Candida albicans Vrp1 is required for polarized morphogenesis and interacts with Wal1 and Myo5

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Recently, a link between endocytosis and hyphal morphogenesis has been identified in *Candida albicans* via the Wiskott–Aldrich syndrome gene homologue *WAL1*. To get a more detailed mechanistic understanding of this link we have investigated a potentially conserved interaction between Wal1 and the *C. albicans* WASP-interacting protein (WIP) homologue encoded by *VRP1*. Deletion of both alleles of *VRP1* results in strong hyphal growth defects under serum inducing conditions but filamentation can be observed on Spider medium. Mutant *vrp1* cells show a delay in endocytosis – measured as the uptake and delivery of the lipophilic dye FM4-64 into small endocytic vesicles – compared to the wild-type. Vacuolar morphology was found to be fragmented in a subset of cells and the cortical actin cytoskeleton was depolarized in *vrp1* daughter cells. The morphology of the *vrp1* null mutant could be complemented by reintegration of the wild-type *VRP1* gene at the *BUD3* locus. Using the yeast two-hybrid system we could demonstrate an interaction between the C-terminal part of Vrp1 and the N-terminal part of Wal1, which contains the WH1 domain. Furthermore, we found that Myo5 has several potential interaction sites on Vrp1. This suggests that a Wal1–Vrp1–Myo5 complex plays an important role in endocytosis and the polarized localization of the cortical actin cytoskeleton to promote polarized hyphal growth in *C. albicans*.

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INTRODUCTION

Candida albicans is a pathogenic yeast that can respond to certain environmental cues by forming hyphal filaments. This morphogenetic switch is regarded as one of several attributes that enable *C. albicans* to cause disease (Sudbery *et al.*, 2004; Whiteway & Oberholzer, 2004; Whiteway & Bachewich, 2007). Hyphal growth is an extreme form of polarized morphogenesis that requires constant delivery of vesicles to support tip growth and remodelling of the cell wall at the tip. The actin cytoskeleton plays an important role by providing tracks for the delivery of vesicles to the tip along actin cables, and actin patches at sites of endocytosis (Pruyne & Bretscher, 2000; Kaksonen *et al.*, 2005). A balance between secretion and endocytosis is also

important for the maintenance of polarized morphogenesis, although a mechanistic link has not yet been established (Aghamohammadzadeh & Ayscough, 2009). Two genes that play a crucial role in endocytosis in *C. albicans* are *CaMYO5*, encoding myosin I, and the Wiskott–Aldrich syndrome homologue *CaWAL1* (Oberholzer *et al.*, 2004; Walther & Wendland, 2004). In *Saccharomyces cerevisiae*, the corresponding homologues *ScMYO3/5* and *LAS17* have been shown to activate the Arp2/3 complex, promoting actin polarization at sites of endocytosis (Evangelista *et al.*, 2000; Machesky, 2000; D’Agostino & Goode, 2005). Deletion of *CaMYO5* leads to viable mutant strains that cannot undergo hyphal development. Yeast cells of *Camyo5* mutants show depolarization of the actin cytoskeleton, which also affects their budding pattern (Oberholzer *et al.*, 2002, 2004). Similarly, deletion of *CaWAL1* results in mutant strains that are unable to generate hyphal filaments. During yeast growth of these mutants, depolarization of the actin cytoskeleton leads to the formation of round cells that show an increase in random budding. Additionally, loss of *CaWAL1* leads to defects in the endocytosis of the lipophilic dye FM4-64 as well as defects in vacuolar fusion. Fragmented vacuoles have been observed in other mutant

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Abbreviations: SH3, Src homology domain 3; WASP, Wiskott–Aldrich syndrome protein; WH2, WASP homology 2; WIP, WASP-interacting protein.

strains, e.g. *vac1* or *vps11*. These strains were also shown to be defective in hyphal morphogenesis (Palmer *et al.*, 2003; Franke *et al.*, 2006). Characteristically, during hyphal growth large vacuoles are formed in the germ cell and in the rear parts of the hyphal filaments. An unequal distribution of vacuoles was also shown to influence the timing of branch emergence (Veses *et al.*, 2008); however, fragmented vacuoles, per se, do not abolish polarized morphogenesis, which was recently also shown in a *Caboi2* mutant (Reijntj *et al.*, 2010).

The functional overlap of *C. albicans* Myo5 and Wall1 and their rather similar mutant phenotypes suggests that the two proteins can function in a complex in *C. albicans*. In mammalian cells, Wiskott–Aldrich syndrome protein (WASP) was shown to interact with the WASP-interacting protein (WIP) (Ramesh *et al.*, 1997; Thrasher & Burns, 2010). WIP suppresses growth defects of the *S. cerevisiae* *end5/vrp1* mutant (Vaduva *et al.*, 1999). ScEnd5/Vrp1 is a very proline-rich protein that is involved in cytoskeletal organization and can interact with both Las17 and Myo5 (Anderson *et al.*, 1998; Evangelista *et al.*, 2000; Munn & Thanabal, 2009). The temperature sensitivity and loss of viability of an *end5-1/vrp1* mutant can be suppressed by the overexpression of ScLAS17 (Naqvi *et al.*, 1998). And finally, loss of ScEnd5/Vrp1 results in severe defects in cytokinesis and Hof1 cannot be recruited to the bud neck (Ren *et al.*, 2005).

Here we describe the analysis of the *C. albicans* VRP1 homologue. The mutant strain shows defects in hyphal growth, endocytosis, organization of the actin cytoskeleton and budding pattern similar to, but less pronounced than, the *wal1* and *myo5* mutant strains. Two-hybrid studies in *S. cerevisiae* showed that Vrp1 interacts strongly with the N-terminal domain of Wall1 and also with Myo5. The data suggest that a Wall–Vrp1–Myo5 complex is crucial for endocytosis and polarized morphogenesis in *C. albicans*.

METHODS

Strains and media. *C. albicans* strain SN148 (Noble & Johnson, 2005) was used to generate the *vrp1* heterozygous and homozygous mutant strains. For the yeast two-hybrid experiment, the following strains were used: PJ69-4a (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2p-ADE2 met2::GAL7-lacZ*) and PJ69-4alpha (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2p-ADE2 met2::GAL7-lacZ*). Strains were grown either in yeast extract/peptone/dextrose medium [YPD; 1% yeast extract, 2% peptone, 2% dextrose (glucose)], or in minimal synthetic defined media [6.7 g l⁻¹ yeast nitrogen base (YNB) with ammonium sulphate and without amino acids, 20 g l⁻¹ glucose] supplemented with 0.69 g l⁻¹ complete supplement mixture (CSM), or with the addition of required amino acids and uridine. Hyphal formation was induced with 10% serum at 37 °C or by incubation on Spider medium. *Escherichia coli* strain DH5α served as a host for plasmid propagation.

Transformation and strain construction. *S. cerevisiae* and *C. albicans* were transformed using the lithium acetate procedure (Walther & Wendland, 2003; Gietz & Schiestl, 2007). Independent

homozygous mutant strains were constructed and verified following standard PCR-based gene targeting methods based on the use of pFA plasmids for cassette generation (Walther & Wendland, 2008). Deleting both ORFs of VRP1 by sequential transformation of SN148 with PCR-generated cassettes resulted in the heterozygous strains CAB9 (*VRP1/vrp1::ARG4*) and CAB10 (*VRP1/vrp1::URA3*), and then the homozygous strains CAB12 (*vrp1::ARG4/vrp1::CdHIS1*) and CAB13 (*vrp1::URA3/vrp1::CdHIS1*). To complement the *Δvrp1* phenotype, VRP1 was amplified from genomic DNA and ligated into cloning vector pDrive, generating #C597. The insert was cloned in #C873, which contains the BUD3 locus for integration and CmlEU2 as selectable marker, using *Sall* and *Bam*HI restriction sites. This generated #C598. The *Δvrp1* homozygous mutant strain CAB13 was transformed with *Spe*I-linearized #C598, generating CAP225.

Strain CAT41 was generated by targeting a *GFP-HIS1* cassette to the *C. albicans* TEF1 locus. All primers were obtained from biomers.net and their sequences will be made available upon request.

Plasmid constructs. For the yeast two-hybrid experiments, freely replicating plasmids were generated using pGAD424 and pGBT9 (Clontech) as backbones. These plasmids contained the Gal4-transcription-factor-activation domain or the Gal4-DNA-binding domain, respectively. Restriction fragments of *WAL1*, *VRP1* and the region encompassing the SH3 domain of *MYO5* were amplified from genomic DNA or plasmid clones and cloned into the corresponding restriction sites of pGAD424 or pGBT9. Correct cloning was verified by sequencing (Eurofins MWG Operon).

Microscopy and staining procedures. Microscopic analyses were done with an Axio-Imager microscope (Zeiss) using Metamorph 7 software tools (Molecular Devices) to drive the automated image-acquisition procedures. Images were acquired with a MicroMax1024 CCD camera (Princeton Instruments). Fluorescence microscopy was performed using the appropriate filter combinations for FM4-64 imaging and actin staining as described previously (Walther & Wendland, 2004; Martin *et al.*, 2005). Samples were analysed by generating either single images or Z-stacks of up to 20 images that were processed into single-plane projections using Metamorph software.

Yeast two-hybrid analysis. *S. cerevisiae* was transformed with two plasmids expressing constructs fused to either the Gal4-DNA-binding domain, based on plasmid pGBT9, or the Gal4-activation domain, based on plasmid pGAD424. Transformants were grown on media selecting for the maintenance of both plasmids (–Trp –Leu). White colonies revealed an interaction of the two expressed fusion proteins, which results in the expression of the *ADE2* reporter gene, whereas red colonies appeared when the *ADE2*-reporter could not be activated. For quantitative analysis, liquid-culture β-galactosidase assays were performed. To this end, strains were incubated overnight at 30 °C. Cells were harvested by centrifugation, protein extracts were prepared using a liquid nitrogen/glass-bead method and the conversion of ONPG (*o*-nitrophenyl β-D-galactopyranoside) was measured photometrically (Rose & Botstein, 1983).

RESULTS

Sequence comparisons

The *C. albicans* homologue of *S. cerevisiae* END5/VRP1 has been identified as orf19.2190. *C. albicans* VRP1 encodes a very proline-rich protein of 664 aa, of which 154 residues are proline. Sequence comparisons with other fungal homologues were done using the CLUSTAL W alignment

tool (Fig. 1). The N-terminal region of CaVrp1p contains a proline stretch present in most fungi and only annotated to be absent in *Ashbya gossypii*. Reinspection of the *VRP1*-locus in *A. gossypii*, however, indicates that there is a polyproline region upstream of the annotated start codon. Furthermore, a Vrp1 homologue in the closely related species *Eremothecium cymbalariae* also contains this polyproline region at the N terminus of Ecy_m-Vrp1 (our unpublished results). Downstream of the polyproline region in Vrp1, two putative WASP homology 2 (WH2) domains are located. Here, the filamentous ascomycete *Neurospora crassa* lacks the second putative WH2 domain. In *S. cerevisiae*, a short region after the second WH2 domain has been characterized as a docking site for Hof1 (Ren *et al.*, 2005). This Hof1-trap (HOT) domain seems to be rather specific for *S. cerevisiae* as it is not found in the other fungal species analysed (Fig. 1a). The central part of Vrp1 orthologues exhibits only a low degree of amino acid sequence conservation, not regarding the many proline-rich stretches, whereas the C-terminal regions in fungal Vrp1 proteins show better conservation. This domain has been characterized as the Las17p-binding domain (Naqvi *et al.*, 1998; Madania *et al.*, 1999; Fig. 1b).

Generation of *C. albicans* *vrp1* mutant strains

To delete both alleles of *C. albicans* *VRP1*, a PCR-based gene targeting approach was applied (Walther & Wendland, 2008). Initially, independent heterozygous mutant strains were generated in which the ORF of one allele of *VRP1* was deleted by either the *C. albicans* *ARG4* or *URA3* marker gene. To generate homozygous mutant strains based on these heterozygous strains, the remaining copy of *VRP1* was deleted using the *Candida dubliniensis* *HIS1* gene. Verification of correct gene targeting and the

absence of the *VRP1* ORF in the homozygous mutant strains CAB11 and CAB13 was done by diagnostic PCR and reintegration of *VRP1* at the *BUD3* locus was used for complementation.

Phenotypic assay of growth morphology of mutant strains

The heterozygous and homozygous *VRP1*-deletion strains were compared to the SC5314 wild-type strain, the SN148 strain used as a host for transformation and a *wal1* mutant strain deleted for the *C. albicans* homologue of the human WASP, described previously (Walther & Wendland, 2004). Hyphal induction was tested on Spider medium, which contains mannitol as the primary carbon source. The wild-type showed strong filamentation at the edge of the colony, whereas the *wal1* strain was a filamentous. The SN148 precursor strain also showed a strong increase in colony wrinkling, which was also found in the heterozygous *VRP1/vrp1* strain. The homozygous *vrp1* strain did not show this colony-wrinkling phenotype; however, the colony edges of the mutant *vrp1* strain did show invasive filamentous growth (Fig. 2a). Hyphal induction in liquid media was done using serum as an inducing cue. Here, the wild-type showed abundant filamentation. SN148 showed slightly less filamentation (due to the *ura3* deletion) and *wal1* again showed no hyphal formation. The heterozygous *VRP1/vrp1* strain showed filamentation similar to that of the wild-type, whereas the *vrp1* mutant strain showed a strong reduction in filamentation and produced instead a large number of pseudohyphal cells and new yeast cells (Fig. 2a). The distribution of cell types after hyphal induction in the various strains used was quantified by counting >100 cells for each strain (Fig. 2c). These analyses indicated a strong defect in hyphal formation of the *vrp1* mutant, which was,

Fig. 1. Alignment of fungal Vrp1 homologues. Amino acid residues corresponding to the majority of analysed sequences are shaded. (a) The N-terminal actin-binding sequences are boxed in all Vrp1 proteins. *S. cerevisiae* Vrp1 harbours non-conserved regions with the consensus sequence 'PxSS' (boxed) that were shown to interact with ScHof1. (b) Alignment of the Vrp1 C-terminal regions harbouring the conserved Las17-binding domain. Protein information was obtained from the following sources: *A. gossypii*, the *Ashbya* Genome Database (<http://agd.vital-it.ch/index.html>); *C. albicans*, the *Candida* Genome Database (<http://www.candidagenome.org>); *Kluyveromyces lactis*, XP_451805; *Neurospora crassa*, XP_963859; and *S. cerevisiae*, AAB67263.

Fig. 2. Characterization of the growth defects of the *vrp1* mutant SN148. (a) The indicated strains were grown on Spider plates for 5 days at 37 °C prior to photography (top row). Microscopic images of the colony edges show the degree of filamentation (middle row). Hyphal formation in liquid medium was induced by using 10 % serum. Images were taken after 6 h of induction (bottom row). (b) Cells induced by serum were counted after 6 h and classified according to their morphology. (c) Complementation of the *vrp1* filamentation defect by reintegration of *VRP1* at the *C. albicans* *BUD3* locus.

however, slightly less severe than in the *wal1* mutant. This result is in line with the filamentation assay on Spider medium. To demonstrate that these filamentation defects are solely due to the deletion of the *VRP1* gene, we reintegrated the *VRP1* gene at the *BUD3* locus in a *vrp1/vrp1* mutant strain (see Methods). This reintegrant was phenotypically like the wild-type, for example, when assayed for germ tube production (Fig. 2b).

Analysis of the actin cytoskeleton in the *vrp1* mutant

Hyphal growth defects may be associated with an altered organization of the actin cytoskeleton. Therefore, we used rhodamine-phalloidin staining of fixed cells to analyse the distribution and polarization of the cortical actin cytoskeleton. Wild-type cells show a polarization of cortical actin in

the emerging bud and at the hyphal tip. The actin cytoskeleton of the *wal1* mutant was shown to be largely depolarized during all growth stages. In the *vrp1* mutant such a depolarization could also be observed in yeast and pseudohyphal cells. Remarkably, in both yeast and hyphal stages the apical growth region showed more intense staining indicating an accumulation of actin at sites of polarized growth (Fig. 3a). Analysis of the budding pattern via fluorescence microscopy of the bud scars showed that the *vrp1* mutant is able to generate a bipolar budding pattern, as found in the wild-type (Fig. 3b).

VRP1 mutants show defects in vacuolar fusion and endocytosis

Altered polarization of the actin cytoskeleton may also affect endocytosis. To study vacuolar morphology and endocytosis

Fig. 3. Analysis of the actin cytoskeleton and budding pattern. (a) Strains were grown to form yeast and hyphal stages, fixed by formaldehyde and stained using rhodamine-phalloidin. Bar, 10 µm. (b) Representative images of calcofluor-stained cells.

we employed the lipophilic dye FM4-64. Using time-lapse microscopy we compared, at the same time, uptake of FM4-64 between the *vrp1* mutant and a wild-type strain expressing cytoplasmic GFP. Within 20 min the dye had been taken up via endocytosis and delivered to the vacuole in the wild-type. This staining of wild-type vacuoles further increased over time. Compared to this the *vrp1* mutant showed a delayed uptake and only after more than 1 h did small endocytic vesicles, and possibly vacuoles, become stained (Fig. 4). Quantification of the vacuoles showed a slight increase in the *vrp1* mutant compared to the wild-type. This *vrp1* phenotype is thus somewhat intermediate between the *wal1* mutant and the wild-type (Fig. 5).

Two-hybrid analyses reveal interaction of Vrp1 with Myo5 and Wal1

In *S. cerevisiae* Vrp1 was shown to interact with Src homology domain 3 (SH3) of the yeast type I myosin Myo5p and the WASP homologue Las17 (Evangelista *et al.*, 2000). To determine if CaVrp1 interacts with both Las17 and Myo5 we performed yeast two-hybrid analyses (Fig. 6). In this assay we found that the C-terminal part of Vrp1 interacts strongly with the N-terminal part of Wal1 containing the WH1-B domains. Interaction of Vrp1 with the full-length Wal1 protein was somewhat weaker. Highest β -galactosidase activity was obtained with a Wal1 fragment in which the central part of *WAL1*, encoding several proline-rich regions, was removed for the assay. To assay the interaction of the myosin I SH3 domain with Vrp1 we used two fragments containing the N and C termini of Vrp1 and a fragment containing only the SH3 domain of Myo5. Here, the Myo5 SH3 domain interacted strongly with the C-terminal part of Vrp1 (Fig. 6).

DISCUSSION

In this report we have characterized the function of the *C. albicans* *VRP1* gene in the polarized morphogenesis and endocytosis of this dimorphic human pathogen. Cell polarization in *C. albicans* is important for budding, filamentation and mating. The establishment of cell polarity occurs either due to intrinsic factors (during budding) or in response to environmental stimuli (during filamentation and mating) (Whiteway & Bachewich, 2007). One result of this polarity is the polarized organization of the actin cytoskeleton, which results in the apical positioning of cortical actin patches and the generation of actin cables emanating from the cell apex (Smith *et al.*, 2001). Generation of actin cables from the emerging bud or the tip of the hyphae has been fairly well characterized. A cascade from locally activated Rho-type GTPases, most notably Cdc42, triggers downstream effector genes, such as the formin Bni1, which nucleates actin filaments (Evangelista *et al.*, 2002; Sagot *et al.*, 2002). Bni1 is part of a complex termed the polarisome, which in *S. cerevisiae* also contains Pea2, Spa2 and Bud6 (Sheu *et al.*, 1998).

Clustered assembly of actin patches occurs at sites of polarized growth. Mutants of *S. cerevisiae* and *C. albicans* that are affected in the position of actin patches, e.g. in the *LAS17/WAL1* or *MYO3/5* genes, show defects in polarized growth (Li, 1997; Lechler *et al.*, 2000; Oberholzer *et al.*, 2002; Walther & Wendland, 2004). Las17 and Myo3/5 have been shown to stimulate actin filament formation via the Arp2/3 complex (Lechler *et al.*, 2000). Mutants affected in these genes show defects in the assembly and organization of the actin cytoskeleton and since the actin cytoskeleton is essential for endocytosis in *S. cerevisiae* they also show defects in clathrin-mediated endocytosis (Munn, 2001; Kaksonen *et al.*, 2003). Most of the proteins known to be involved in endocytosis co-localize with actin patches.

Fig. 4. Time-lapse analysis of endocytosis of the lipophilic dye FM4-64. Wild-type cells carrying a cytoplasmic GFP label (on the right side of each panel) were mixed with *vrp1/vrp1* cells (on the left side of each panel). Microscopy slides with wells were filled with 0.75 ml 0.5× YPD and 0.75 ml 3.4 % agarose. To this mixture 1 μ l FM4-64 (200 μ g ml⁻¹ in DMSO) was added. Image acquisition started 10 min after preparation of the slide for a duration of 3 h with a frequency of 1 image min⁻¹. Selected frames are shown at the indicated time points, starting with a GFP image identifying the wild-type cells followed by a bright-field differential interference contrast (DIC) image of all cells. Bar, 10 μ m.

Fig. 5. Analysis of vacuolar morphology. Strains were grown overnight and then stained with FM4-64 for 2 h prior to photography. Cells were counted and characterized according to the number of vacuoles they contained. Representative images of cells displaying one, two to three, or more than four vacuoles are shown.

Thus actin patches are sites of endocytosis (Kaksonen *et al.*, 2005).

Deletion of *S. cerevisiae* *VRP1* results in temperature sensitivity and the depolarization of actin patches. Specifically, actin patches do not cluster in emerging buds (Lambert *et al.*, 2007). We have also observed a depolarization of actin patches in both mother and daughter cells of the *C. albicans* *vrp1* mutant. Furthermore, hyphal morphogenesis in the *vrp1* strains was inhibited – although not abolished as in *wal1* cells. Nevertheless, during growth of *vrp1* germ tubes, actin appeared to accumulate in a cap-like structure.

Our two-hybrid analysis suggests that a Wal1–Vrp1–Myo5 complex may be formed in *C. albicans* similar to that identified in *S. cerevisiae* (Evangelista *et al.*, 2000). This could provide an explanation of the mechanism responsible for the similar phenotypes of the *wal1* and *myo5* mutants observed previously. Both of these genes are activators of the Arp2/3 complex, and loss of either of these genes may be more detrimental to cells than loss of *VRP1*. Consequently, the observed defects in endocytosis and vacuole formation were less severe in the *vrp1* strains compared to *wal1*. Interestingly, *S. cerevisiae* Vrp1 contains a region characterized as the Hof1-trap domain, which is essential for binding the Hof1-SH3 domain (Ren *et al.*, 2005). A Hof1-trap domain could not be identified in *C. albicans* Vrp1. Our attempts to identify a two-hybrid interaction of the *C. albicans* SH3 domain of Hof1 with Vrp1 were unsuccessful, which may suggest that this interaction is occurring either

Thus, our analysis contributes to our understanding of the mechanistic link between Wall and Myo5 in *C. albicans*. With the defects in hyphal morphogenesis and endocytosis of the *vrp1* mutant strain we have identified another player partaking in the yeast-to-hyphal switch in *C. albicans*.

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