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

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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)-glucanotransferases 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.

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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,

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Abbreviations: GPI, glycosylphosphatidylinositol; qRT-PCR, real-time quantitative RT-PCR; RHE, reconstituted human epithelium.

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 laminarioligosaccharide 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, $\Delta phr1$ cells have the same phenotype as the wild-type. As pH increases above 6, $\Delta phr1$ cells exhibit a defective phenotype which also becomes more severe at higher pH values. In the yeast form, at pH 7.5 or 8, $\Delta phr1$ cells appear round, larger than the wild-type, with a swollen aspect similarly to that of *S. cerevisiae* cells deleted in *GAS1* (Popolo *et al.*, 1993; Saporito-Irwin *et al.*, 1995). In conditions of hyphal growth, $\Delta phr1$ 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/ura3Δ::imm434*), derived from CAF-3 (Fonzi & Irwin, 1993); CAS-10 (*phr1Δ::hisG/phr1Δ URA3/ura3Δ::imm434*), derived from CAS-8, a *Phr1*⁺ derivative of CAF3-1 (Saporito-Irwin *et al.*, 1995); and CAS-11 (*phr1Δ::hisG/PHR1-pUC18-URA3-phr1Δ ura3Δ::imm434/ura3Δ::imm434*), a *Phr1*⁺ derivative of CAS-8 (Saporito-Irwin *et al.*, 1995). The same strains have previously been used to test the virulence of $\Delta phr1$ mutants (De Bernardis *et al.*, 1998). For the adhesion and invasion assays, cells were cultured 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% heat-inactivated fetal calf serum (FCS) and 1 mM sodium pyruvate, was used. Cells were cultivated at 37 °C under 5% CO₂ until they reached confluence.

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 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 × DMEM, 0.25 M HEPES, 0.8% NaHCO₃, 20% FCS, 0.22 M NaOH, 0.1 mg gentamicin ml⁻¹) to form a solid gel of pH ~7.4. Collagen mixture with gelling solution (300 µl) was left to solidify in 24-well plate inserts (ThinCert, Greiner) for 15 min at 37 °C under 5% CO₂. Each insert was transferred to a well of a 24-well plate containing 0.5 ml DMEM supplemented with 10% FCS and 1 mM sodium pyruvate (pH 7.4), and overlaid with 100 µl supplemented DMEM containing 1 × 10⁵ Caco-2 cells. After 2 days of cultivation the inserts were infected with 10 µl (~2.8 × 10⁴ cells) of stationary phase *Candida* cells in YPD/150 mM HEPES, pH 6.0, diluted into deionized H₂O (dH₂O) to OD₆₀₀ 0.1. After 7 and 24 h the cultures were fixed for 1 h in Bouin's solution, embedded into paraffin blocks and sectioned (thickness 3 µm, Leica RM 2145 microtome). Samples were stained with periodic acid-Schiff stain and evaluated under a light microscope (Zeiss, Axiovert 200M).

Adhesion assays to abiotic surfaces and cell monolayers. The adhesion assays were set up in 24-well polystyrene plates (Greiner). The cavities were either untreated or pre-treated to establish a confluent monolayer of Caco-2 or TR146 cells on the bottom of the well, using supplemented DMEM. Once the plates were ready for the experiment, the culture medium was removed and 250 µl 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 buffered 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 × 10² 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 Axiovert 25 microscope and photographed with a Zeiss AxioCam MRm digital camera. For chitin 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 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 RNeasy 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 ΔC_T value was calculated by subtracting the

Table 1. Oligonucleotide sequences used

Gene	Left primer (5'–3')	Right primer (5'–3')
<i>TDH3</i>	gccgtcaacgatccattc	agaatcgattttgaacatgtaagca
<i>HWPI</i>	attgctccaggtgctgaaac	ttccggaatagtaatagcaccac
<i>ECE1</i>	tcaagctgccatcatccac	gcagctggagcaacatctct
<i>ALS3</i>	tgaatattggtctcaatcatttgc	gtgtttctggtgttcctcagt
<i>EAPI</i>	ccactccaattcaaccaaca	tcgtgtaggaggtagattcaacaa

mean C_T value for *TDH3* from the mean C_T value of the gene of interest. For relative quantification of the abundance of *ALS3*, *EAP1*, *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 $2^{-\Delta\Delta C_T}$ 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

($\Delta phr1/\Delta phr1$) 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 $\Delta phr1$ phenotype; (ii) the phenotype of $\Delta phr1$ is not yet manifested at pH 6. As described in detail in a previous work (Saporito-Irwin *et al.*, 1995), the phenotype of $\Delta phr1$ 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

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 $\Delta phr1$ 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 $\Delta phr1$ cells in adhesion assays.

A. C. albicans $\Delta phr1$ 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 $\Delta phr1$ 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 $\Delta phr1$ mutant is pH-dependent, and there is a phenotypic lag in expression

Fig. 2. Adhesion of CAI-10, CAS-10 and CAS-11 strains to polystyrene surfaces. The adhesion to polystyrene assay was performed as described in Methods. (a) DMEM supplemented with 10% FCS at 37 °C, pH 7.4. (b) DMEM buffered with 25 mM MES at pH 6 supplemented with 10% FCS at 37 °C. The results shown are the mean $\pm$ SD of three separate experiments.

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 $\Delta phr1$ 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 $\Delta phr1$ mutant is not manifested at pH 6 (Saporito-Irwin *et al.*, 1995). Thus, the adhesion defect was expected to be absent. At this

Fig. 3. Morphology of *C. albicans* strains adhering to polystyrene surfaces. Cells of the indicated strains were photographed at time zero and at 2 h after their transfer from a pre-culture in YPD at pH 6 and 30 °C 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. Plates were kept static and both adherent and sedimented cells are shown. (a, b and c) YPD-pH 6, (d, e and f) suspension at 2 h, (g, h and i) adhesion at 2 h.

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 $\Delta phr1$ 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 $\Delta phr1$ 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. $\Delta phr1$ 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 $\Delta phr1$ 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

Fig. 4. Adhesion of CAI-10 (Phr1 +/+), CAS-10 (Phr1 -/-) and CAS-11 (Phr1 +/-) strains to monolayers of human enterocytes (Caco-2) (a) and buccal epithelial cells (TR146) (b). The adhesion assay was carried out as described in Methods. The data are the mean $\pm$ SD of three independent experiments.

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 $\Delta phr1$ 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). *EAP1* 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 hypha 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. Modulation of *HWP1* and *ECE1* expression in adherent $\Delta phr1$ cells. Transcriptional profiling of wild-type strain CAI-10 (white bars), $\Delta phr1$ 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. As 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 $\pm$ 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*.

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 $\Delta phr1$ 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 $\Delta phr1$ 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 $\Delta phr1$ 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

Fig. 6. Analysis of the position of the first septum in $\Delta phr1$ cells. Cells were pre-grown in YPD-pH 6 at 30 °C and shifted to supplemented DMEM, pH 7.4, as described in the legend to Fig. 5. (a) $\Delta phr1$ cells from a culture in suspension conditions for 2 h. Cells were mildly sonicated and stained with Calcofluor White. The inset shows wild-type cells (CAI-10). Bar, 12 μ m. (b) Cells grown in adhesion conditions for 2 h were scraped, mildly sonicated and stained with Calcofluor White. The micrographs show the details of the presence of septa in the indicated strains. Arrows indicate the first septum. Bar, 10 μ m.

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 & Klis, 2006). In an attempt to explore the adhesion defects of a $\Delta phr1$ 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 $\Delta phr1$ cells to cell monolayers or abiotic surfaces could result from an altered level of *Hwp1p* and *Ece1p* that together could affect the adhesin surface layer and also disturb the cell wall structure. However, other adhesin-encoding genes, namely *ALS3* and *EAP1*, were not affected in the $\Delta phr1$ 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 $\Delta phr1$ mutation may affect their surface localization as a consequence of the lack of β -(1,3)-glucanotransferase 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 $\Delta phr1$ 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 $\Delta phr1$ mutant cells. The finding that the first septum is formed in many $\Delta phr1$ 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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