Characterization of a cell-wall acid phosphatase (PhoAp) in *Aspergillus fumigatus*

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In the filamentous fungus *Aspergillus fumigatus*, the vast majority of the cell-wall-associated proteins are secreted proteins that are in transit in the cell wall. These proteins can be solubilized by detergents and reducing agents. Incubation of a SDS/β-mercaptoethanol-treated cell-wall extract with various recombinant enzymes that hydrolyse cell-wall polysaccharides resulted in the release of a unique protein in minute amounts only after incubation of the cell wall in the presence of 1,3-β-glucanase. Sequence analysis and biochemical studies showed that this glycoprotein, with an apparent molecular mass of 80 kDa, was an acid phosphatase (PhoAp) that was active on both phosphate monoesters and phosphate diesters. PhoAp is a glycosylphosphatidylinositol-anchored protein that was recovered in the culture filtrate and cell-wall fraction of *A. fumigatus* after cleavage of its anchor. It is also a phosphate-repressible acid phosphatase. The absence of PhoAp from a phosphate-rich medium was not associated with a reduction in fungal growth, indicating that this cell-wall-associated protein does not play a role in the morphogenesis of *A. fumigatus*.

Keywords: GPI protein, 1,3-β-glucan

INTRODUCTION

The vast majority of proteins associated with the fungal cell wall are secreted proteins that are transiently found in the cell wall before they are secreted into the extracellular environment (Klis, 1994). Most of these proteins have enzymic functions required for the fungus to grow in different natural environments. Recent studies have also suggested that in yeast several proteins are covalently linked to the cell-wall β-glucan (Kapteyn et al., 1999). Some of these proteins (i.e. the Pir proteins) are released from the cell wall by β-elimination and are supposed to be O-linked directly to 1,3-β-glucan (Mrsa et al., 1997; Kandasamy et al., 2000). Other 1,3-β-glucan-linked proteins are released from the cell wall by glucanase treatment and are also sensitive to hydrofluoric acid treatment. Initially, these proteins are anchored by glycosylphosphatidylinositol (GPI) to the plasma membrane and are linked to 1,3-β-glucan via 1,6-β-glucan through GPI remnants (van der Vaart et al., 1995; Kapteyn et al., 1995, 1996; Fujii et al., 1999). Anchoring of these proteins to the cell-wall polysaccharides is functionally important, since it ensures their localization at the surface of the cell wall. In most cases, even in an extended configuration, a cell surface location will never be achieved if these proteins remain bound to the plasma membrane by their GPI anchor (Stratford, 1994). The cell-wall-associated protein that has been studied most is the sexual agglutinin (AGz1) that is required for sexual conjugation in *Saccharomyces cerevisiae* (Lipke et al., 1989; Lu et al., 1994). Other cell-wall-associated proteins are thought to be involved in fungal cell wall–host cell interactions, e.g. the agglutinin-like sequences (Als proteins) of *Candida albicans* (Hoyer et al., 1999; Kapteyn et al., 2000). It has been suggested repeatedly that at least some cell-wall-associated proteins play a role in cell-wall organization, but mutants lacking these proteins are perfectly viable and are unaffected in their vegetative growth (van der Vaart et al., 1995). As the studies described above have been performed in yeast and because cell-wall-associated

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**Abbreviations**: AfPhoAp, acid phosphatase of *A. fumigatus*; GPI, glycosylphosphatidylinositol; P, inorganic phosphate; PNGase, peptide-N-glycosidase.

The GenBank accession number for the *A. fumigatus* PHOA sequence reported in this paper is AF462065.
proteins have not been carefully investigated in filamentous fungi (Schoffelmeer et al., 2001; Cao et al., 1998), we have characterized an acid phosphatase (PhoAp) that is the only protein released upon treatment of the cell wall of the opportunistic fungal pathogen *Aspergillus fumigatus* with glycosylhydrolases.

**METHODS**

**Preparation of the mycelium and cell wall.** *A. fumigatus* CBS 144-89 was grown in a 15 l fermenter in liquid medium containing 2% glucose and 1% mycopeptone (Biokar) as described previously (Latgé et al., 1994). After 24 h of culture (within the exponential growth phase), the mycelium was collected by filtration and washed extensively with water. The mycelium was then resuspended in a 200 mM Tris-HCl (pH 7.8) buffer containing 20 mM EDTA and 1 mM PMSF. One-millimetre-diameter glass beads were added to the suspension and the mycelium was disrupted by using a Dyno-mill cell homogenizer at 4°C. The disrupted mycelial suspension was centrifuged at 5500 r.p.m. for 10 min. The cell-wall pellet was recovered, washed five times in the buffer described above and then stored at −20°C.

**Protein isolation from culture medium.** Proteins were precipitated from the culture filtrates by the addition of 4 vols of ethanol to the medium, and the culture filtrates were left overnight at 4°C. The pellet was then recovered by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.5) containing 20 mM EDTA.

**Protein extraction from the cell wall.** Non-covalently bound proteins were removed from the cell-wall pellet by boiling 1 g of wet cell walls (150 mg dry weight) in 10 ml of a 50 mM Tris-HCl (pH 7.4) buffer containing 5 mM sodium azide and 500 U of Quantzyme (recombinant endo-1,3-β-glucanase; Quantum Biogene) and incubated for 16 h at 37°C; (ii) 100 mg of dry cell wall was treated with 0.06 U of recombinant chitinase A from *Serratia marcescens* produced in *Escherichia coli* and purified as previously described (Vorgias et al., 1993) for 3 days at 37°C in a 50 mM Tris-HCl (pH 8.0) buffer containing 1 mM EDTA and 5 mM sodium azide; (iii) 100 mg of dry cell wall was treated with 8 mg (16 U mg⁻¹) of recombinant 1,3-α-glucanase from *Trichoderma harzianum* produced in *Aspergillus oryzae* (Fuglsang et al., 2000) for 16 h at 37°C in a 50 mM sodium acetate (pH 5.6) buffer containing 5 mM sodium azide; and (iv) 100 mg of dry cell wall was resuspended in 50 mM sodium acetate (pH 5.0) buffer and digested with 5 U of 1,6-β-glucanase (Glyko) for 16 h at 37°C. For each of the glycosylhydrolase treatments, enzymic hydrolysis was stopped by boiling the samples for 10 min in the extraction buffer. Solubilized proteins were recovered in the supernatant after centrifugation at 4000 r.p.m. for 10 min.

The SDS/β-mercaptoethanol-extracted cell-wall sample was also treated with ice-cold hydrofluoric acid (50%, v/v) on ice for 3 days in a cold room at 4°C (Ferguson, 1992). The hydrofluoric acid was then removed from the sample by centrifugation. The pellet was washed seven times with methanol, dried under N₂ after each wash and then extracted with SDS/β-mercaptoethanol.

**Protein extraction from intact mycelium.** The mycelium was incubated in a 50 mM Tris/HCl (pH 7.5) buffer containing 50 mM DTT, 2 mM EDTA and 1 mM PMSF for 2 h at 4°C, with shaking. Intact mycelium was also incubated in the same buffer with 1 µg trypsin (Sigma) (mg mycelium)⁻¹ for 2 h at 25°C. Solubilized proteins were separated from the mycelium by filtration and stored at −20°C.

**Protein analysis.** Solubilized material was precipitated with 4 vols ethanol, lyophilised and boiled in Laemmli buffer (Laemmli, 1970), before undergoing electrophoresis on a 10% polyacrylamide separating gel or a pre-packed 4–12% polyacrylamide gel (NuPAGE Birst gel from Novex). Proteins were visualized by Coomassie blue or silver nitrate staining. Preliminary assays have shown that no difference was seen between SDS-PAGE patterns of a soluble extract precipitated by ethanol or concentrated under vacuum and dialysed. To estimate the amount of protein released by the different treatments, soluble material was precipitated with 4 vols of ethanol, dried under vacuum and resuspended in water. The protein concentration was quantified using the Bio-Rad Protein Assay with BSA as a standard either directly or after subtraction of the amount of protein corresponding to the different glycosylhydrolases (when added).

For Western-blot analysis, proteins were blotted onto a nitrocellulose membrane. The membrane was blocked with 5% defatted milk powder (Regilait) in TBS containing 0.05% Tween 20 and 5 mM EDTA and incubated with an anti-1,3-β-glucan (Australia Biosupplies) mouse mAb, an anti-cross-reactive-determinant rabbit polyclonal antibody (Oxford Glycosystem) or rabbit antisera against catalase, dipeptidyl peptidase V, RNase and acid phosphatase. Immunolabelling was visualized using the ECL Chemiluminescence Detection Kit, following the manufacturer’s instructions (Amersham Pharmacia Biotech).

The anti-1,3-β-glucan, a mouse mAb, was diluted to 1/10000 in TBS containing 0.05% Tween 20, 5 mM EDTA and 5% milk. Anti-phoA is a polyclonal antibody that was raised by Eurogentec (Herstal, Belgium) in rabbits against two internal peptides of PhoAP (TFDDEGTYSKSNKI and PDELKGTQD-DFTFYT) that were coupled to an m-maleimidobenzenoyl N-hydroxysuccinimide ester through a cysteine residue. It was diluted to 1/1000. The anti-cross-reactive-determinant antibody was diluted to 1/100. The polyclonal rabbit antisera was raised against purified 18 kDa RNase (Latgé et al., 1991) and the purified recombinant catalase and dipeptidyl peptidase V proteins produced in *Pichia pastoris* (Calera et al., 1997; Beauvais et al., 1997). For immunization, 250 µg of protein was injected intradermally into the rabbits in Freund’s complete adjuvant. The animals were boosted (1–3 times) at 2-week intervals with the same amount of protein in Freund’s incomplete adjuvant.

**Sequencing.** After in-gel digestion of the proteins that had been separated by gel electrophoresis (see above) with endolysin C, internal peptide sequencing was performed by J. d’Alayer (Laboratoire de microsequencage des protéines, Institut Pasteur, Paris) on an Applied Biosystems 470 gas-phase sequencer, as described previously (Beauvais et al., 1997).

The position of the *A. fumigatus* PHOA (APHOA) introns was determined after amplification of a cDNA clone obtained from a cDNA library of *A. fumigatus* (kindly provided by Dr Monod) by PCR, using primers deduced from the genomic
DNA sequence of clone 719 (cl719). The forward primer P1 (5’-ATGAGGCTTCCGGTGGG-3’) complementary to nucleotides 459–476 of the genomic DNA of cl719 and the reverse primer P1 (5’-AAGCAGTGGTTAGCCGA-3’) complementary to nucleotides 1895–1912 of the genomic DNA of cl719 were used in the amplification, as described by Mouyna et al. (2002). Homologues of PhoAp were searched for in the SWISS-PROT databases (Worley et al., 1995). The nucleotide sequence of AIPHOA reported in this paper has been deposited in GenBank under accession no. AF462065.

**Glycopeptidase (PNGase) treatment.** Deglycosylation of the protein samples was carried out using a recombinant PNGase F (Roche). After protein denaturation by boiling in 1% SDS for 20 min, the samples were adjusted to 0.8% n-octyl glucoside, 0.1% SDS and 100 mM β-mercaptoethanol and incubated overnight at 37 °C with PNGase F (5 U per 100 µl of trypsin extract or Quantzyme extract containing 1–2 µg protein). Digestion was terminated by precipitating the proteins with 4 vols of ethanol.

**Enzyme assays.** Acid phosphatase activity in the samples was assayed as described for the samples. For each sample, the amount of inorganic phosphate (P<sub>i</sub>) released was measured by using 5 mM bis-(p-nitrophenyl) phosphate (Sigma) as a substrate. The reaction mixture contained (in a total of 100 µl) 5 mM Mg<sub>2</sub>O, 50 µM sodium acetate buffer (pH 6.0) and 2 µl of the acid phosphatase solution containing 10–20 ng protein. Incubation was performed at 37 °C for 30 min. The reaction was stopped by the addition of 100 µl of 1 M Na<sub>2</sub>CO<sub>3</sub> to the sample; for each sample, the amount of p-mercaptoethanol released by the monoesterase activity was determined by measuring the absorbance value at 414 nm.

Phosphodiesterase activity within the samples was measured by using 5 mM bis-(p-nitrophenyl) phosphate sodium salt (Sigma) (O’Brien et al., 2001) or 5 mM thymidine 5’-monophosphate p-nitrophenyl ester sodium salt (Sigma) as a substrate (Gijsbers et al., 2001). The protocol was the same as that described above, except that the reactions were stopped by the addition of 100 µl of 5% Na<sub>2</sub>CO<sub>3</sub> to the samples.

The release of inorganic phosphate (Pi) from glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, ATP, UMP and UDP by the proteins within the samples was assayed as described for p-nitrophenyl phosphatase in a 50 mM sodium acetate (pH 6.0) buffer containing 5 mM substrate. Released P<sub>i</sub> was measured by the method of Ames (1966).

Compounds tested as inhibitors (EDTA, MgCl<sub>2</sub>, CaCl<sub>2</sub>, ZnCl<sub>2</sub>, HgCl<sub>2</sub>, NaF, sodium molybdate, sodium orthovanadate, ammonium tartrate and DTT) of the proteins within the samples were used at a concentration of 10 mM. SDS was also tested as an inhibitor of protein activity (1% and 0.1%, w/v).

**HPLC purification.** The trypsin extract of the mycelium was analysed by gel filtration to isolate PhoAp. Gel filtration chromatography was performed on a Superdex 75 HR 10/30 column (Pharmacia) in 50 mM Tris/HCl (pH 7.5) containing 120 mM NaCl at a flow rate of 0.5 ml min<sup>-1</sup>.

**RESULTS**

**Extraction of an 80 kDa polypeptide from the cell wall of A. fumigatus by treatment with 1,3-β-glucanase**

Treatment of the cell wall of A. fumigatus with 2% SDS and 40 mM β-mercaptoethanol released non-covalently associated proteins (Fig. 1). The first SDS/β-mercaptoethanol treatment released about 3% of the cell-wall dry weight. Protein release from the cell-wall sample increased continuously following successive SDS/β-mercaptoethanol treatments. By the fourth treatment, some soluble proteins were still released from the cell wall, but they were not detectable by Coomassie blue staining (data not shown). The total amount of protein released from the cell-wall sample of A. fumigatus by the four successive SDS/β-mercaptoethanol treatments accounted for a maximum of 3.5% of the cell wall (by dry weight). Western blots with antiserum directed against three secreted proteins (namely 90 kDa catalase, 88 kDa dipeptidyl peptidase and 18 kDa RNase) showed three bands which corresponded to the molecular masses of these proteins (Fig. 1). These results showed that among the proteins extracted from the cell wall of A. fumigatus by SDS/β-mercaptoethanol treatments there are secreted proteins that are transiently associated with the cell wall.

The SDS/β-mercaptoethanol-extracted cell-wall sample was enzymically hydrolysed by treatment with a 1,3-β-glucanase, a chitinase, a 1,3-α-glucanase or a 1,6-β-glucanase. Treatments with the chitinase, 1,3-α-glucanase or 1,6-β-glucanase did not release any specific proteins (Fig. 2). Unexpectedly, proteins were released during incubation of the cell-wall extract in the buffer alone at 37 °C and no difference could be seen between the pattern of the proteins released in the buffer alone and the patterns generated after the addition of the glycosylhydrolases to the buffer (Fig. 2). The only differences in the banding patterns were due to the added glycosylhydrolases themselves (bands highlighted by solid circles in Fig. 2). In addition, the longer the incubation time the higher the amount of proteins released, when the cell-wall sample was incubated in buffer alone and in the presence of the glycosylhydrolases. However, the protein patterns produced were

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**Fig. 1.** Analysis of proteins released from A. fumigatus by SDS/β-mercaptoethanol treatment and separated on a 10% polyacrylamide gel. Each lane was loaded with the amount of protein extracted from 0.7 mg of dry cell wall. Lanes: 1, proteins stained with Coomassie blue; 2, protein immunoblotted with anti-catalase; 3, protein immunoblotted with anti-dipeptidyl peptidase; 4, protein immunoblotted with anti-RNase.
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generally similar with or without the addition of the glycosylhydrolases. These results show that in spite of several SDS/β-mercaptoethanol treatments, soluble proteins still remain associated with the cell wall of *A. fumigatus*. Western blots with antisera directed towards the three secreted proteins (i.e. catalase, dipeptidyl peptidase and RNase) confirmed that these proteins were also present in the extracts from the cell wall treated with the enzymes and buffer (data not shown).

A single diffuse band between 75 and 90 kDa was seen when the SDS/β-mercaptoethanol-extracted cell-wall pellet was digested with 1,3-β-glucanase (Fig. 2, lane 4a, indicated by an arrow head). This band could be stained by Coomassie blue but was barely seen with the silver nitrate stain, and it represented less than 0-1% of the cell-wall dry weight compared to the soluble proteins which accounted for 3–4% of the cell-wall dry weight. The whole of this diffuse band was used for peptide sequencing and only one protein was identified from the band. Hence, the band was not contaminated by other proteins.

An antibody directed against 1,3-β-glucan showed reactivity between 75 and 90 kDa, when used for a Western blot of the diffuse protein band (Fig. 3a). Thus, the protein extractable by treatment of the cell wall with 1,3-β-glucanase seemed to be strongly associated with the cell-wall 1,3-β-glucan. The wide band seen with Coomassie blue staining was associated with the heterogeneity of the glucan hydrolysed by the 1,3-β-glucanase Quantazyme. If the Quantazyme extract was then processed using another recombinant 1,3-β-glucanase (Zverlov *et al*., 1997), a single 80 kDa band was seen (data not shown).

Deglycosylation of the Quantazyme extract with a PNGase F was associated with a shift in the 1,3-β-glucan immunolabelled band to 58 kDa (Fig. 3a), showing that PhoAp is N-glycosylated but that glucan binding to PhoAp is not associated with N-glycosylation.

**Molecular characterization of the 80 kDa cell-wall-associated protein**

Amino-acid sequencing of the entire polypeptide band produced by electrophoresis of the Quantazyme-treated cell-wall sample showed that the band contained only one protein. An internal amino-acid sequence was obtained – IFSVLLGAIPDELK. This polypeptide sequence was found in one clone, cl719, in the TIGR genomic database of *A. fumigatus* (http://www.tigr.org/cgi-bin/BlastSearch). The ORF deduced from the cDNA sequence of cl719 was 1341 nt long and had two putative introns of 53 and 60 bp which started at nucleotides 187 and 302, respectively. The positions of the putative introns were confirmed by PCR amplification. The ORF encoded a polypeptide of 447 aa residues with a theoretical molecular mass of 53 kDa (Fig. 4). Twelve potential N-glycosylation sites were located at amino-acid residues 119, 150, 177, 186, 208, 217, 234, 240, 315, 332, 382 and 405 of the 447 aa polypeptide. The high number of N-glycosylation sites was in agreement with the shift observed when the 1,3-β-glucanase-extracted protein was treated with PNGase F. After treatment with PNGase F the polypeptide migrated with an apparent molecular mass of 58 kDa, which is in accordance with its theoretical molecular mass of 53 kDa (Fig. 3). The hydrophathy profile of the protein showed the presence of a signal peptide at its amino terminus. Using the (−3, −1) rule of Von Heijne (1986),
A cell-wall acid phosphatase in *A. fumigatus*

**Fig. 4.** Comparison of the predicted amino-acid sequence of AfPhoAp with its closest relatives. Aniger, P34724 of *A. niger*; Kmarxianus, JC7179 of *K. marxianus*; Kmarxianus2, P08540 of *K. marxianus*; Pchrysogenum, P37274 of *P. chrysogenum*; Mtuberculosis, E70842 of *M. tuberculosis*. Identical residues are indicated by solid boxes; similar residues are indicated by grey boxes. The amino hydrophobic terminus of AfPhoAp is indicated by (1). Its putative peptide cleavage site is indicated by an arrow. The 12 potential N-glycosylation sites of AfPhoAp are each indicated by an asterisk. The putative active site containing histidine and aspartic acid is indicated by §. The peptide that was sequenced is indicated by (2).

the protein from *A. fumigatus* was predicted to have a signal peptide cleavage site at residue 20, an alanine. One hydrophobic domain at the carboxy terminus of the *A. fumigatus* protein was associated with a serine/threonine-rich region, a characteristic of GPI-anchored proteins. Indeed, recent biochemical studies have shown that this membrane protein can be labelled with an anti-cross-reactive determinant, a characteristic of all GPI-anchored proteins (Bruneau et al., 2001). Based on the consensus predicted cleavage of the GPI anchor (Gerber et al., 1992), the ω, ω + 1, ω + 2 site for GPI attachment could be N<sub>448</sub> A<sub>422</sub> A<sub>425</sub> (Fig. 4).

BLAST searches of the SWISS-PROT databases showed that the acid phosphatase protein of *A. fumigatus* (AfPhoAp) has significant identity with fungal acid phosphatases (Fig. 4), namely acid phosphatase P34724 of *Aspergillus niger* (62% identity; Ehrlich, 1994), acid phosphatase P37274 of *Penicillium chrysogenum* (60% identity; Haas et al., 1991, 1992) and acid phosphatases JC7179 and P08540 of *Kluyveromyces marxianus* (56 and 51% identity, respectively; Yoda et al., 2000). AfPhoAp also showed a lower level of identity with bacterial acid phosphatases, such as E70842 of *Mycobacterium tuberculosis* (Saleh & Belisle, 2000).
peptides of the two polypeptides and of the PhoAp molecular species of PhoAp. The sequences of the major polypeptides were sequenced and were shown to be two reacted positively with the anti-phoA antibody. These The two polypeptides contained within these bands trypsin, two bands, with apparent molecular masses of culture filtrate or mycelial extract was treated with form, AfPhoAp was resistant to trypsin. When a total culture filtrate of actively growing cultures. In its native absence of DTT; and (iii) PhoAp was found in the was readily released from the intact mycelium when it wet mycelium (6 mg dry mycelium) in a Tris buffer at 4 °C during active growth (Fig. 5a). Evidence for Localization of AfPhoAp Western-blot analysis using an anti-phoA serum showed that PhoAp was secreted into the external medium by A. fumigatus during active growth (Fig. 5a). Evidence for this includes the following: (i) PhoAp was present in the SDS/β-mercaptoethanol cell-wall extract; (ii) PhoAp was readily released from the intact mycelium when it was incubated at 4 °C in a Tris buffer in the presence or absence of DTT; and (iii) PhoAp was found in the culture filtrate of actively growing cultures. In its native form, AfPhoAp was resistant to trypsin. When a total culture filtrate or mycelial extract was treated with trypsin, two bands, with apparent molecular masses of 75 and 60 kDa, were detected after SDS-PAGE (Fig. 5b). The two polypeptides contained within these bands reacted positively with the anti-phoA antibody. These polypeptides were sequenced and were shown to be two molecular species of PhoAp. The sequences of the major peptides of the two polypeptides and of the PhoAp released by 1,3-β-glucanase from the cell wall of A. fumigatus were identical (IFSVLLGGAIPDELK). These forms of PhoAp released into the culture filtrate did not react with the anti-1,3-β-glucan antibody. None of the PhoAp species isolated from the cell wall or secreted into the culture filtrate reacted with the anti-cross-reactive-determinant antibody (data not shown), in contrast to membrane-associated species released by an endogenous phosphatidylinositol-specific phospholipase C (PI-PLC) treatment (Bruneau et al., 2001), suggesting that extra-cellular and cell-wall-associated forms of PhoAp were released from the membrane by proteolytic cleavage.

Analysis of the phosphatase activity of AfPhoAp

The acid phosphatase activity of AfPhoAp was analysed by using mycelial extracts that had been treated with trypsin, since the phosphatase activity due to AfPhoAp was easy to recover from a complex mixture following a trypsin digest.

AfPhoAp was active against both mono- and diphosphate esters, producing $K_m$ values of 1.45 and 2.3 mM for $p$-nitrophenyl phosphate and bis-($p$-nitrophenyl) phosphate sodium salt, respectively. These data suggest that PhoAp had both a phosphomonoesterase and a phosphodiesterase activity.

The enzyme was active from pH 3 to 7, with its optimum activity occurring between pH 4 and 6. A slight difference in the optimum pH was observed for the two substrates tested. AfPhoAp was most active at pH 5.5 when $p$-nitrophenyl phosphate was the substrate, whereas it was most active at pH 5 when bis-($p$-nitrophenyl) phosphate sodium salt was the substrate. The tendency of the optimum pH of AfPhoAp to change depending on the substrate used was confirmed by testing the phosphodiesterase activity of AfPhoAp on thymidine 5′-monophosphate $p$-nitrophenyl ester sodium salt, where its activity was maximal at pH 4. AfPhoAp had no significant activity above pH 7, confirming that it is an acid phosphatase.

The phosphatase activity of AfPhoAp was not substrate-dependent, since it was able to cleave a broad range of phosphate esters, including glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, ATP, UMP, UDP and thymidine 5′-monophosphate $p$-nitrophenyl.

AfPhoAp did not require metal ion cofactors and was not inhibited by thiol reagents (such as DTT), SDS or metal chelators at the concentrations tested. It was partially inhibited by heavy metal ions such as Zn$^{2+}$ (68% inhibition) and Hg$^{2+}$ (56% inhibition), totally inhibited by 10 mM vanadate, and 60 and 85% inhibited by 10 mM NaF and molybdate, respectively. It was not inhibited by tartrate.

The secretion of PhoAp into the culture medium was repressed when A. fumigatus was grown in the presence of $P_i$. As shown in Fig. 6, PhoAp was not produced when the culture medium contained a concentration of $P_i$ between 1 and 100 mM. In contrast, PhoAp was secreted in high amounts at low $P_i$ concentrations (100 and 10 μM). No A. fumigatus growth was observed at $P_i$ concentrations below 10 μM. These results indicate that AfPhoAp is a phosphate-repressible acid phosphatase.
The extraction protocols used to recover PhoAp from the cell wall of *A. fumigatus* suggested that this protein is strongly associated with the cell-wall 1,3-β-glucans. This association could result from covalent linkages, strong ionic interactions or lectin interactions. There are several arguments that favour a covalent linkage between PhoAp and the 1,3-β-glucans. (i) Release of PhoAp from the cell wall was seen after 1,3-β-glucanase treatment. (ii) A band migrating with the apparent molecular mass of PhoAp was also labelled by the anti-1,3-β-glucan antibody; this labelling remained after PNGase F treatment and was associated with a shift in the molecular mass corresponding to the size of the N-glycan removed by the PNGase F treatment. (iii) PhoAp is a GPI-anchored protein and most proteins shown to be covalently linked to the cell wall of *S. cerevisiae* wall are GPI-anchored proteins. The release of PhoAp from the cell wall of *A. fumigatus* by hydrofluric acid (data not shown) is in agreement with a yeast-like configuration.

Other findings suggest that the strong association between PhoAp and 1,3-β-glucan is not covalent. These include the following. (i) PhoAp is released from the cell wall of *A. fumigatus* by 1,3-β-glucanase in minute amounts (as shown by Western-blot analysis; Fig. 5a), whereas the majority of PhoAp is extracted from the cell wall by the SDS/β-mercaptoethanol treatment or is actively secreted. A longer incubation time, of up to 3–4 days, or higher glycosylhydrolase concentrations did not modify the pattern and concentration of proteins released from the cell wall (data not shown). (ii) It was impossible to release all of the soluble proteins from the cell wall before 1,3-β-glucanase treatment. Indeed, as shown in Fig. 2, incubation of the SDS/β-mercaptoethanol-treated cell-wall sample in a buffer at 37 °C overnight resulted in the passive release of soluble proteins that remained associated to the cell wall. This phenomenon was observed even when the SDS/β-mercaptoethanol treatment was repeated 10 times (data not shown). Soluble PhoAp could thus remain non-covalently associated with the cell wall and then be released by the 1,3-β-glucanase because this treatment disrupts the constitutive polysaccharide network of the cell wall. (iii) The acid phosphatase of *S. cerevisiae* is a secreted protein that remains located at the surface of the cell (Linnemans et al., 1977) and is strongly associated with cell-wall mannans and β-glucans. Mech-
Table 1. Comparative genomics of GPI-anchored proteins in *S. cerevisiae* and *A. fumigatus*

Genes that gave a different cell-wall or membrane localization for the encoded proteins in the Caro *et al.* (1997) and Hamada *et al.* (1998) studies or which were only tested by Caro *et al.* (1997) were not included in this table.

<table>
<thead>
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<th><em>S. cerevisiae</em> genes</th>
<th>Localization in <em>S. cerevisiae</em></th>
<th>Homologues in <em>A. fumigatus</em></th>
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<td>YOL 011W (PLB3)</td>
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<td>YCR 061W</td>
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<tr>
<td>YPL 261C</td>
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<td>YAL 063C (FLO9)</td>
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<td>YAR 050W (PLO1)</td>
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<td>YIR 019C (MUC1)</td>
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Table 1. (cont.)

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<tr>
<th>S. cerevisiae genes</th>
<th>Localization in S. cerevisiae</th>
<th>Homologues in A. fumigatus*</th>
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<td>YOR 009W</td>
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<td>YOR 010C</td>
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<td>Agglutinin family</td>
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<td>YNR 044 (AGA1)</td>
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<td>YOR 214C</td>
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<td>YLR 110C (CCW12)</td>
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<td>YNL 300W</td>
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<td>YCR 089W (FIG2)</td>
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<td>YDR 534C</td>
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<tr>
<td>YNL 327W (EGT2)</td>
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</table>

* – , Absence of a homologue of S. cerevisiae in the A. fumigatus database; nos 1–6 indicate the number of A. fumigatus homologues; (P), a protein also detected by proteome analysis in Bruneau et al. (2001).

anical disruption of the cell wall or its digestion with glucanase are necessary to release soluble Pho5p efficiently (Arnold, 1972). Pho5p was even considered to be covalently linked to the yeast cell wall (Arnold, 1972). AfPhoAp could facilitate strong interactions with the cell wall 1,3-β-glucans. (iv) A genomic and proteomic analysis has suggested that the A. fumigatus GPI proteins are membrane-bound and not covalently linked to the cell wall.

The putative localization of the GPI-anchored proteins of S. cerevisiae in either the membrane or the cell wall has been investigated by Caro et al. (1997) and Hamada et al. (1998). Caro et al. (1997) based their results on the fact that plasma-membrane GPI proteins possess before their predicted GPI-attachment site a dibasic residue motif that is absent in cell-wall-associated GPI proteins. Hamada et al. (1998) constructed fusion proteins of 40 C-terminal amino acids for each predicted GPI protein with a reporter protein (z-galactosidase) and considered that only cell-wall-associated GPI proteins were released by treatment of the cell wall with laminarinase; fusion proteins that were not released from the cell wall by the laminarinase treatment were exclusively classified as membrane-bound GPI proteins. Fifty-two GPI proteins had localizations that were in concordance in the two studies (Table 1). The search for homologues of these yeast GPI-anchored proteins in A. fumigatus was done by using the BLAST tool and the TIGR unfinished sequence database (http://www.tigr.org/cgi-bin/Blast-Search). This survey identified 22 A. fumigatus genes that were homologous to genes of S. cerevisiae encoding GPI-anchored proteins (Table 1). Three of these genes (YDR 061C, YMR 200W and YCR 061W) were unique and the others belonged to the GAS, SPS2, PLB, CRH and YAP families. These results are in agreement with a recent proteome study of GPI-anchored proteins of A. fumigatus (Bruneau et al., 2001): only the YDR 061C, YMR 200W, YCR 061W and YAP family genes were not identified in this electrophoretic analysis of GPI-bound proteins. Most of the A. fumigatus genes (18 out of 22) were homologues of genes that encoded membrane-bound proteins in S. cerevisiae. Among the genes encoding proteins with a putative cell-wall localization in S. cerevisiae (Caro et al., 1997; Hamada et al., 1998) only four homologues were found in the A. fumigatus databases. These genes belong to the S. cerevisiae CRH family. The four genes of this family have sequence signatures suggesting a 1,3-β-glucanase activity (Rodriguez-Pena et al., 2000), a finding that would question their covalent association with the cell wall.

Our comparative genomic analysis of A. fumigatus genes is in agreement with our biochemical analysis of the cell-wall proteins of A. fumigatus, in that it has shown a lack of proteins covalently associated with the cell wall of A. fumigatus. It has also suggested that GPI-anchored proteins in A. fumigatus seem to play only an enzymic role (GEL family) in cell-wall biogenesis (Mouyna et al., 2000), without being covalently linked to the cell wall. These data show that the structural organization of the cell wall of the yeast S. cerevisiae and the fungus A. fumigatus is different. This difference is also seen at the level of the polysaccharide composition of the cell wall (Fontaine et al., 2000).
The type of association that PhoAp has with the cell-wall 1,3-β-glucans will only be elucidated by a chemical analysis of the C-terminal peptide of PhoAp, similar to that performed for Tip1 of S. cerevisiae (Fujii et al., 1999). The low amount of protein obtained from that performed for Tip1 of S. cerevisiae, similar to analysis of the C-terminal peptide of PhoAp, since its absence from medium with a high concentration of P1 is not associated with a reduction in fungal growth or in perturbation of cell-wall integrity.

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REFERENCES

A cell-wall acid phosphatase in *A. fumigatus*


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