PHR1, a pH-regulated gene of Candida albicans encoding a glucan-remodelling enzyme, is required for adhesion and invasion

Julia Calderon, Martin Zavrel, Enrico Ragni, William A. Fonzi, Steffen Rupp and Laura Popolo

1Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, 20133 Milano, Italy
2Fraunhofer IGB, Nobelstrasse 12, D-70569 Stuttgart, Germany
3Department of Microbiology and Immunology, Georgetown University, Washington, DC, USA

The fungal cell wall plays a crucial role in host–pathogen interactions. Its formation is the result of the coordinated activity of several extracellular enzymes, which assemble the constituents, and remodel and hydrolyse them in the extracellular space. Candida albicans Phr1 and Phr2 proteins belong to family GH72 of the β-(1,3)-glucanosyltransferases and play a crucial role in cell wall assembly. PHR1 and PHR2, homologues of Saccharomyces cerevisiae GAS1, are differently regulated by extracellular pH. PHR1 is expressed when ambient pH is 5.5 or higher, whereas PHR2 has the reverse expression pattern. Their deletion causes a pH-conditional defect in morphogenesis and virulence. In this work we explored whether PHR1 deletion affects the ability of C. albicans to adhere to and invade human epithelia. PHR1 null mutants exhibited a marked reduction in adhesion to both abiotic surfaces and epithelial cell monolayers. In addition, the mutant was unable to penetrate and invade reconstituted human epithelia. Transcription profiling of selected hyphal-specific and adhesin-encoding genes indicated that in the PHR1 null mutant, HWP1 and ECE1 transcript levels were similarly reduced in both adhesion and suspension conditions. These results, combined with microscopy analysis of the septum position, suggest that PHR1 is not required for the induction of hyphal development but plays a key role in the maintenance of hyphal growth. Thus, the β-(1,3)-glucan processing catalysed by Phr1p is of fundamental importance in the maintenance of the morphological state on which the adhesive and invasive properties of C. albicans greatly depend.

INTRODUCTION

Candida albicans, normally a harmless fungal commensal, colonizes different compartments of the human body such as the oral cavity, the intestinal epithelia and the vaginal mucosa. When the defence mechanisms of the host are compromised, or in the presence of physical injuries of the epithelia and perturbations of the mucosal microenvironment, this micro-organism can switch to being a fungal pathogen. Regulation of gene expression in response to environmental signals, morphological plasticity, and a capacity for phenotypic switching and adherence to and invasion of tissues are required to confer virulence upon C. albicans (Calderone & Fonzi, 2001). However, the extent and severity of disease are greatly dependent on the immune response of the host. In immunocompromised individuals with neutrophil depletion as a result of chemotherapy or with advanced HIV infection, C. albicans can invade the organs and become a serious threat to the life of the patient (Mavor et al., 2005).

In order to survive and grow within the host, C. albicans exploits its striking ability to respond to changes in nutrient availability, ion or serum concentrations, osmotic pressure and environmental pH. In particular, the adaptability to allow growth over a wide range of ambient pH has been recognized as a crucial property for colonizing different niches of the host (Davis et al., 2000). The pH can be slightly alkaline, such as in the bloodstream (pH 7.3), close to neutrality, such as in organs like the kidney, liver and duodenum, or acidic as in the stomach (pH ~2) or vagina (pH ~4.5). Several C. albicans genes are regulated by the extracellular pH through Rim101-dependent or -independent pathways (Bensen et al., 2004; Lotz et al., 2004; Porta et al., 1999; Ramon et al., 1999). Among the genes regulated by Rim101p, albeit in opposite directions,
are PHR1 and PHR2 (pH-responsive genes 1 and 2), which were also the first identified pH-regulated genes in C. albicans (Muhlschlegel & Fonzi, 1997; Saporito-Irwin et al., 1995). PHR1 is expressed at pH values of 5.5 or higher, whereas PHR2 is expressed at pH values of 5.5 or lower. This pH-dependent regulation is crucial for pathogenesis in the respective host niches (De Bernardis et al., 1998). PHR1 and PHR2 encode highly similar glycoproteins which are predicted to be anchored to the plasma membrane through a glycosylphosphatidylinositol (GPI) (Fonzi, 1999). Clues to the identification of their biochemical activity came from in vitro studies on recombinant Phr1 and Phr2 proteins as well as their homologous proteins, Gas1 from the budding yeast Saccharomyces cerevisiae and Gel1 from the fungal pathogen Aspergillus fumigatus. In vitro, Phr/Gas/Gel proteins cleave internally a laminari-oligosaccharide of >10 glucose residues and then transfer the new reducing end to the non-reducing end of another laminarioligosaccharide. This transferase activity results in the elongation of β-(1,3)-glucan chains (Hurtado-Guerrero et al., 2009; Mouyna et al., 2000; Ragni et al., 2007a). This activity has a great importance in fungal biology. All yeast and fungal species so far sequenced have homologues of the PHR/GAS/GEL gene families (Ragni et al., 2007a). In addition, the domain organization and the 3D structure of a Gas enzyme have recently been reported (Hurtado-Guerrero et al., 2009; Popolo et al., 2008).

In each fungal species, GH72 enzymes constitute a redundant family of enzymes. In S. cerevisiae, five proteins are present. The Gas1–Gas5 protein pair is active during vegetative growth, whereas the Gas2–Gas4 protein pair is essential for spore wall formation. The function of Gas3p remains unknown (Ragni et al., 2007a, b). In C. albicans, three other PHR homologues are present: PHR3, PGA4 and PGA5. These genes appear to function in conditions which differ from those of PHR1 and PHR2, and the single deletion of these genes does not show a relevant effect on growth, dimorphism or virulence (Eckert et al., 2007).

The cell walls of C. albicans and S. cerevisiae have a very similar composition and architecture. Glucans and mannoproteins are the two major constituents, with twice as much β-(1,6)-glucan in C. albicans compared with S. cerevisiae, whereas chitin is a minor component (Mio et al., 1997). The outermost shield of mannoproteins regulates permeability and interactions with the environment, whereas the inner layer of glucans is crucial in determining the cell shape (De Groot et al., 2005). Chitin is important for morphogenesis, being required for septum formation. The primary in vivo role of GH72 enzymes is presumed to be the elongation of β-(1,3)-glucan chains as an alternative to, or in synergy with, β-(1,3)-glucan synthase. Moreover, this activity is also important to create anchoring sites for the attachment of other cell wall components such as chitin and cell wall mannoproteins (Fonzi, 1999; Mouyna et al., 2000).

As expected from the fact that cell wall biogenesis and morphogenesis are two tightly linked processes, the lack of GH72 activity in S. cerevisiae and C. albicans affects cell shape, causing a loss of the ellipsoidal shape and defects in cell separation, bud maturation and filament elongation (Kang & Jiang, 2005; Popolo et al., 1993; Saporito-Irwin et al., 1995). A profound change in cell wall composition and architecture, which involves a hyperaccumulation of chitin and increased cross-linking of mannoproteins with chitin, occurs in the absence of GH72 enzyme activity (Fonzi, 1999; Popolo et al., 1997; Ram et al., 1998). These changes are secondary responses aimed at counteracting the weakening of the cell wall and preventing lysis (Popolo et al., 2001). A great deal of evidence indicates that these changes are triggered by the constitutive activation of signalling pathways, which culminates in the upregulation of cell wall-related and stress-responsive genes (Fonzi, 1999; Garcia et al., 2004; Lagorce et al., 2003; Popolo & Vai, 1998; Ram et al., 1998).

The lack of Phr1p or Phr2p affects C. albicans morphogenesis and cell wall biogenesis in a manner that is dependent on ambient pH (Fonzi, 1999). At pH 6 or lower, Δphr1 cells have the same phenotype as the wild-type. As pH increases above 6, Δphr1 cells exhibit a defective phenotype which also becomes more severe at higher pH values. In the yeast form, at pH 7.5 or 8, Δphr1 cells appear round, larger than the wild-type, with a swollen aspect similar to that of S. cerevisiae cells deleted in GAS1 (Popolo et al., 1993; Saporito-Irwin et al., 1995). In conditions of hyphal growth, Δphr1 cells produce shorter and enlarged germ tubes (Saporito-Irwin et al., 1995). Additionally, in restrictive pH conditions, PHR1 null mutants have a reduction in β-(1,3)-glucan and an increase of chitin accumulation (Fonzi, 1999). Conversely, deletion of PHR2 results in a morphogenetic defect which is expressed at acidic pH (Muhlschlegel & Fonzi, 1997). Consistent with the dependence of phenotype on pH, a PHR1 null mutant is avirulent in a mouse model of systemic infection but uncompromised in its ability to cause vaginal infection in rats (De Bernardis et al., 1998). The virulence phenotype of a PHR2 null mutant is the inverse.

It is conceivable that an alteration in cell wall organization can affect key processes in C. albicans pathogenicity, as the cell surface is the first interface between the pathogen and the host. One of the early phases of infection consists of the attachment of the pathogen to the surface of host tissues. Cell wall proteins with adhesive properties or that act as ligands for host receptors play a relevant role in mediating this early event (Chaffin et al., 1998). The capacity to adhere is also crucial for the persistence of the pathogen in the host and for attachment to artificial substrates. Indeed, the adhesion and the formation of biofilms on medical devices, such as catheters, is currently a serious problem in hospitals (Douglas, 2003; Nobile et al., 2006). In addition, virulence also depends on the ability to invade human epithelia. The main port of entry in systemic infections is through the epithelial layer of the gut. In addition, adhesion to the endothelial lining of blood vessels and
transmigration across the endothelium cause bloodstream invasion and penetration into the tissues (Grubb et al., 2008).

To dissect the contribution of PHR1 to C. albicans pathobiology we tested the ability of PHR1 null mutants to adhere to different substrates and to invade human epithelia. Toward this aim we exploited in vitro adhesion assays and reconstituted human epithelium (RHE) model systems.

METHODS

Strains and growth conditions. The C. albicans strains used in this study were CAI-10 (URA3/ura3::imm434), derived from CAF-3 (Fonzi & Irwin, 1993); CAS-10 (phr1Δ::hisG/phr1Δ URA3/ura3::imm434), derived from CAS-8, a Phr1Δ derivative of CAF3-1 (Saporito-Irwin et al., 1995); and CAS-11 (phr1Δ::hisG/PHR1-pUC18-URA3-phr1Δ ura3Δ::imm434/ura3::imm434), a Phr1Δ derivative of CAS-8 (Saporito-Irwin et al., 1995). The same strains have previously been used to test the virulence of Δphr1 mutants (De Bernardis et al., 1998). For the adhesion and invasion assays, cells were cultivated overnight at 30 °C on yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% Bacto-peptone, 2% glucose) supplemented with 150 mM HEPES, pH 6.0, before sterilization by filtration. Agar at 2% was added to solid medium.

Cell cultures. Two human cell lines, Caco-2 (ATCC HTB-37), an intestinal epithelial cell line, and TR146, a buccal epithelial cell line derived from the oral cavity (Rupniak et al., 1985), were grown in 75 cm² tissue-culture flasks (Greiner). For both cell lines, Dulbecco’s Modified Eagle’s medium (DMEM; with NaHCO₃), supplemented with 10% FCS, was used. Cells were cultivated at 37 °C under 5% CO₂ with continuous stirring. In addition, cells from 1 ml of the cell suspension were collected immediately by centrifugation and quickly frozen in liquid nitrogen. After 2 h, adherent cells were detached from the plastic surface with a cell scraper and collected together with the non-adherent cell fraction.

Invasion assay. To characterize the tissue-invasive properties of the strains, we used a 3D model system of the human intestinal cell line Caco-2 that has been described previously (Hernandez & Rupp, 2008). The system is composed of collagen type I matrix, overlaid by a confluent monolayer of Caco-2 epithelial cells. Acidic collagen solution (collagen type I from rat tail tendons dissolved in 0.1 M acetic acid) was mixed with an appropriate volume of gelling solution (2% mixture with gelling solution (300 mg/ml fresh DMEM (pre-warmed at 37 °C) was added to each well. Adhesion to polystyrene was also monitored at pH 6 using the same medium supplemented with 25 mM MES buffer at pH 6. C. albicans cells were grown overnight at 30 °C in YPD buffered at pH 6.0 and diluted to OD₆₀₀ 0.2 with dH₂O. After serial dilution, ~3 × 10⁵ cells (in 50 µl) were added to each well (for five time points in duplicate for each strain). Plates were incubated at 37 °C under 5% CO₂ without agitation. After 30, 60, 120 and 240 min, the plates were shaken at 200 r.p.m. for 2 min to detach loosely bound cells, and then the culture medium was removed to plate the non-adherent C. albicans cells onto YPD agar plates. The wells were then washed with 300 µl PBS and after the addition of fresh PBS, C. albicans adherent cells were scraped off and spread onto agar plates. As a reference time point, adherent and non-adherent C. albicans cells were plated immediately after inoculation (time zero). YPD plates were incubated at 30 °C for 24 h. c.f.u. were counted for both adherent and non-adherent cells, and the mean percentage of adherent cells (± sd) from four independent experiments was calculated.

Microscopy. The morphology of cells growing in suspension or adhering to plastic was examined directly using a Zeiss Axiosvert 25 microscope and photographed with a Zeiss AxiosCam MRm digital camera. For chin staining, adherent cells were scraped from the plastic surface, mildly sonicated and collected by centrifugation at 4 °C. Cells were stained for 5 min in a 0.1% aqueous solution of Calcofluor White (Sigma) and washed three times with PBS.

Quantification of mRNA using real-time quantitative RT-PCR (qRT-PCR). Stationary phase cells from an overnight culture in YPD/150 mM HEPES, pH 6.0, at 30 °C and an agitation speed of 160 r.p.m. were pelleted and suspended in sterile dH₂O (OD₆₀₀ 1). A 0.1 ml volume of this suspension was immediately added to 10 ml fresh pre-warmed supplemented DMEM medium, pH 7.4, in a cell-culture flask to induce adhesion to the polystyrene surface. The flask was placed in an incubator at 37 °C under 5% CO₂. A second 1 ml aliquot of the same cell suspension was added to another 10 ml supplemented DMEM, pH 7.4, in a 50 ml Erlenmeyer flask and incubated at 37 °C under CO₂ with continuous stirring. In addition, cells from 1 ml of the cell suspension were collected immediately by centrifugation and quickly frozen in liquid nitrogen. After 2 h, adherent cells were detached from the plastic surface with a cell scraper and collected together with the non-adherent cell fraction.

Cells growing in suspension were collected by centrifugation. Cell pellets from both conditions were also frozen in liquid nitrogen.

Frozen pellets were broken mechanically by grinding in a pre-cooled grinder using a Retsch mill. RNA was further isolated using an RNAasy Mini kit (Qiagen). Up to 1 µg isolated RNA was transcribed into cDNA using a Quantitect Reverse Transcription kit (Qiagen). Samples of cDNA were diluted 1:50 and used for qRT-PCR in a LightCycler 480 system (Roche) with a universal probe library and respective oligonucleotides (Table 1). For elaboration of the data, the Livak method was used (Livak & Schmittgen, 2001). Briefly, from each triplicate reaction, a ΔΔCT value was calculated by subtracting the

<table>
<thead>
<tr>
<th>Gene</th>
<th>Left primer (5’–3’)</th>
<th>Right primer (5’–3’)</th>
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<tbody>
<tr>
<td>TDF3</td>
<td>gcccgtcagactgcttacatc</td>
<td>aagatgttgaatgactgagaac</td>
</tr>
<tr>
<td>HWP1</td>
<td>atttgttccaggtgtgccaaac</td>
<td>tctggcaataagtaagacacac</td>
</tr>
<tr>
<td>ECE1</td>
<td>tcagctgcagtttagcatac</td>
<td>gcagcttggaagcaacatctt</td>
</tr>
<tr>
<td>ALS3</td>
<td>tgaatgtgctcaatctttgc</td>
<td>ggttgcctggttagtgcgac</td>
</tr>
<tr>
<td>EAPI</td>
<td>cccacctcacttaccaacaca</td>
<td>tctgtagagggtagatcaacaa</td>
</tr>
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Table 1. Oligonucleotide sequences used
mean CT value for TDH3 from the mean CT value of the gene of interest. For relative quantification of the abundance of ALS3, EAPI, HWP1 and ECE1 transcripts in the pre-culture in YPD medium buffered to pH 6 (YPD-pH 6), at 2 h after the shift to hyphal growth-inducing conditions in suspension and adhesion, the 2−ΔΔCT of each gene transcript in suspension was set as equal to 1. Then, the mean and SD of the normalized values from four different experiments were calculated.

RESULTS

A PHR1 null mutant of C. albicans cannot penetrate an RHE

The ability to invade human epithelia is a crucial phenotypic trait for the virulence of C. albicans. To evaluate the contribution of the PHR1 gene to invasion we exploited an RHE as a model system (Dieterich et al., 2002). Cells from strains CAI-10 (parental), CAS-10 (Δphr1/Δphr1) and CAS-11, wherein the wild-type allele was reintroduced, were pre-grown in YPD-pH 6 until reaching stationary phase. The pre-growth at pH 6 was chosen for two reasons: (i) the expression of PHR2 is already reduced and therefore also possible interference from remaining amounts of Phr2p in the assessment of the Δphr1 phenotype; (ii) the phenotype of Δphr1 cells is indistinguishable from that of the wild-type at pH 6 and becomes apparent with a progressive increase of the pH, becoming marked at pH 7.5 and peaking at pH 8 (Saporito-Irwin et al., 1995).

RHEs were exposed to a defined number of cells and the behaviour of the three strains was scored at 24 h post-infection. Fig. 1(a) shows that cells from CAI-10 penetrated the entire epithelium layer very efficiently and visibly invaded the underlying collagen matrix. Fig. 1(b) shows

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Fig. 1. Infection of an RHE with CAI-10 (Phr1+/+), CAS-10 (Phr1−/−) and CAS-11 (Phr1+/−) strains. (a) Penetration of the enterocytic cell layer and invasion into the collagen matrix by CAI-10 cells. (b) CAS-10 is not able to penetrate the model system. (c) CAS-11 shows a phenotype similar to that of CAI-10. (d) At 7 h, individual hyphae of CAI-10 are clearly seen penetrating into the epithelial cell layer, while (e) CAS-10 cells are visible on the enterocytic cell layer and a few penetrate, but do not invade, the underlying space. (f) At 24 h, CAI-10 has penetrated and invaded the matrix, while (g) CAS-10 cells are still in the proximity of the cell monolayer and a few cells have partially penetrated the layer. The cells are round and no formation of filaments has occurred. Bars: (a–c), 100 μm; (d, f), 25 μm; (e, g), 25 μm.
that incubation with the PHR1 null mutant did not result in penetration of the epithelium layer and no invasion of the matrix was detectable. Reintroduction of the PHR1 gene resulted in penetration of the tissue and invasion of the matrix, indicating that the defect in the mutant was not due to other mutations present in the deletion strain. However, the reconstituted strain appeared to invade less efficiently than the isogenic strain CAI-10. This phenotypic difference was observed in three independent experiments. The reproducibility of this observation suggests that a gene dosage effect may be present, which is consistent with the phenotype observed in in vivo studies (De Bernardis et al., 1998).

In order to monitor the process in more detail we analysed the cell morphology of penetrating cells at 7 and 24 h. Fig. 1(d, e) shows that at 7 h, cells of the parental strain showed elongated hyphae that penetrated through the epithelium layer, and that many cells resided in the proximity of the epithelium layer or were attached to it. The null mutant cells appeared round and larger, and at some sites they clustered and occasionally penetrated into the epithelial cells without invading the matrix. No cells were detected in the collagen matrix in repeated experiments. At 24 h, the parental strain had completely invaded the matrix, whereas the mutant cells appeared very similar to those reported at 7 h (Fig. 1f, g).

These results indicate that the Δphr1 mutant is strongly defective in penetration and invasion of epithelia. Moreover, the lack of cells in proximity to the epithelium layer suggests that adhesion is also affected. Consequently, we assayed the behaviour of Δphr1 cells in adhesion assays.

**A C. albicans Δphr1 mutant is defective in adhesion to abiotic surfaces**

Based on the foregoing observations, we examined the capacity of the phr1 mutant to adhere to abiotic surfaces. Cells were pre-grown at 30 °C in YPD-pH 6 before being transferred to hyphal growth-inducing conditions in polystyrene plates. Adherent and non-adherent cells were counted at different time points after inoculation, and the percentage of total adherent cells was calculated. In the conditions used, DMEM supplemented with 10 % FCS, 5 % CO₂, pH 7.4, adherence occurred rapidly and was nearly maximal within 30 min for both control and mutant strains (Fig. 2a). About 80 % of the wild-type control cells of strain CAI-10 adhered within 30 min, increasing to about 95 % by 4 h. Although Δphr1 mutant cells (CAS-10) adhered with similar kinetics, the extent of adherence was greatly reduced: only 20 % adhered by 30 min, increasing to about 30 % by 4 h. At 4 h, adhesion did not show a further increase. To verify the association of the adherence defect with loss of PHR1, the genetically rescued strain CAS-11 was tested, and restoration of a wild-type PHR1 allele was found to restore normal levels of adherence (Fig. 2a).

The morphological phenotype of the Δphr1 mutant is pH-dependent, and there is a phenotypic lag in expression following a shift from permissive to restrictive pH (Saporito-Irwin et al., 1995). Cell to cell variability in this lag period may have resulted in a subpopulation of Δphr1 mutant cells that was not yet phenotypically Phr1⁺ and thus able to adhere. To assess this possibility, cells in suspension or on polystyrene plates were examined microscopically (Fig. 3). Whereas the control cells displayed elongated and thin hyphae, PHR1 null mutant cells exhibited short and enlarged germ tubes, in agreement with the previously described phenotype (Saporito-Irwin et al., 1995). The morphology was similar for cells growing in suspension and adhesion conditions, although planktonic cells tended to aggregate (Fig. 3d, e and f). In addition, a similar morphology was also observed for both adherent and non-adherent cells (Fig. 3g, h and j), indicating that the mutant retains a limited ability to adhere to plastic.

As an additional test of the association of the adherence phenotype with loss of PHR1, the experiment was repeated at pH 6. The morphological phenotype of the Δphr1 mutant is not manifested at pH 6 (Saporito-Irwin et al., 1995). Thus, the adhesion defect was expected to be absent. At this
pH the kinetics and extent of adhesion of the control strain were essentially identical to those observed in the preceding experiment (Fig. 2b). Moreover, adhesion of the Δphr1 cells was indistinguishable from that of the control strain (Fig. 2b). Together, these results indicate that inactivation of PHR1 causes a marked reduction in the ability of cells to adhere to polystyrene surfaces at the restrictive pH. Loss of Phr1p reduces adhesion of C. albicans cells to monolayers of epithelial cells

We tested the ability of Δphr1 cells to adhere to monolayers of human epithelial cells. For this purpose we used an intestinal epithelial cell line (Caco-2) and a buccal epithelial cell line (TR146). As shown in Fig. 4(a), the control strain CAI-10 rapidly adhered to the cells, reaching a value of 60% adhesion in about 30 min and a maximal value of about 90% in 2 h. Δphr1 cells reached a level of adhesion of about 25% in the first 30 min, then adhesion slowly increased, reaching a maximal value of 30% at about 60 min. A very similar pattern of adhesion was observed with the TR146 cell monolayer (Fig. 4b). The reintroduction of PHR1 into the Δphr1 mutant restored the adhesive properties in both assays (Fig. 4a, b). These results indicate that PHR1 deletion greatly affects the adhesive properties of C. albicans cells. The data do not show whether the majority of the mutant cells fail to adhere entirely or whether they exhibit a loose attachment unable to withstand washing. Unlike the invasion of epithelial cells, a single copy of the wild-type allele is sufficient for full adhesion on epithelia, indicating the lack of a gene dosage effect.

Expression of HWP1 and ECE1 is affected in the Δphr1 mutant

To determine whether the adhesion defects were associated with changes in the expression of genes involved in adhesion we monitored the expression of a few selected genes by qRT-PCR. We assayed the expression of two cell surface adhesin-encoding genes, ALS3 and EAP1. ALS3 is a member of the agglutinin-like ALS gene family, and is known to encode a differentially regulated cell surface glycoprotein that promotes fungal adhesion (reviewed by Chaffin, 2008). ALS3 is specifically expressed during hyphal development, and it is involved in cell aggregation and adhesion to epithelial and endothelial cells (Chaffin, 2008; Oh et al., 2005). Als3p is uniformly distributed on the germ tube and hyphal surfaces (Coleman et al., 2009; Hoyer et al., 1998). EAPI was chosen because it is required for binding
of *C. albicans* cells to polystyrene surfaces and to human epithelial cells (Li et al., 2007). In addition to these genes, we tested the expression of two hyphal growth-associated genes, *HWP1* and *ECE1*. *HWP1* is a target of mammalian transglutaminases and is required for adhesion to the mammalian cell surface (Staab et al., 1999). *ECE1* is highly expressed during hyphal formation and to an extent that depends on hyphal elongation (Chaffin, 2008; Li & Palecek, 2003, 2008). *ECE1* may also contribute to biofilm formation (Nobile et al., 2006). *ALS3*, *EAP1*, *HWP1* and *ECE1* are not essential for cell elongation or hypha formation (Chaffin, 2008; Sharkey et al., 2005). *ALS3*, *EAP1* and *HWP1* genes encode GPI-containing proteins, whereas the *ECE1* gene product has a secretory signal peptide but no GPI attachment signal.

To distinguish between a change of expression due to the shift to the hyphal growth-inducing conditions and a specific effect of adhesion to an abiotic surface, total RNA was isolated from cultures pre-grown to stationary phase in YPD-pH 6 (time zero) and 2 h after the shift to hyphal growth-inducing medium, in both suspension and adhesion conditions. As shown in Fig. 2(a), at this time the adhesion process was complete. For qRT-PCR analysis, *TDH3* was used as a reference transcript. This gene encodes glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme which is not affected by the loss of *PHR1*. The relative transcript levels were calculated as described in Methods. The data for the different strains in each of four different experiments were normalized to the wild-type (CAI-10) in suspension. The mean of the normalized values is reported in Fig. 5(a, b). *HWP1* and *ECE1* were specifically induced upon the shift to hyphal growth conditions, in agreement with previous results (Birse et al., 1993; Nobile & Mitchell, 2005; Sharkey et al., 1999), but the levels of their transcripts were reproducibly lower in the adhesion condition than in planktonic cells in all the strains. In comparison with the wild-type in suspension, the mutant showed a significant reduction of *HWP1* and *ECE1* transcript levels in both suspension and adhesion conditions. When the data were compared with the wild-type in the same condition, in the mutant the *HWP1* transcript in suspension and adhesion conditions was reduced to 35 and 44 %, respectively, and *ECE1* transcript levels to 52 and 32 %, respectively. Reintroduction of the wild-type copy of Phr1p restored the transcript levels, although with a higher reproducibility for *HWP1* than for *ECE1*. The exposure of wild-type cells to hyphal growth-inducing conditions triggered the expression of *ALS3* and increased the level of *EAP1* transcript. However, no significant differences were found between the wild-type and the mutant in both suspension and adhesion, and these genes were not further analysed (data not shown).

In conclusion, the transcription profiling of *ALS3*, *EAP1*, *HWP1* and *ECE1* revealed a similar response in adherent and planktonic cells. The reduction of *HWP1* and *ECE1* transcript levels at 2 h after the shift to hyphal growth-inducing conditions seems to be a consequence of the lack of *PHR1* and is not influenced by growth on surfaces.

**Deletion of *PHR1* affects the maintenance of hyphal growth**

To further understand the adhesion and invasion defects of the *PHR1* mutant, we re-examined the morphogenic

![Fig. 5](image-url)

**Fig. 5.** Modulation of *HWP1* and *ECE1* expression in adherent *Aphr1* cells. Transcriptional profiling of wild-type strain CAI-10 (white bars), *Aphr1* mutant strain CAS-10 (dark-grey bars) and its respective *phr1::PHR1* revertant strain CAS-11 (light-grey bars) after 2 h of adhesion to a polystyrene surface under hyphae-inducing conditions or in suspension culture. Cells were grown overnight in YPD-pH 6 at 30 °C and then shifted to DMEM supplemented with 10% FCS at 37 °C and 5% CO2 pH 7.4, to grow in suspension in a flask or on polystyrene plates for adhesion for 2 h. A calibrator, gene *TDH3*, encoding glyceraldehyde-3-phosphate dehydrogenase, was used (data not shown). All data from different strains in YPD-pH 6, suspension or adhesion were normalized to the wild-type in suspension, set as equal to 1. The mean ± SD of the normalized values obtained from four independent experiments are shown. For the reference strain, the sds from qRT-PCR were used. (a) *HWP1*, (b) *ECE1*. 

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defects of the mutant. To distinguish more accurately between true hyphae and pseudohyphae and to assess whether the mutant was defective in hyphal development or in maintenance of hyphal growth, we stained the cells for chitin and examined the position of the first septum, an observation that to our knowledge has never been described before. If the first septum was present in the mutant and it was in an advanced position in the germ tube, this would mean that the induction of the development of hyphae was normal, although the maintenance of hyphal growth was impaired.

As shown in Fig. 6(a), the germ tubes produced by the Δphr1 mutant at pH 7.4 at 2 h were shorter than those of the wild-type and were expanded laterally, giving rise to curved and distorted growth projections. It is clearly visible in Fig. 6(a) that the first septum is present in some cells and that it is placed in an advanced position. Moreover, the hypha was enlarged in the apical region proximal to the first septum, indicating an inability of the mutant to maintain hyphal elongation and the proper diameter of the hypha. This can be seen in more detail in Fig. 6(b). In addition, an intense fluorescence was noticed. This is consistent with the increase of chitin content previously reported in isolated cell walls of the Δphr1 mutants (Fonzi, 1999). The morphological defects were similar in adherent and planktonic cells, and Fig. 6 provides examples of cells in both conditions.

These results suggest that deletion of PHR1 in hyphal growth-inducing conditions does not affect the induction of hyphal development but severely impairs hyphal elongation, probably as a consequence of cell wall weakening.

**DISCUSSION**

Adhesion and invasion are two fundamental steps in C. albicans infections. In this work we have tested the ability of a PHR1 null mutant to invade an RHE. The mutant manifested a complete inability to penetrate the epithelium and invade the underlying matrix space. This particular behaviour provides a strong phenotypic trait that is important in assessing the role of PHR1 in C. albicans pathobiology. Despite the artificiality of the model system, it has been reported that the majority of genes upregulated in the RHE are also upregulated in C. albicans infecting the oral cavity of HIV-positive patients (Zakikhany et al., 2007). Moreover, the assay of in vitro invasion of epithelia represents an invaluable tool to dissect some properties of single mutants in a context which is devoid of detectable components of the immune system.

In this work we have also demonstrated that PHR1 null mutant cells display a remarkable reduction in the ability to adhere to both plastic surfaces and epithelial cell monolayers. The combined defects in adhesion and invasion that we report here are likely to contribute to the avirulence of the mutant that has been reported for a mouse model of systemic infection (De Bernardis et al., 1998). Consistent with a crucial role for PHR1 in the attachment to and invasion of epithelia, a recent large-scale analysis of the C. albicans transcriptome has highlighted the occurrence of the PHR1 transcript among the most induced genes during the progression of C. albicans infections of human oral epithelial cells (Wilson et al., 2009).

Other processes that are dependent on cell-mediated immunity are likely to contribute to the reported avirulence of a Δphr1 mutant. In this regard, it has been shown that the changes in composition and architecture of the cell wall caused by the lack of Phr2p activity unmask cell wall determinants that are recognized by receptors of immune cells (Wheeler & Fink, 2006; Wheeler et al., 2008). The accessibility of PHR2 null mutants to binding of anti-β-(1,3)-glucan antibodies, a property not shared by
wild-type cells, implicates β-(1,3)-glucan in a rapid clearance of the pathogen due to the increased immune response that it elicits (Coleman et al., 2009; Wheeler & Fink, 2006).

Adhesion and hyphal morphogenesis are two tightly linked processes in C. albicans and contribute to the establishment and progression of infections. On the cell wall surface, C. albicans exposes ligands of receptors present on human cells, as well as other molecules which are responsible for adhesion to epithelial and endothelial cells and to plastic, cell–cell aggregation and biofilm formation (Chaffin et al., 1998). Moreover, a general hydrophobicity of the cell wall surface also mediates the process of adhesion (Verstrepen 1998). Moreover, a general hydrophobicity of the cell wall surface may be dramatically modified and could affect the surface also mediates the process of adhesion (Verstrepen & Klis, 2006). In an attempt to explore the adhesion defects of a Δphr1 mutant at the molecular level, we tested the expression of a few marker genes. HWP1 is a well-known marker of hyphal-specific expression (Sharkey et al., 2005, 1999; Tsuchimori et al., 2000). ECE1, which shares a partial common regulation at the transcriptional level with HWP1 (Sharkey et al., 1999), encodes a cell surface protein and is upregulated as the hypha elongates. Both ECE1 and HWP1 are dispensable for hyphal development (Birse et al., 1993; Sharkey et al., 2005). We detected a reduction of HWP1 and ECE1 transcript levels in the PHR1-deleted strain irrespective of whether growth was in suspension or adhesion conditions, indicating that lack of PHR1 causes a general defect in the expression of both genes during hyphal growth-inducing conditions.

Interestingly, reduced expression of hyphal-specific genes has so far been reported for many null mutants that are defective in transcription factors, such as Efg1p or Cph1p, but has never been reported to our knowledge for a cell wall-related mutant. Future investigation will assess whether the decrease in HWP1 and ECE1 transcript levels is a consequence of a failure to sustain hyphal morphogenesis or of the activation of a repressive pathway. The reduced adhesion of Δphr1 cells to cell monolayers or abiotic surfaces could result from an altered level of Hwp1p and Ece1p that together could affect the adhesion surface layer and also disturb the cell wall structure. However, other adhesin-encoding genes, namely ALS3 and EAP1, were not affected in the Δphr1 mutant compared with the wild-type, both in adherent and planktonic cells. Although we did not detect differences in the transcript levels of ALS3 in the PHR1 null mutant, further experiments would be necessary to define whether ALS3 expression is unaffected in the mutant or whether a higher stability of ALS3 mRNA, compared with HWP1 and ECE1 mRNA, masks a reduction in ALS3 expression.

Regarding the cell surface proteins, it should be recalled that the Δphr1 mutation may affect their surface localization as a consequence of the lack of β-(1,3)-glucanprocessing activity. In the absence of Phr1p activity, the proper anchoring and specific localization of surface mannoproteins is altered as mannoprotein linkages are diverted to chitin (Fonzi, 1999). As a consequence, the structure of the cell wall surface may be dramatically modified and could affect the complex molecular mechanism of adhesion. In this regard, it has recently been shown that an anti-Als3p monoclonal antibody can block the adhesion of C. albicans to host cells, suggesting that exposure of the protein epitopes is crucial for the adhesion process (Coleman et al., 2009). A deep analysis of the hyphal wall structure in the Δphr1 mutant would be necessary to definitively confirm this point.

In addition, Phr1p has been identified as a component of the cell wall proteome in vegetative cells (de Groot et al., 2004), and can be detected as an extractable cell wall protein (Urban et al., 2003). This suggests that Phr1p is anchored to the plasma membrane through GPI, although a fraction could undergo a transglycosylase reaction at the cell surface and be cross-linked to the glucan network, as occurs for other cell wall mannoproteins and for the Gas1 homologous protein (De Groot et al., 2005; Rolli et al., 2009). Phr1p could be exposed to the cell surface and remodel the outer surface layer, thereby affecting the position of other cell wall mannoproteins also.

Finally, the microscopy analyses provided evidence for the presence of the first septum in germ tubes emerging from Δphr1 mutant cells. The finding that the first septum is formed in many Δphr1 mutant germ tubes at 2 h after the shift to hyphal growth-inducing conditions suggests that the mutant is not defective in the induction of hyphal development but in the maintenance of hyphal growth.

In conclusion, the inability of the mutant to sustain elongation and hyphal growth suggests that β-(1,3)-glucan processing catalysed by GH72 enzymes is of fundamental importance to the maintenance of the morphological state on which the adhesive and invasive properties of C. albicans greatly depend.

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