Microbiology (2002), **148**, 2819–2829 Printed in Great Britain

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

Muriel Bernard,[1] Isabelle Mouyna,[1] Guy Dubreucq,[1]† Jean-Paul Debeaupuis,[1] Thierry Fontaine,[1] Constantinos Vorgias,[2] Claus Fuglsang[3] and Jean-Paul Latgé[1]

Author for correspondence: Muriel Bernard. Tel: +33 1 45 68 82 25. Fax: +33 1 40 61 34 19. e-mail: mbernard@pasteur.fr

[1] Unité des Aspergillus, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France

[2] National and Kapodistrian University of Athens, 15784 Athens, Greece

[3] Novozymes A/S, Krogshoejvej 36, 2880 Bagsvaerd, Denmark

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 1,3-β-glucans (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 (AGα1) 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

†**Present address:** Biodex, Saint Amand les Eaux, France.

Abbreviations: AfPhoAp, acid phosphatase of *A. fumigatus*; GPI, glycosylphosphatidylinositol; P_i, 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 50 mM EDTA, 2% SDS and 40 mM β-mercaptoethanol for 10 min. The extracted cell wall was recovered by centrifugation. This procedure was repeated four times. The four successive SDS/β-mercaptoethanol supernatants were stored separately at 4 °C for further analysis. The residual cell-wall pellet was washed five times with deionized water and then freeze-dried.

The SDS/β-mercaptoethanol-extracted cell-wall sample was subjected to various glycosylhydrolase treatments: (i) 100 mg of lyophilized cell wall was resuspended in 3 ml of 50 mM Tris/HCl (pH 7·4) buffer containing 5 mM sodium azide and 500 U of Quantazyme (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 5·4 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 N_2 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, lyophilysed 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 Bistris 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 vaccum 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 Chemioluminescence 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 (TFDEDGTYSKSNKI and PDELKGTQDDTFYT) that were coupled to an *m*-maleimidobenzoyl *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 were 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 microséquençage 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* (Af*PHOA*) 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′-ATGAAGCCTTCCGTCGCG-3′) complementary to nucleotides 459–476 of the genomic DNA of cl719 and the reverse primer P1 (5′-AAGCAGGTTTAGAGCGAG-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 Af*PHOA* 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 Quantazyme 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 measured by using *p*-nitrophenyl phosphate (Sigma) as a substrate. The reaction mixture contained (in a total of 100 µl) 5 mM substrate, 50 mM 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 NaOH to the sample; for each sample, the amount of *p*-nitrophenol 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_2CO_3 to the samples.

The release of inorganic phosphate (P_i) 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 phosphate in a 50 mM sodium acetate (pH 6·0) buffer containing 5 mM substrate. Released P_i was measured by the method of Ames (1966).

Compounds tested as inhibitors (EDTA, $MgCl_2$, $CaCl_2$, $ZnCl_2$, $HgCl_2$, 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^{-1}.

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 decreased 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 antisera 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.

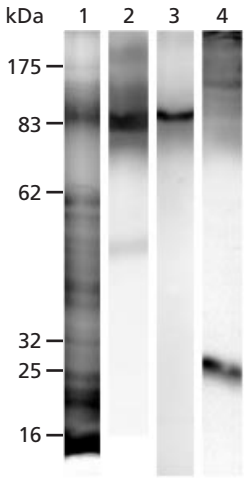

Fig. 1. Analysis of proteins released from *A. fumigatus* by SDS/β-mercaptoethanol treatment and separated on a 10 % polyacrylamide gel. Each lane was loaded with the amount of protein extracted from 0·7 mg of dry cell wall. Lanes: 1, proteins stained with Coomassie blue; 2, protein immunoblotted with anti-catalase; 3, protein immunoblotted with anti-dipeptidyl peptidase; 4, protein immunoblotted with anti-RNase.

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 glucosylhydrolases. However, the protein patterns produced were

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.

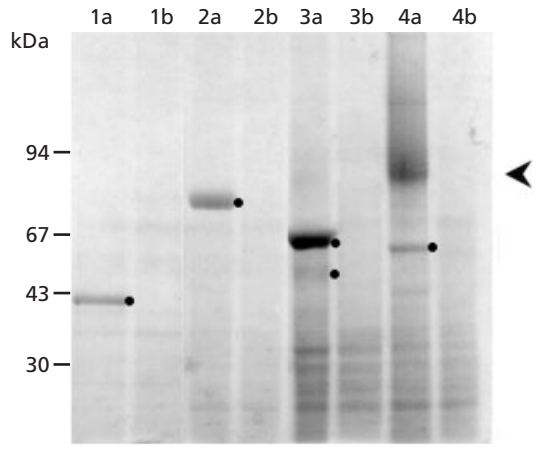

Fig. 2. Cell-wall proteins released after enzymic treatment of the *A. fumigatus* cell-wall extract. Proteins were separated on a 4–12% polyacrylamide gel and stained with Coomassie blue. Each lane was loaded with the amount of protein extracted from 7 mg of dry cell wall by treatment with 1,6-β-glucanase (lane 1a), 1,3-α-glucanase (lane 2a), chitinase (lane 3a) or 1,3-β-glucanase (lane 4a). Lanes 1b, 2b, 3b and 4b correspond to the protein extract obtained from the cell-wall sample incubated at 37 °C without the hydrolase. ●, Bands corresponding to the recombinant hydrolases. Protein material released by treatment of the sample with 1,3-β-glucanase is shown by an arrow head.

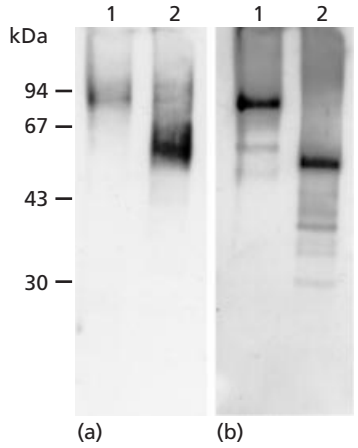

Fig. 3. Analysis of the cell-wall-associated protein released by treatment of the sample with 1,3-β-glucanase. An aliquot (100 µl) of the Quantazyme extract was treated with 10 U of PNGase F (Roche) (lane 2) or was left untreated (lane 1). The different extracts were analysed by Western blot either with an anti-1,3-β-glucan antibody (a) or with an anti-phoA antibody (b).

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 – IFSVLLGGAIPDELK. 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 hydropathy profile of the protein showed the presence of a signal peptide at its amino terminus. Using the (−3, −1) rule of Von Heijne (1986),

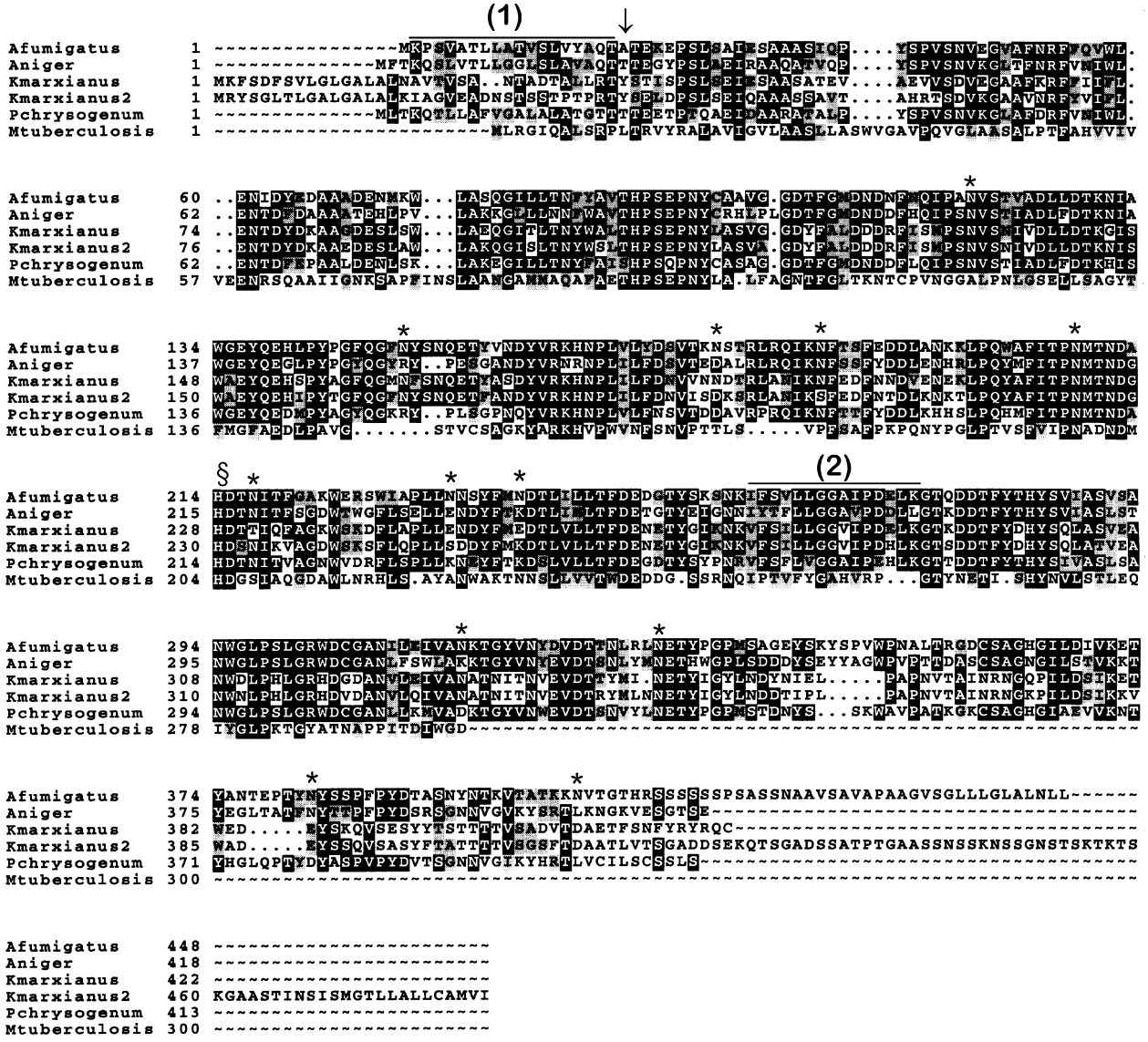

Fig. 4. Comparison of the predicted amino-acid sequence of AfPhoAp with its closest relatives. Aniger, P34724 of *A. niger*; Kmarxianus, JC7179 of *K. marxianus*; Kmarxianus2, P08540 of *K. marxianus*; Pchrysogenum, P37274 of *P. chrysogenum*; Mtuberculosis, E70842 of *M. tuberculosis*. Identical residues 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, $\omega+1$, $\omega+2$ site for GPI attachment could be N_{423} A_{424} A_{425} (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). The

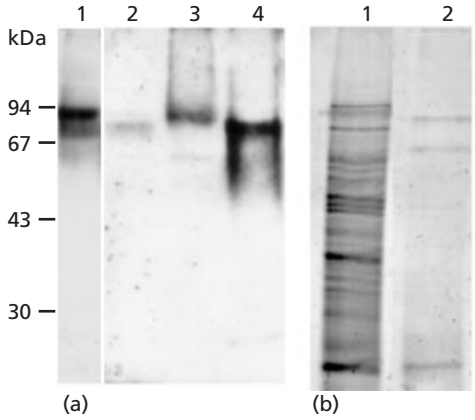

Fig. 5. (a) Detection of PhoAp in different extracts. Western-blot analysis was performed with the anti-phoA antibody. The amount of sample loaded into each lane is indicated in parentheses for each lane. Lanes: 1, culture filtrate (3·6 ml of culture filtrate); 2, mycelium extract [incubation of 40 mg intact wet mycelium (6 mg dry mycelium) in a Tris buffer at 4 °C for 4 h]; 3, Quantazyme extract (1·5 mg dry cell wall); 4, SDS/β-mercaptoethanol extract (1·5 mg dry cell wall). (b) Resistance of PhoAp to trypsin. Analysis of a mycelium extract [see lane 2, (a)] before (lane 1) or after trypsin treatment (lane 2). Proteins were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie blue.

RHGXRXP motif typical of many phosphatase enzymes (Van Etten *et al*., 1991) was not present in any of these phosphatases, but histidine 214 and aspartic acid 215, conserved in all of these proteins, could be part of the catalytic active site (Ostanin *et al*., 1992).

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 extracellular 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 di-phosphate 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.

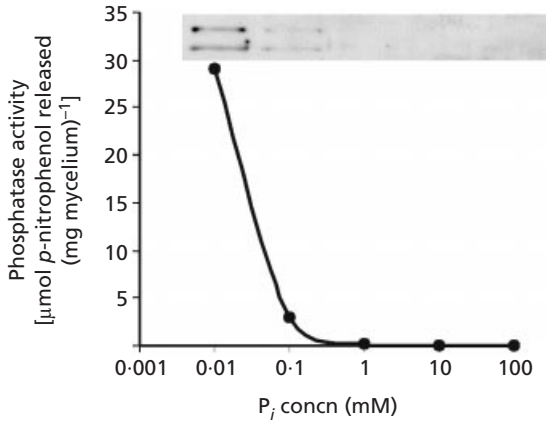

Fig. 6. Repression of PhoAp activity by the presence of P_i in the culture medium. Mycelium was grown at 37 °C for 24 h in a defined medium supplemented with various amounts of phosphate (100 mM to 10 µM). Phosphatase activity was measured in the culture filtrate and is expressed as µmol *p*-nitrophenol released (mg mycelium)$^{-1}$. The gel image (inset) shows the analysis of the different extracts by Western blotting with the anti-phoA antibody.

DISCUSSION

The AfPhoAp described in this manuscript (PhoAp) has several specific characteristics. (i) When in its native form, PhoAp is resistant to trypsin. (ii) As with the acid phosphatase from *P. chrysogenum* (Haas *et al*., 1991), PhoAp is not inhibited by high concentrations of tartrate, which is a competitive inhibitor of acid phosphatases from animal sources. However, contrary to the acid phosphatase from *P. chrysogenum*, PhoAp is not inhibited by high concentration of SDS. (iii) PhoAp is active on both mono- and di-phosphate esters. Phosphatases that share both phosphatase and phosphodiesterase activities have been described to date only in bacteria (O'Brien *et al*., 2001). An acid phosphatase that hydrolyses both phosphomonoesters and phosphodiesters has been found in *Penicillium funiculosum*, but this enzyme is only able to cleave aryl phosphates (Yoshida *et al*., 1989). (iv) PhoAp is a GPI-anchored protein. Most other known acid phosphatases are secreted; examples of these secreted acid phosphatases are the Pho3p and Pho5p proteins of *S. cerevisiae* (Vogel & Hinnen, 1990), as well as numerous *Aspergillus* spp. phosphatases, including those from *Aspergillus ficuum*, *A. niger* and *Aspergillus nidulans* (Ehrlich *et al*., 1994; MacRae *et al*., 1988), and a phytase from *A. fumigatus* (Wyss *et al*., 1999; Ullah *et al*., 2000; Rodriguez *et al*., 2000). The only other GPI-anchored phosphatase described is Pho610p from *K. marxianus* (Yoda *et al*., 2000); this protein shares sequence similarity with AfPhoAp and is released from the cell wall of *K. marxianus* after its treatment with laminarinase. AfPhoAp also has enzymic features that are common to the fungal acid phosphatases described to date: (i) it has an acid pH optimum; (ii) it is inhibited by NaF, molybdate and vanadate; (iii) it is highly glycosylated; and (iv) its synthesis is repressed by high concentrations of P_i (1 mM) in the extracellular medium. Phosphate-repressed acid phosphatases have been described in *S. cerevisiae* (Vogel & Hinnen, 1990), *A. niger* (MacRae *et al*., 1988) and *P. chrysogenum* (Haas *et al*., 1991, 1992). Phosphate is essential for the growth of all fungi (Jennings, 1995), and *A. fumigatus* was unable to grow at a P_i concentration lower than 10 µM. PhoAp is part of an enzymic arsenal that allows *A. fumigatus* to utilize P_i from its environmental medium. Other enzymes which have a similar role in *A. fumigatus* include a phospholipase C recently characterized in our laboratory (Buitrago, unpublished data) and a phytase (Wyss *et al*., 1999; Ullah *et al*., 2000; Rodriguez *et al*., 2000).

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 *S. cerevisiae* cell wall are GPI-anchored proteins. The release of PhoAp from the cell wall of *A. fumigatus* by hydrofluoric 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.

***S. cerevisiae* genes**	**Localization in *S. cerevisiae***	**Homologues in *A. fumigatus****
GAS family		
YMR 307W (*GAS1*)	Membrane	*GEL* 1 (P)
YLR 343W (*GAS2*)	Membrane	*GEL* 2
YMR 215W (*GAS3*)	Membrane	*GEL* 3
YOL 132W (*GAS4*)	Membrane	*GEL* 4 (P)
YOL 030W (*GAS5*)	Membrane	*GEL* 5
		GEL 6
YAP family		
YDR 144C (*MKC7*)	Membrane	1
YDR 349C (*YAP6*)	Membrane	1
YLR 120C (*YPS1*)	Membrane	1
YLR 121C (*YPS3*)	Membrane	1
YIR 039C (*YPS6*)	Membrane	1
SPS2 family		
YBR 078W (*ECM33*)	Membrane	1 (P)
YCL 048W	Membrane	—
YDR 055W (*PST1*)	Membrane	—
YDR 522C	Membrane	—
PLB family		
YMR 006C (*PLB2*)	Membrane	1
YMR 008C (*PLB1*)	Membrane	1
YOL 011W (*PLB3*)	Membrane	1 (P)
Others (non-clustered as family)		
YDR 261C (*EXG2*)	Membrane	1
YMR 200W (*ROT1*)	Membrane	1
YNL 190W	Membrane	—
YCR 061W	Membrane	1
YNL 322C (*KRE1*)	Membrane	—
YPL 261C	Membrane/cell wall	—
CRH family		
YEL 040C (*CRH2*)	Cell wall	1 (P)
YGR 189C (*CRH1*)	Cell wall	3 (P)
FLO family		
YAL 063C (*FLO9*)	Cell wall	—
YAR 050W (*PLO1*)	Cell wall	—
YHR 211W (*FLO5*)	Cell wall	—
YKR 102W	Cell wall	—
YIR 019C (*MUC1*)	Cell wall	—
CWP family		
YKL 096W	Cell wall	—
YKL 097W	Cell wall	—
TIR family		
YBR 067C (*TIP1*)	Cell wall	—
YER 011W (*TIR1*)	Cell wall	—
YIL 011W	Cell wall	—
YJR 150C	Cell wall	—
YJR 151C	Cell wall	—
YLR 040C	Cell wall	—

Table 1. (*cont.*)

S. cerevisiae genes	Localization in *S. cerevisiae*	Homologues in *A. fumigatus**
YOR 009W	Cell wall	–
YOR 010C	Cell wall	–
SED family		
YDR 077W (*SED1*)	Cell wall	–
YER 150W	Cell wall	–
Agglutinin family		
YJR 004C (*AGα1*)	Cell wall	–
YNR 044 (*AGA1*)	Cell wall	–
Others (non-clustered as family)		
YOR 214C	Cell wall	–
YLR 110C (*CCW12*)	Cell wall	–
YJL 078C (*PRY3*)	Cell wall	–
YNL 390W (*ECM19*)	Cell wall	–
YNL 300W	Cell wall	–
YCR 089W (*FIG2*)	Cell wall	–
YDR 534C	Cell wall	–
YNL 327W (*EGT2*)	Cell wall	–

* –, 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 is highly glycosylated, and has 12 putative *N*-glycosylation sites. As has been suggested for the yeast phosphatase, high amounts of mannosylation of 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 (α-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/BlastSearch). 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 *A. fumigatus* and its contamination with other cell-wall proteins have made these analyses inconclusive to date. Whatever the type of linkage between PhoAp and the cell wall, it is evident that this protein does not play a role in the organization of the cell wall of *A. fumigatus*, since its absence from medium with a high concentration of P_i is not associated with a reduction in fungal growth or in perturbation of cell-wall integrity.

ACKNOWLEDGEMENTS

This work was supported by a studentship from Aventis Pharma (M.B.) and a research and development grant from Aventis Pharma/Institut Pasteur (J.-P.L.). We are grateful to R. Calderone for reviewing our manuscript.

REFERENCES

Ames, B. N. (1966). Assay of inorganic phosphate and phosphatases. *Methods Enzymol* **8**, 115–118.

Arnold, W. N. (1972). Location of acid phosphatase and β-fructofuranosidase within yeast cell envelopes. *J Bacteriol* **112**, 1346–1352.

Beauvais, A., Monod, M., Debeaupuis, J.-P., Diaquin, M., Kobayashi, H. & Latgé, J.-P. (1997). Biochemical and antigenic characterization of a new dipeptidyl-peptidase isolated from *Aspergillus fumigatus*. *J Biol Chem* **272**, 6238–6244.

Bruneau, J. M., Magnin, T., Tagat, E., Legrand, R., Bernard, M., Diaquin, M., Fudali, C. & Latgé, J.-P. (2001). Proteome analysis of *Aspergillus fumigatus* identifies glycosylphosphatidylinositol-anchored proteins associated to the cell wall biosynthesis. *Electrophoresis* **22**, 2812–2823.

Calera, J. A., Paris, S., Monod, M., Hamilton, A. J., Debeaupuis, J.-P., Diaquin, M., Lopez-Medrano, R., Leal, F. & Latgé, J.-P. (1997). Cloning and disruption of the antigenic catalase gene of *Aspergillus fumigatus*. *Infect Immun* **65**, 4718–4724.

Cao, L., Chan, C. M., Lee, C., Wong, S. S. Y. & Yuen, K. Y. (1998). *MP1* encodes an abundant and highly antigenic cell wall mannoprotein in the pathogenic fungus *Penicillium marneffei*. *Infect Immun* **66**, 966–973.

Caro, L. H. P., Tettelin, H., Vossen, J. H., Ram, A. F. J., van den Ende, H. & Klis, F. M. (1997). *In silicio* identification of glycosylphosphatidylinositol-anchored plasma-membrane and cell wall proteins of *Saccharomyces cerevisiae*. *Yeast* **13**, 1477–1489.

Ehrlich, K. C., Montalbano, B. G., Mullaney, E. J., Dishinger, H. C., Jr & Ullah, A. H. J. (1994). An acid phosphatase from *Aspergillus ficuum* has homology to *Penicillium chrysogenum* PhoA. *Biochem Biophys Res Commun* **204**, 63–68.

Ferguson, M. A. (1992). Colworth medal lecture. Glycosylphosphatidylinositol membrane anchors: the table of a tail. *Biochem Soc Trans* **20**, 243–256.

Fontaine, T., Simenel, C., Dubreucq, G., Adam, O., Delepierre, M., Lemoine, J., Vorgias, C. E., Diaquin, M. & Latgé, J.-P. (2000). Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. *J Biol Chem* **275**, 27594–27607.

Fuglsang, C. C., Berka, R. M., Wahleithner, J. A., Kauppinen, S., Shuster, J. R., Rasmussen, G., Halkier, T., Dalboge, H. & Henrissat, B. (2000). Biochemical analysis of recombinant fungal mutanases. A new family of α-1,3-glucanases with novel carbohydrate-binding domains. *J Biol Chem* **275**, 2009–2018.

Fujii, T., Shimoi, H. & Iimura, Y. (1999). Structure of the glucan-binding sugar chain of Tip1p, a cell wall protein of *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1427**, 133–144.

Gerber, L. D., Kodukula, K. & Udenfriend, S. (1992). Phosphatidylinositol glycan (PI-G) anchored membrane proteins. Amino acid requirements adjacent to the site of cleavage and PI-G attachment in the COOH-terminal signal peptide. *J Biol Chem* **267**, 12168–12173.

Gijsbers, R., Ceulemans, H., Stalmans, W. & Bollen, M. (2001). Structural and catalytic similarities between nucleotide pyrophosphatases/phosphodiesterases and alkaline phosphatases. *J Biol Chem* **276**, 1361–1368.

Haas, H., Redl, B., Leitner, E. & Stöffler, G. (1991). *Penicillium chrysogenum* extracellular acid phosphatase: purification and biochemical characterization. *Biochim Biophys Acta* **1074**, 392–397.

Haas, H., Redl, B., Friedlin, E. & Stöffler, G. (1992). Isolation and analysis of the *Penicillium chrysogenum phoA* gene encoding a secreted phosphate-repressible acid phosphatase. *Gene* **113**, 129–133.

Hamada, K., Fukuchi, S., Arisawa, M., Baba, M. & Kitada, K. (1998). Screening for glycosylphosphatidylinositol (GPI)-dependent cell wall proteins in *Saccharomyces cerevisiae*. *Mol Gen Genet* **258**, 53–59.

Hoyer, L. L., Clevenger, J., Hecht, J. E., Ehrhart, E. J. & Poulet, F. M. (1999). Detection of Als proteins on the cell wall of *Candida albicans* in murine tissues. *Infect Immun* **67**, 4251–4255.

Jennings, D. H. (1995). Phosphorus. In *The Physiology of Fungal Nutrition*, pp. 252–287. Cambridge: Cambridge University Press.

Kandasamy, R., Vediyappan, G. & Chaffin, W. L. (2000). Evidence for the presence of pir-like proteins in *Candida albicans*. *FEMS Microbiol Lett* **186**, 239–243.

Kapteyn, J. C., Montijn, R. C., Dijkgraaf, G. J. P., Van den Ende, H. & Klis, F. M. (1995). Covalent association of β-1,3-glucan with β-1,6-glucosylated mannoproteins in cell walls of *Candida albicans*. *J Bacteriol* **177**, 3788–3792.

Kapteyn, J. C., Montijn, R. C., Vink, E., de la Cruz, J., Llobell, A., Douwes, J. E., Shimoi, H., Lipke, P. N. & Klis, F. M. (1996). Retention of *Saccharomyces cerevisiae* cell wall proteins through a phosphodiester-linked β-1,3/β-1,6-glucan heteropolymer. *Glycobiology* **6**, 337–345.

Kapteyn, J. C., Van den Ende, H. & Klis, F. M. (1999). The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochim Biophys Acta* **1426**, 373–383.

Kapteyn, J. C., Hoyer, L. L., Hecht, J. E., Müller, W. H., Andel, A., Verkleij, A. J., Makarow, M., Van den Ende, H. & Klis, F. M. (2000). The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. *Mol Microbiol* **35**, 601–611.

Klis, F. M. (1994). Review: cell wall assembly in yeast. *Yeast* **10**, 851–869.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Latgé, J.-P., Moutaouakil, M., Debeaupuis, J.-P., Bouchara, J. P., Haynes, K. & Prévost, M. C. (1991). The 18-kilodalton antigen secreted by *Aspergillus fumigatus*. *Infect Immun* **59**, 2586–2594.

Latgé, J.-P., Kobayashi, H., Debeaupuis, J.-P., Diaquin, M., Sarfati, J., Wieruszeski, J. M., Parra, E. & Fournet, B. (1994). Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. *Infect Immun* **62**, 5424–5433.

Linnemans, W. A. M., Boer, P. & Elbers, P. F. (1977). Localization of acid phosphatase in *Saccharomyces cerevisiae*: a clue to cell wall formation. *J Bacteriol* **131**, 638–644.

Lipke, P. N., Wojciechowicz, D. & Kurjan, J. (1989). *AGα1* is the structural gene for the *Saccharomyces cerevisiae* α-agglutinin, a cell surface glycoprotein involved in cell–cell interactions during mating. *Mol Cell Biol* **9**, 3155–3165.

Lu, C. F., Kurjan, J. & Lipke, P. N. (1994). A pathway for cell wall anchorage of *Saccharomyces cerevisiae* α-agglutinin. *Mol Cell Biol* **14**, 4825–4833.

MacRae, W. D., Buxton, F. P., Sibley, S., Garven, S., Gwynne, D. I., Davies, R. W. & Arst, H. N., Jr (1988). A phosphate-repressible acid phosphatase gene from *Aspergillus niger*: its cloning, sequencing and transcriptional analysis. *Gene* **71**, 339–348.

Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, W. A., Diaquin, M., Popolo, L., Hartland, R. P. & Latgé, J.-P. (2000). Glycosylphosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. *J Biol Chem* **275**, 14882–14889.

Mouyna, I., Sarfati, J., Recco, P., Fontaine, T., Henrissat, B. & Latgé, J.-P. (2002). Molecular characterization of a cell wall associated β(1-3)endoflucanase of *Aspergillus fumigatus*. *Med Mycol* (in press).

Mrsa, V., Seidl, T., Gentzsch, M. & Tanner, W. (1997). Specific labelling of cell wall proteins by biotinylation. Identification of four covalently linked O-mannosylated proteins of *Saccharomyces cerevisiae*. *Yeast* **13**, 1145–1154.

O'Brien, P. J. & Herschlag, D. (2001). Functional interrelationships in the alkaline phosphatase superfamily: phosphodiesterase activity of *Escherichia coli* alkaline phosphatase. *Biochemistry* **40**, 5691–5699.

Ostanin, K., Harms, E. H., Stevis, P. E., Kuciel, R., Zhou, M. M. & Van Etten, R. L. (1992). Overexpression, site-directed mutagenesis, and mechanism of *Escherichia coli* acid phosphatase. *J Biol Chem* **267**, 22830–22836.

Rodriguez, E., Mullaney, E. J. & Lei, X. G. (2000). Expression of the *Aspergillus fumigatus* phytase gene in *Pichia pastoris* and characterization of the recombinant enzyme. *Biochem Biophys Res Commun* **268**, 373–378.

Rodriguez-Pena, J. M., Cid, V. J., Arroyo, J. & Nombela, C. (2000). A novel family of cell wall-related proteins regulated differently during the yeast life cycle. *Mol Cell Biol* **20**, 3245–3255.

Saleh, M. T. & Belisle, J. T. (2000). Secretion of an acid phosphatase (SapM) by *Mycobacterium tuberculosis* that is similar to eukaryotic acid phosphatases. *J Bacteriol* **182**, 6850–6853.

Schoffelmeer, E. A. M., Vossen, J. H., van Doorn, A. A., Cornelissen, B. J. C. & Haring, M. A. (2001). FEM1, a *Fusarium oxysporum* glycoprotein that is covalently linked to the cell wall matrix and is conserved in filamentous fungi. *Mol Genet Genomics* **265**, 143–152.

Stratford, M. (1994). Another brick in the wall? Recent developments concerning the yeast cell envelope. *Yeast* **10**, 1741–1752.

Ullah, A. H. J., Sethumadhavan, K., Lei, X. G. & Mullaney, E. J. (2000). Biochemical characterization of cloned *Aspergillus fumigatus* phytase (PhyA). *Biochem Biophys Res Commun* **275**, 279–285.

van der Vaart, J. M., Caro, L. H. P., Chapman, J. W., Klis, F. M. & Verrips, C. T. (1995). Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. *J Bacteriol* **177**, 3104–3110.

Van Etten, R. L., Davidson, R., Stevis, P. E., MacArthur, H. & Moore, D. L. (1991). Covalent structure, disulfide bonding and identification of reactive surface and active site residues of human prostatic acid phosphatase. *J Biol Chem* **266**, 2313–2319.

Vogel, K. & Hinnen, A. (1990). The yeast phosphatase system. *Mol Microbiol* **4**, 2013–2017.

Von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* **14**, 4683–4690.

Vorgias, C. E., Tews, I., Perrrakis, A., Wilson, K. J. & Oppenheim, A. B. (1993). Purification and characterization of the recombinant chitin degrading enzymes, chitinase A and chitobiase from *Serratia marcescens*. In *Chitin Enzymology*, pp. 417–422. Edited by R. A. A. Muzzarelli. Antona, Italy: European Chitin Society.

Worley, K. C., Wiese, B. A. & Smith, R. F. (1995). BEAUTY: an enhanced BLAST-based search tool that integrates multiple biological information resources into sequence similarity search results. *Genome Res* **5**, 173–184.

Wyss, M., Pasamontes, L., Friedlein, A. & 13 other authors (1999). Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance. *Appl Environ Microbiol* **65**, 359–366.

Yoda, K., Ko, J. H., Nagamatsu, T., Lin, Y., Kaibara, C., Kawada, T., Tomishige, N., Hashimoto, H., Noda, Y. & Yamasaki, M. (2000). Molecular characterization of a novel yeast cell-wall acid phosphatase cloned from *Kluyveromyces marxianus*. *Biosci Biotechnol Biochem* **64**, 142–148.

Yoshida, H., Oikawa, S., Ikeda, M. & Reese, E. T. (1989). A novel acid phosphatase excreted by *Penicillium funiculosum* that hydrolyzes both phosphodiesters and phosphomonoesters with aryl leaving groups. *J Biochem* **105**, 794–798.

Zverlov, V. V., Volkov, I. Y., Velikodvorskaya, T. V. & Schwarz, W. H. (1997). Highly thermostable endo-1,3-β-glucanase (laminarinase) LamA from *Thermotoga neapolitana*: nucleotide sequence of the gene and characterization of the recombinant gene product. *Microbiology* **143**, 1701–1708.

Received 7 May 2002; revised 17 May 2002; accepted 10 June 2002.