strains of *C. albicans* were grown in Sabouraud dextrose [glucose] broth at 28 °C until late exponential phase. Cells were washed three times and resuspended in 0.9% NaCl. Samples (200 µl) of suspension containing 5×10^6 cells were used to infect immunocompetent mice by intravenous injection into the lateral tail vein. Survival of the animals was monitored for 20 days. For comparison of survival curves the log-rank test was used (Peto *et al.*, 1977). A *P* value ≤ 0.05 was considered significant.

RESULTS

Disruption and reintroduction of *VAC1*

A *vac1* null mutant strain of *C. albicans* CAI-4 was generated in order to characterize the function of Vac1p as a potential vacuolar vesicle transport protein. The 4.6 kb *SacI-PstI* fragment of *VAC1* contained in plasmid pV1 (Fig. 1a) was used to transform the *C. albicans* Ura⁻ strain CAI-4. Southern blot analysis of *C. albicans* DNA from a heterozygous *vac1* mutant (CV1) identified two bands (Fig. 1c). The 2.7 kb fragment represents the *VAC1* wild-type allele (Fig. 1c, lane 1), while the 5.7 kb fragment can be identified as the *hisG-URA3-hisG* cassette that replaced the *VAC1* gene (lane 2). The excision of one copy of *hisG* and the *URA3* gene in the Ura⁻ derivatives results in the 2.9 kb band (CV2, lane 3). A second transformation with the same disruption construct generated *vac1* null mutant strain CV3. The loss of the 2.7 kb wild-type fragment and the presence of 5.7 kb and 2.9 kb bands are consistent with the replacement of the second *VAC1* allele (lane 4). Southern blot analysis of a representative Ura⁻ segregant identified a 2.9 kb band that represents two *hisG* disrupted alleles (CV4, lane 5). For reintegration of *VAC1*, the 4.3 kb *SacI-KpnI* fragment of plasmid pUCVU (*VAC1* and *URA3*) was used to transform strain CV4. Ura⁺ clones were selected, and proper introduction of *VAC1* and *URA3* was recognized by the appearance of a 3.6 kb *PstI-HindIII* fragment, which represents the integrated *VAC1* and *URA3* genes (CV5, lane 6).

Vac1p is required for the transport and fusion of prevacuolar endocytic compartments

Vps34p is required for the transport of prevacuolar vesicles to the vacuole in both *C. albicans* and *S. cerevisiae*. This function plays a role in endocytosis and in the CPY (carboxypeptidase Y) protein transport pathway from the late Golgi to the vacuole (Bruckmann *et al.*, 2001; Wurmser & Emr, 1998). This action can be analysed by following the distribution of the fluorescent lipophilic dye FM4-64, as it is transported in yeast cells via the endocytotic uptake and vesicle-mediated transport to the vacuole (Vida & Emr, 1995). The endocytic transport of the lipophilic dye was analysed in the *vac1* null mutant strain CV3 by fluorescence microscopy (Fig. 2). The null mutant strain showed weak staining of the vacuolar membrane, whereas prevacuolar endocytic compartments in the cytoplasm were stained. The heterozygous mutant strain CV1 and the revertant strain CV5 showed similar staining to the wild-type strain SC5314, where the dye is contained in the vacuolar membrane and

Fig. 2. Characterization of the endocytic protein transport pathway by following the distribution of the lipophilic dye FM4-64 in *C. albicans* wild-type strain SC5314, heterozygous mutant strain CV1, *vac1* null mutant strain CV3, and *VAC1* revertant strain CV5. Cells were pulsed for 30 min with the fluorescent endocytic dye FM4-64 and then chased for 60 min. Fluorescence microscopy and differential interference contrast (DIC) microscopy show that the lyophilic dye FM4-64 accumulated in mutant strain CV3 in presumptive prevacuolar compartments (arrows) in the cytoplasm. The wild-type strain SC5314, the heterozygous mutant strain CV1 and the revertant strain CV5 show typical ring staining pattern of the vacuole membrane. v, vacuoles; pvc, prevacuolar compartments. Bar, 10 μ m (applies to all photographs).

shows a typical ring staining pattern. Therefore we conclude that deletion of *VAC1* in the *C. albicans vac1* null mutant strain results in defective transport/fusion functionality of the prevacuolar endocytic vesicle.

Role of Vac1p in metal ion homeostasis

Metal ion homeostasis is affected in the *vps34* null mutant. Therefore, we investigated the influence of metal ions on the growth of the *C. albicans vac1* null mutant strain, which would indicate a role of Vac1p in metal ion homeostasis. *C. albicans* strains were grown on solid medium supplemented with Cu^{2+} , Zn^{2+} or Ni^{2+} . The *vac1* null mutant strain CV3 showed inhibited growth in the presence of CuCl_2 at a concentration of 8 mM, ZnCl_2 at 15 mM, and

NiSO_4 at 4 mM. The heterozygous mutant strain CV1 and the revertant strain CV5 were similar in growth to the wild-type strain SC5314. The *vac1* null mutant showed increased sensitivity to high concentrations of the tested metal ions, therefore demonstrating a role of Vac1p in resistance to metal ions (Fig. 3).

Vac1p influences dimorphic growth of *C. albicans*

Dimorphism is considered an important virulence factor of *C. albicans* (Lo *et al.*, 1997). Filamentous growth of *C. albicans* is induced by several signalling pathways that are triggered by several environmental signals, and which are activated experimentally by Spider medium supplemented with mannitol as a carbon source, serum, microaerophilic conditions, growth in a solid agar matrix, and pH-shift. These various methods were used to induce hyphal growth of the *vac1* null mutant strain CV3. When solid Spider medium was used for hyphal induction, the colonies of the

Fig. 3. The *C. albicans vac1* null mutant shows increased sensitivity to metal ions. The growth of wild-type strain SC5314, heterozygous mutant strain CV1 ($\Delta vac1/VAC1$), *vac1* null mutant strain CV3 ($\Delta vac1/\Delta vac1$), and *VAC1* revertant strain CV5 ($\Delta vac1/VAC1-URA3$) in the presence of CuCl_2 , ZnCl_2 and NiSO_4 is shown.

Fig. 4. Vac1p is involved in hyphal development. Hyphal growth of *C. albicans* wild-type strain SC5314, heterozygous mutant strain CV1, *vac1* null mutant strain CV3, and *VAC1* revertant strain CV5 using solid Spider medium containing mannitol as carbon source (a), solid medium supplemented with 20% serum (b), under microaerophilic/embedded conditions in an agar matrix (c), and after shift of pH from 4.5 to 6.5 (d).

vac1 null mutant strain did not form mycelial structures. However, the colonies of the heterozygous mutant strain CV1 and the revertant strain CV5 were similar to the

wild-type strain SC5314, which showed mycelial structures indicating hyphal growth (Fig. 4a). The hyphal cells in the colonies were counted microscopically. The colonies of the

null mutant strain contained nearly 100 % yeast cells. Those of SC5314, CV1 and CV5 contained about 95 % true hyphae.

Defective hyphal growth of the *vac1* null mutant strain CV3 was also observed on solid medium supplemented with serum. Under these conditions the colonies do not form mycelial structures, but show a rough surface (Fig. 4b). The different cell types that form these colonies were counted. About 20 % of the null mutant cells formed true hyphae, 60 % pseudohyphae, and 20 % yeast cells (data not shown). Again, colonies of the wild-type strain and strains CV1 and CV5 showed about 95 % true hyphae.

In liquid Spider medium as well as liquid medium supplemented with serum, hyphal growth of the *C. albicans vac1* null mutant strain was induced and the time-course of hyphal development did not show significant differences from the wild-type strain (data not shown).

Hyphal development is also induced by microaerophilic conditions in an agar matrix. Following growth in such a matrix for 120 h, the *vac1* null mutant strain CV3 showed hyperfilamentation (Fig. 4c): 100 % mycelium-forming colonies were observed. The wild-type strain SC5314 showed 12 %, the heterozygous mutant strain CV1 8 %, and the revertant strain CV5 6 % mycelium-forming colonies (Fig. 4c).

In addition, a shift in pH was used for hyphal induction. Under these conditions the *vac1* null mutant strain CV3 showed delayed hyphal induction. After 2 h the null mutant strain showed 25 % true hyphae, as compared to 85 % for the wild-type strain. After 3 h the percentages of hyphae for the *vac1* null mutant strain (81 %) and wild-type strain (95 %) were similar (Fig. 4d).

The dimorphic growth was restored in all tested conditions by reintroducing one *VAC1* allele into the *vac1* null mutant strain (CV5, Fig. 4a–d). This reconstitution clearly confirms that the defective phenotypes of the *vac1* null mutant strain CV3 are associated with deletion of *VAC1*. The nearly identical phenotype of the wild-type strain, the heterozygous mutant strain and the revertant strain indicates that the different position of the *URA3* gene and a possible reduction of transcription of *URA3* gene does not affect the phenotypes of the null mutant strain, because the position of

the *URA3* gene in the null mutant strain is identical to the position in the heterozygous strain.

Vac1p contributes to chlamydospore formation in *C. albicans*

The affected hyphal growth of the *vac1* null mutant strain inspired us to investigate the potential involvement of Vac1p in another morphogenetic switch in *C. albicans*. Nutrient-limited conditions induce the formation of thick-walled cells, called chlamydospores (Odds, 1988); these form at the end of elongated suspensor cells that are attached to hyphae. Growth of chlamydospores is induced in the dark under nutrient-deprived oxygen-limited conditions at low temperature on rice agar. In addition, chlamydospore induction occurs upon blockage of hyphae formation (Torosantucci & Cassone, 1983; Nobile *et al.*, 2003). The *vac1* null mutant CV3 was completely unable to form either chlamydospores or hyphae on rice agar (Fig. 5). These morphogenetic defects were also observed in liquid rice agar. The heterozygous mutant strain CV1 was similar to the wild-type strain SC5314 in chlamydospore formation, and the ability to form chlamydospores was restored by introduction of the *VAC1* gene into the mutant strain (CV5).

Adhesion to epithelial human cells and secretion of aspartyl proteinases are decreased in the *vac1* null mutant

Adhesion of *C. albicans* to host epithelial and endothelial cells is considered a prerequisite for virulence and commensal existence. To investigate an involvement of Vac1p in adhesion, binding of the *vac1* null mutant strain CV3 to human vaginal epithelial cells was tested. The *vac1* null mutant strain showed reduced adhesion ($38 \pm 12 \%$) compared to the wild-type strain SC5314 ($100 \pm 19 \%$) (Fig. 6a). This attenuation indicates a role of Vac1p in yeast adhesion to host cells. The adhesion of the heterozygous mutant strain CV1 ($90 \pm 25 \%$) and the revertant strain CV5 ($80 \pm 16 \%$) did not show significant differences from the wild-type strain (Fig. 6a).

Secretion of aspartyl proteinases (Saps) is considered relevant for virulence (Naglik *et al.*, 2003; Schaller *et al.*, 2003). Therefore, secretion of Saps, mainly Sap2p, was

Fig. 5. Chlamydospore formation is defective in the *vac1* null mutant strain. Chlamydospore formation in wild-type strain SC5314, heterozygous mutant strain CV1, *vac1* null mutant strain CV3 and *VAC1* revertant strain CV5 is shown.

Fig. 6. The *C. albicans vac1* null mutant shows affected adhesion and Sap secretion. (a) Adhesion of *C. albicans vac1* mutant strains on vaginal cell line SK-LMS-1 detected by a microtest plate fluorescence assay. Adhesion values were determined after 2 h co-incubation of *C. albicans* and epithelial cells by Calcofluor white staining and fluorescence measurement. For comparison, adhesion of wild-type strain SC5314 on SK-LMS-1 cells was set to 100% in each experiment. For the wild-type strain and *vac1* null mutant strain CV3, the bars represent the means $\pm$ SD of 60 separate readings in three experiments; for CV1 and CV5, the bars represent the means $\pm$ SD for 18 separate readings in one experiment. The difference between wild-type strain SC5314 and strain CV3 was considered significant by Student's *t*-test ($P < 0.025$). No significant difference was found between SC5314 and strain CV1 or strain CV5. (b) Sap secretion of wild-type strain SC5314, heterozygous mutant strain CV1, *vac1* null mutant strain CV3, and heterozygous revertant strain CV5 under Sap-inducing conditions, examined by an ELISA assay. One representative experiment out of two. The error bars represent SD.

analysed in the *vac1* null mutant strain CV3. Sap secretion was reduced in this strain, whereas the heterozygous mutant strain CV1 and the revertant strain CV5 showed similar secretion to the wild-type strain SC5314 (Fig. 6b). These results suggest that Vac1p affects the secretion of Sap proteinases, probably by regulating their vesicular transport and/or exocytosis.

Vac1p is required for virulence of *C. albicans* in a mouse model of systemic candidiasis

Three factors that play an important role in pathogenesis of *C. albicans* are affected in the *vac1* null mutant strain. The virulence of the *vac1* mutant strains CV1, CV3 and CV5 was therefore assayed in a mouse model of systemic candidiasis. The *vac1* null mutant strain CV3 was avirulent: mice infected with 5×10^6 CV3 cells survived during the complete course of the experiment (Fig. 7a). In contrast, all mice infected with the wild-type strain SC5315 died after 8 days. The heterozygous mutant strain CV1 and the revertant strain CV5 showed virulence comparable to that of the wild-type strain (Fig. 7a).

To rule out that the defective virulence is due to a growth defect, growth of the null mutant strain was tested under various conditions. In Sabouraud medium the *vac1* null mutant strain and the wild-type strain SC5314 showed

Fig. 7. The avirulence of the *vac1* null mutant is linked to defective virulence factors and not to reduced growth. (a) Vac1p is involved in pathogenicity of *C. albicans*. Wild-type strain SC5314, heterozygous mutant strain CV1, *vac1* null mutant strain CV3 and *VAC1* revertant strain CV5 were tested in a mouse model of systemic candidiasis. Survival of mice infected with 5×10^6 cells was monitored for 20 days ($n = 10$). A *P* value of < 0.05 was considered as significant (Peto *et al.*, 1977). (b) Wild-type strain SC5314, heterozygous mutant strain CV1, null mutant strain CV3 and *VAC1* revertant strain CV5 show equal growth in Sabouraud medium.

identical growth rates (Fig. 7b). Similarly, identical growth was observed in YPD medium (data not shown).

DISCUSSION

In this study we characterized the vacuolar transport protein Vac1p of *C. albicans* as being involved in protein transport, metal ion resistance, hyphal growth, chlamydospore formation, adhesion, secretion of Saps, and virulence. Vac1p is clearly involved in a virulence-determining pathway, and therefore represents a useful target for the screening of antifungal drugs.

The lipid kinase, but not the protein kinase activity of the multifunctional vacuolar transport protein Vps34p affects virulence of *C. albicans* (Günther *et al.*, 2005). In order to elucidate the signalling pathway that mediates virulence, we analysed whether proteins with high homology to PtdIns(3)P-binding proteins of *S. cerevisiae* like Fab1p and Vac1p are associated with virulence. A *C. albicans fab1* null mutant strain has the same virulence as the wild-type strain (Augsten *et al.*, 2002). Our work shows that another potential lipid-binding protein, Vac1p, is involved in virulence. The *vac1* null mutant strain is avirulent and shows defective yeast-to-hyphae transition.

Vac1p has been shown to mediate endosomal docking of vesicles in *S. cerevisiae* (Peterson & Emr, 2001). We show here that Vac1p also affects the docking of prevacuolar vesicles to the vacuole in *C. albicans*. This is concluded from the accumulation of the endocytotic fluorescent dye marker in prevacuolar vesicles in the *vac1* null mutant strain. At the stage of prevacuolar vesicles, endocytosis is linked to the CPY pathway from the Golgi to the vacuole, transiting through the prevacuolar vesicles. Thus in *C. albicans* Vac1p may influence both the CPY pathway and the endocytosis.

The role of Vac1p in endocytosis as shown here for *C. albicans* is in agreement with results reported for the human Vac1p homologue, which regulates fusion of endosomal vesicles with the early endosome (Stenmark *et al.*, 1996). Further evidence for a role of Vac1p in endocytosis comes from the interaction of *S. cerevisiae* Vac1p and Vps33p, a protein involved in endocytosis (Subramanian *et al.*, 2004).

A *vps34* null mutant strain of *C. albicans* exhibits an increased sensitivity to metal ions (Kitanovic *et al.*, 2005). The *vac1* null mutant strain also shows an increased metal ion sensitivity. Thus, Vac1p and Vps34p, and consequently the vacuolar protein transport, evidently play a role in resistance to metal ions.

Vac1p influences virulence by affecting the virulence factors hyphal growth, chlamydospore formation, adhesion to host cells, and secretion of Saps. In this work we have shown that Vac1p is involved in control of hyphal growth under microaerophilic/embedded conditions. In addition, Vac1p has a mild effect on hyphal growth induced by serum or mannitol (Spider medium), or by shift to neutral pH.

However, when *vac1* null mutant cells were cultivated in liquid media no significant reduction in hyphal growth was observed, indicating that Vac1p-dependent hyphal induction pathways are induced by environmental stress, such as starvation, limited supply of oxygen or shift in pH. The response of yeast cells to such stress conditions requires the transport and accumulation of compounds in the vacuole. The defective last step in vacuolar transport, the fusion of prevacuolar vesicles to the vacuole, in *vac1* null mutant cells may prevent induction of hyphal development and of several other stress-induced signalling pathways. The *in vitro* stress conditions that induce Vac1p-dependent pathways of hyphal growth are also found during pathogenesis of candidiasis. Therefore, the affected adaptation of the *vac1* null mutant to these stress conditions, and the defect in hyphal growth, results in the avirulence of the null mutant in the mouse model of systemic candidiasis.

In addition, Vac1p is required for the morphogenetic pathway of chlamydospore formation. The *vac1* null mutant strain is completely unable to form chlamydospores either on a slide with rice agar covered with a coverslip or in liquid rice agar. This result, together with the hyperfilamentation of embedded cells of the *vac1* null mutant, may indicate that the morphogenetic regulator Efg1p is affected in the *vac1* null mutant, because Efg1p is required for chlamydospore development and repressed the filamentation of embedded cells (Sonneborn *et al.*, 1999; Giusani *et al.*, 2002). In addition, hyphal growth in response to alkaline pH requires Efg1p (El Barkani *et al.*, 2000). Therefore, the affected hyphal growth under alkaline conditions seems to further indicate that Vac1p influences pathways that are regulated by Efg1p.

Vac1p plays a decisive role in the secretion of Saps. The defective Saps activity in the *vac1* null mutant strain is probably caused by defects in the secretory pathway from the Golgi apparatus, or the pathway from the vacuole to the cell surface, because in *S. cerevisiae* the secretory pathway leads to both the Golgi and the vacuole (Gurunathan *et al.*, 2002). Such a link between the vacuolar transport and exocytosis was also shown in the *C. albicans vps34* null mutant strain, where exocytotic vesicles accumulated in the periplasm (Bruckmann *et al.*, 2001). In addition, the Vps34p and Vps11p vacuolar transport proteins are also involved in Sap secretion in *C. albicans* (Kitanovic *et al.*, 2005; Palmer *et al.*, 2003). In *S. cerevisiae* Vps11p, as well as Vac1p, mediates the docking of transport vesicles to the vacuolar and endosomal membrane. The synthetic interaction between a *vps11ts* allele and *VAC1* in *S. cerevisiae* (Srivastava *et al.*, 2000) indicates a cooperation of Vac1p and Vps11p in Sap secretion of *C. albicans*. Sap2p contributes to both tissue damage and invasion during systemic candidiasis (Schaller *et al.*, 2003). Therefore, the defective Sap2p secretion very probably contributes to the avirulence of the *vac1* null mutant strain.

The *vac1* null mutant strain is avirulent in the mouse model of systemic candidiasis. This avirulence phenotype is

independent of defective cell growth, since the null mutant shows almost identical growth to the wild-type strain SC5314.

In summary, the potential PtdIns(3)P-binding protein Vac1p is involved in the vacuolar vesicle transport and probably acts downstream of the PI 3-kinase Vps34p. Both the *vps34* and *vac1* null mutants are avirulent, and they show some similar phenotypes. Together with knowledge from *S. cerevisiae* these results indicate that Vac1p and Vps34p are involved in the same virulence-determining pathway in *C. albicans*.

Environmental stress affects protein transport into the vacuole of *C. albicans*. This process may induce signals for the activation of virulence signalling pathways and the start of the pathogenic life cycle. Therefore we conclude that as key regulators of vacuolar transport, Vac1p, Vps34p and Vma7p could influence virulence of *C. albicans*. Such proteins can be termed virulence-determining signal proteins.

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