Cell wall protein and glycoprotein constituents of *Aspergillus fumigatus* that bind to polystyrene may be responsible for the cell surface hydrophobicity of the mycelium

M. Carmen Peñalver, Manuel Casanova, José P. Martínez and M. Luisa Gil

Author for correspondence: José P. Martínez. Tel: +34 6 3864770. Fax: +34 6 3864770.
e-mail: Jose.Pedro.Martinez@uv.es

Departamento de Microbiología y Ecología, Facultad de Farmacia, Universitat de València, Avda. Vicente Andrés Estellés, s/n, 46100-Burjasot, Valencia, Spain

Cell surface hydrophobicity (CSH) of *Aspergillus fumigatus* grown both in complex medium (yeast extract/peptone/dextrose; YPD) and minimal (Vogel's N) medium was monitored by assessing attachment of polystyrene microspheres to the cell surface. It was found that mature mycelium was hydrophobic. Treatment of intact mycelium with β -mercaptoethanol (β ME) abolished binding of the microspheres to hyphal elements, and coating of the microspheres with β ME extracts from mycelium inhibited their attachment to intact mycelial cells. *A. fumigatus* mycelium was tagged *in vivo* with biotin and treated with β ME. The β ME extracts were analysed by SDS-PAGE and Western blotting with both peroxidase-conjugated-ExtraAvidin and concanavalin A (ConA). This procedure allowed identification of cell wall surface proteins and glycoproteins. Rabbit polyclonal antisera were raised against β ME extracts obtained from cells grown in YPD and Vogel's N media. These antisera defined some major cell-wall-bound antigens. SDS-PAGE and Western blotting analysis of the cell wall material released by β ME and adsorbed on polystyrene microspheres revealed about 19 protein species with apparent molecular masses ranging from 20 to 70 kDa, and two high-molecular-mass glycoproteins of 115 and 210 kDa. Treatment of cells grown in YPD, but not those grown in Vogel's N medium, with β ME released a 55 kDa polypeptide able to adsorb to polystyrene microspheres that was detectable with the antisera. The ability to bind to polystyrene particles exhibited by several protein and glycoprotein species released by β ME treatment suggested that these cell wall moieties possess exposed hydrophobic domains that could be responsible for the CSH of mycelium.

Keywords: *Aspergillus fumigatus*, cell surface hydrophobicity, cell wall, proteins, glycoproteins

INTRODUCTION

Aspergillus fumigatus is a ubiquitous fungus involved in a variety of allergic and invasive diseases in man such as allergic asthma, allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis (Bodey & Vartivarian, 1989; Kwon-Chung & Bennett, 1991). The most severe form is invasive aspergillosis which has been

reported as an increasingly important cause of mortality in severely immunocompromised patients, especially those with acute leukaemia and other haematological malignancies, and those undergoing organ transplantation (Cohen, 1991). Although the search for virulence factors has been the focus of many studies in recent years (Bouchara *et al.*, 1995), the mechanisms by which this fungus persists in the lung and causes diseases in certain individuals are still unclear.

Hydrophobic interactions are generally considered to have an important role in the adherence of pathogenic micro-organisms to host cell surfaces (Beachey, 1981).

Abbreviations: ConA, concanavalin A; CSH, cell surface hydrophobicity; IF, immunofluorescence; β ME, β -mercaptoethanol; pAb, polyclonal antibody.

Hazen and others have shown that cell surface hydrophobicity (CSH) enhances the ability of *Candida albicans* to adhere to epithelial tissue, since hydrophobic interactions apparently contribute to maintain and/or increase the number of receptor–ligand contacts between the parasite and the host surfaces (Beachey, 1981; Hazen, 1990; Rotrosen *et al.*, 1986; Silva *et al.*, 1995). In addition, hydrophobic cells are not killed as rapidly as hydrophilic cells by polymorphonuclear neutrophils (Antley & Hazen, 1988). A similar observation for hydrophobic versus hydrophilic conidia of *Aspergillus* and *Rhizopus* species has been made by other workers (for a review, see Diamond, 1988). These results suggest that expression of CSH could contribute to successful colonization of host tissues and thus to fatal, disseminated candidiasis and aspergillosis.

The nature of the cell wall of *A. fumigatus* has received considerable attention, partly due to recognition of the potential of its molecular constituents as marker antigens for diagnosis (Drouhet *et al.*, 1972; Hearn, 1984, 1992; Hearn & Sietsma, 1994; Hearn *et al.*, 1990, 1991; Latgé *et al.*, 1994; Reijula *et al.*, 1992; Ste-Marie *et al.*, 1990; Stynen *et al.*, 1992; Wilson & Hearn, 1982). However, although cell-wall-bound molecules that may account for CSH have been characterized in other opportunistic fungal pathogens such as *C. albicans* (Glee *et al.*, 1995; Hazen *et al.*, 1990; López-Ribot *et al.*, 1991), no reports exist on the identification of analogous wall components in *A. fumigatus*.

In this study, we describe for the first time how *A. fumigatus* mycelial filaments grown under different defined, *in vitro* conditions display strong CSH. Consequently, we have attempted characterization of cell wall components that could be related to CSH following the procedure previously used to identify similar molecules in *C. albicans* (López-Ribot *et al.*, 1991). Results presented here indicate that several cell-wall-bound proteins and glycoproteins which are released by treatment of intact cells with β -mercaptoethanol (β ME) may be responsible for CSH in *A. fumigatus* cells. This knowledge may help to establish the role that CSH plays in the virulence and pathogenicity of this fungal species.

METHODS

Micro-organism and culture conditions. *A. fumigatus* strain 2071, obtained from the Spanish Culture Collection (CECT) was used throughout this work. The organism was grown on plates of Vogel's N medium (containing NaNO_3 , glucose, salts and trace elements; Martínez *et al.*, 1991) solidified with Bacto-agar (1.5%, w/v) at 25 °C for 30 d. Mature conidia were harvested from aerial mycelia by washing the plates with sterile 0.05% (v/v) Tween 20 in glass-distilled water. Conidia were pelleted from the suspension by centrifugation at 1500 g for 10 min, washed in sterile water and quantified by haemocytometer counting. Conidia were inoculated at a final concentration of 10^7 ml^{-1} into liquid YPD [1% (w/v) Bacto-yeast extract, 2% (w/v) Bacto-peptone, and 2% (w/v) dextrose] or Vogel's N media. Incubations were carried out at 37 °C for different times for each experiment (see below). Finally, mycelium was harvested from liquid cultures by filtration

(Millipore HA filters, 0.45 μm pore size) and washed thoroughly with double-glass-distilled water. The mycelial pad was used immediately or frozen and kept at –20 °C until required.

Assessment of CSH. CSH of individual hyphal cells was determined by light microscopy observations, following attachment to the surface of the cells of polystyrene microspheres ($0.801 \pm 0.036 \mu\text{m}$ diameter) having a low negative charge density essentially as described by Hazen & Hazen (1987). Briefly, cells from 1.5 ml aliquots of culture medium were harvested and resuspended in 0.15 ml 0.05 M sodium phosphate buffer, pH 7.4 (buffer A) kept at 0–4 °C and mixed in acid-washed glass tubes with 150 μl of a dilution of the microsphere stock solution made in the same chilled buffer A to achieve an approximate concentration of 9×10^8 microspheres ml^{-1} . The mixtures were rapidly equilibrated to 28 °C in a water bath and stirred for 30 s in a vortex mixer. Samples were withdrawn from the mixtures and observed with a phase-contrast Zeiss III photomicroscope. Overall hydrophobicity of the population of resting conidia was determined by an aqueous/hydrocarbon biphasic partition assay based on the procedure of Rosenberg *et al.* (1980) as modified by Hazen & Hazen (1987). Cells were suspended in buffer A to yield an OD_{600} of 0.200 ± 0.020 (measured in a Shimadzu UV-160 spectrophotometer). Samples of 1.2 ml from these suspensions were mixed with 0.3 ml cyclohexane and vigorously vortexed for 3 min. The phases were then allowed to separate and the percentage change in OD_{600} of the aqueous phase was considered the hydrophobicity value of the entire conidial population.

Chemical treatments of cells. Cells present in 1 ml aliquots withdrawn from the liquid cultures were sedimented by centrifugation (3000 g, 10 min) and resuspended in 1 ml buffer containing the following agents: β ME at 1% (v/v) in buffer A, concanavalin A (ConA) at 100 $\mu\text{g ml}^{-1}$ in 10 mM Tris/HCl (pH 7.4) containing 0.9% (w/v) NaCl and 3% (w/v) BSA, or 2% (v/v) paraformaldehyde in buffer A. Treatments were accompanied by gentle shaking for 30 min. After treatment, cells were washed by centrifugation three times with buffer A, and CSH assessed by determination of attachment of polystyrene microspheres as described above.

Generation of polyclonal antibodies (pAbs). Mycelium grown in both liquid YPD or Vogel's N media was harvested, washed with distilled water and resuspended in buffer A containing 1% β ME, 5 μg aprotinin ml^{-1} , 1 mM EDTA and 1 mM PMSF (the cells were resuspended in a volume of buffer equivalent to 1/3 of the original volume of the culture medium in which they were grown), and incubated for 30 min at 37 °C in a rotary incubator. After treatment, the cells were sedimented by centrifugation, and the supernatant fluid was recovered, extensively dialysed against glass-distilled water (four changes) for 48 h at 4 °C, and concentrated by freeze-drying (' β ME extract'). The lyophilized β ME extracts were resuspended in a small volume of buffer A. Samples of the different β ME extracts [obtained from cells grown in YPD or Vogel's N media (β MEYPD extract and β MEV extract, respectively), containing in all cases approximately 1 mg total protein ml^{-1}] were emulsified with an equal volume (1 ml) of complete Freund's adjuvant and injected subcutaneously into adult female New Zealand White rabbits. After 3 weeks, the rabbits were boosted in a similar manner with 1 mg of the corresponding β ME extract in incomplete Freund's adjuvant. Seven days later the rabbits were bled and the immunoglobulin fraction was separated from the crude serum as previously described (Casanova *et al.*, 1992b). Antibody titres were maintained by booster injections of the corresponding immunogen mixed with incomplete Freund's adjuvant every 3 weeks.

Coating of polystyrene microspheres with β ME extract. One millilitre of a commercial stock colloidal suspension [10% (w/v) solids] of polystyrene microspheres was mixed with 0.5 mg β ME extract (expressed as total protein content) in 1 ml 100 mM glycine/saline buffer (pH 8.4). The mixture was incubated overnight at 4 °C. The microspheres were sedimented by centrifugation, washed with buffer A and used in assays to assess CSH as described above. Coating of polystyrene particles was assessed by means of an agglutination assay using the pAb preparations raised towards β ME extracts (see above).

ELISA and immunofluorescence (IF). The reactivity of pAbs was assayed by ELISA and by IF according to the procedures described previously (Casanova *et al.*, 1989). IF observations were made on mycelium grown in liquid YPD or Vogel's N media with a 1:10 dilution of the corresponding antiserum in PBS buffer (10 mM sodium phosphate buffer, pH 7.4, containing 150 mM sodium chloride) containing 1% BSA. Control experiments were performed with preimmune rabbit serum diluted in PBS buffer at a similar concentration.

Surface-labelling of mycelial filaments with biotin. Mycelium was collected, washed with 100 mM sodium phosphate buffer, pH 8, and resuspended in a volume of the same buffer equivalent to 1/3 of the volume of culture medium in which the cells were grown, containing 1 mg 3-sulfo-*N*-hydroxy-succinimide ester (NSH) of biotin ml⁻¹ (NHS-biotin was previously dissolved in dimethyl sulfoxide). After incubation for 1 h at 28 °C in a shaking bath, the cells were recovered and washed with 50 mM phosphate buffer, pH 6 and then with 10 mM phosphate buffer, pH 7.4. Biotinylated cells were also subjected to treatment with β ME to release cell-wall-bound protein moieties as described above.

Analysis of material released by β ME. To assess the existence in the crude materials released by β ME of molecules exhibiting the ability to bind to the surface of the polystyrene microbeads, 2 mg (expressed as total protein content) of the corresponding lyophilized β ME extracts were resuspended in 0.2 ml buffer A and mixed with 0.4 ml stock colloidal suspension of polystyrene microspheres. The mixtures were incubated at 4 °C for 12 h with gentle agitation. The microspheres were extensively washed (10 times) by centrifugation (2 min in a Eppendorf microfuge at 10000 *g*) with buffer A, and the washings were pooled, dialysed, concentrated by freeze-drying and resuspended in 0.2 ml buffer A ('unadsorbed material'). The washed microspheres were immediately resuspended in 0.2 ml electrophoresis sample buffer [35% (v/v) glycerol, 14% β ME, 7% (w/v) SDS and 0.035% (w/v) bromphenol blue in 0.5 M Tris/HCl pH 6.8; Laemmli, 1970] and heated for 5 min in a boiling-water bath. The microspheres were removed by centrifugation and the supernatant mixed with 0.5 vol. buffer A. The crude and unadsorbed materials were also treated with electrophoresis sample buffer (1 vol. sample buffer per 2 vols corresponding sample) at 100 °C for 5 min and subjected in parallel with the polystyrene-adsorbed material to SDS-PAGE, followed by blotting to nitrocellulose paper as described below.

PAGE and Western blot techniques. SDS-PAGE was performed essentially by the procedure of Laemmli (1970), using slab gels (12.5%, w/v, polyacrylamide; with a ratio of acrylamide to bis-acrylamide of 30:0.2) with 3.5 to 4% polyacrylamide stacking gels. Electrophoretic transfer (Western blot) to nitrocellulose paper was carried out as reported previously (Casanova *et al.*, 1989). Indirect ConA-mediated peroxidase staining of mannoproteins in nitrocellulose blots was done as described elsewhere (Casanova *et al.*, 1989). Biotinylated proteins transferred to nitrocellulose paper were detected as reported previously (Casanova *et al.*, 1992a). Immunodetection of proteins transferred to nitrocellulose was done as described

by Casanova *et al.* (1989), using the pAbs raised against β ME extract from cells grown in Vogel's N medium (at a final dilution of 1:1000 in 10 mM Tris/HCl, pH 7.4, containing 0.9% NaCl, 0.05% Tween 20 and 1% BSA; TBSTB) or from cells grown in YPD medium (at a final dilution of 1:500 in TBSTB). Peroxidase-labelled goat anti-rabbit immunoglobulin diluted 1:3000 in TBSTB was used as the indicator antibody. Protein was visualized by the silver staining procedure of Morrissey (1981).

Miscellaneous. The total protein content of the different samples was determined by the Lowry method with BSA as standard. The total sugar content was determined by the method of Dubois *et al.* (1956). Gel electrophoresis and blotting reagents, peroxidase- and fluorescein-isothiocyanate-conjugated goat anti-rabbit immunoglobulin G and molecular mass markers were from Bio-Rad. Polystyrene microspheres were obtained from Sigma. Culture medium components, and complete and incomplete Freund's adjuvants were purchased from Difco. All other chemicals were purchased from Sigma.

RESULTS

Assessment of CSH during growth of conidia to mature mycelia

Resting conidia were found to be highly hydrophobic as determined by an aqueous/hydrocarbon biphasic partition assay (see Methods), that allows determination of overall CSH for a cell population (Hazen & Hazen, 1987; López-Ribot *et al.*, 1991). According to the percentage change in OD₆₀₀ of the aqueous phase, which is proportional to the number of hydrophobic cells that entered the hydrocarbon phase, more than 85% of resting conidia displayed strong CSH, but after 30 min of incubation in YPD medium the hydrophobic status of cells began to change (only 60% of the conidia entered the hydrocarbon phase), and by 3 h of incubation essentially all the conidia, which had still not germinated, appeared to be hydrophilic and remained in the aqueous phase. These results were in agreement with observations made when CSH of individual *A. fumigatus* cells grown in liquid YPD medium was determined by an assay based on the attachment of polystyrene microspheres to the cell surface (Hazen & Hazen, 1987). In this case, swollen, non-germinated conidia and swollen conidia bearing short-length germ tubes exhibited some microspheres non-uniformly attached to their surfaces (Fig. 1a, b; indicated by arrows). However, long mycelial filaments (which appeared after 15 h or more of incubation) were highly hydrophobic since they showed numerous microspheres attached along the whole length of the hyphae (Fig. 1c). No differences in CSH, as determined by the attachment of microspheres to the cell surface, were found between mycelial filaments grown in YPD or Vogel's N media (Fig. 1 and results not shown, respectively).

Effects of different treatments on CSH

Treatment of mycelium grown in YPD or Vogel's N media with β ME abolished attachment of the polystyrene microspheres to the cell surface (Fig. 1d and results not shown, respectively). Coating of polystyrene particles with β ME extract prior to the CSH assessment assay

Fig. 1. Appearance of different cell morphologies of *A. fumigatus* grown in YPD medium after mixing with polystyrene microspheres to assess CSH as described in Methods. (a) Swollen conidia prepared by incubating the resting conidia for about 5 h at 37 °C; (b) germinated swollen conidia bearing short-length hyphae obtained by incubating the swollen conidia for an additional period of 5 h at 37 °C; (c) mature hyphae obtained after 10 h incubation of swollen conidia at 37 °C; (d) β ME-treated mycelial filaments; (e) intact mature mycelial filaments previously coated with β ME extract. Bars, 10 μ m.

Fig. 2. Characterization of proteinaceous components released by β ME from the cell surface of mycelium grown in Vogel's N medium (lanes 1, 3 and 5) or YPD medium (lanes 2, 4 and 6). Components were separated by SDS-PAGE and revealed by silver nitrate staining (lanes 1 and 2; 75 μ g total protein per well) or by indirect ConA-mediated peroxidase staining following blotting onto nitrocellulose paper (lanes 3 and 4; 150 μ g total sugars per well). Biotinylated polypeptides released from the cell surface by β ME treatment were also separated by SDS-PAGE, transferred to nitrocellulose, and detected with ExtrAvidin-peroxidase conjugate (lanes 5 and 6; 75 μ g total protein per well). Molecular masses (in kDa) of standard proteins run in parallel are shown on the left.

by their ability to bind the microspheres, whereas ConA treatment or pre-incubation of mycelium with pAbs raised towards β ME extracts significantly reduced (yet did not abolish) attachment of polystyrene particles to the cell surface (data not shown).

Characterization of protein species released by β ME from the walls of mycelial cells grown in complex or minimal media

The material released by β ME from intact *A. fumigatus* cells grown in YPD or Vogel's N media was first analysed by SDS-PAGE and stained with silver nitrate (Fig. 2, lanes 1 and 2). Treatment with β ME solubilized a complex array of high- to low-molecular-mass species (about 30–35 different polypeptides), randomly distributed throughout the entire molecular mass range (from 10 to > 112 kDa). The polypeptide profiles obtained from the β MEV and β MEYPD extracts (Fig. 2, lanes 1 and 2, respectively) were quite similar, although quantitative differences in the amount of protein in several bands were detected. These patterns were extremely reproducible from one experiment to another.

ConA, a lectin with a high affinity for (1 $\rightarrow$ 2)- α -D-mannooligosaccharides and glucose (Goldstein & Hayes, 1978), was selected as a probe to detect wall glycoproteins released by β ME. Western blot analysis of the β ME extracts with ConA is shown in lanes 3 and 4 of Fig. 2. Again, β MEV and β MEYPD extracts (lanes 3 and 4, respectively) gave similar profiles of glycoprotein species, within an apparent molecular mass range of 50 to > 112 kDa, thus suggesting that the low-molecular-mass polypeptides (< 50 kDa) detected in the polyacrylamide gels by means of silver staining (Fig. 2, lanes 1 and 2) contain no glucose and/or mannose polymers.

In vivo labelling of cells with biotin, a technique that has been previously employed by our group to identify surface cell wall protein and glycoprotein components in *C.*

inhibited their binding to the surface of intact mycelial cells (Fig. 1e). Fixation of cells with 2% paraformaldehyde did not cause any detectable effect on CSH as determined

Fig. 3. Phase-contrast (a) and IF (b) photographs of filamentous growth of *A. fumigatus* stained with antibodies raised against the components released by β ME from the cell surface of mycelia grown in YPD medium. The cells shown were also grown in YPD medium. Arrows point to the location of conidia, which exhibited no fluorescence under UV illumination. Bar, 10 μ m.

albicans (Casanova *et al.*, 1992a), was used in this work to delineate further the wall moieties released by β ME from *A. fumigatus* mycelial cells. Biotinylated proteins were extracted from the cell wall of intact mycelia by treatment with β ME, separated by SDS-PAGE, transferred to nitrocellulose sheets and detected with ExtrAvidin-

peroxidase conjugate as described in Methods (Fig. 2, lanes 5 and 6). Although silver staining of polyacrylamide gels revealed a similar polypeptide pattern in both extracts (β MEV and β MEYPD) as mentioned above (Fig. 2, lanes 1 and 2), biotinylation allowed identification of several polypeptides whose expression seemed to be dependent on the type of culture media where the cells were grown (compare lane 5 versus lane 6 in Fig. 2). In addition, the number of biotinylated moieties was found to be lower in the β MEV extract (Fig. 2, lane 5; 11 bands) than in the β MEYPD extract (Fig. 2, lane 6; 18 bands).

Generation of pAbs against β ME extracts

pAbs against wall components present in β MEV (pAb anti- β MEV) and β MEYPD (pAb anti- β MEYPD) extracts were tested by ELISA and IF. By ELISA, each antibody cross-reacted with the two extracts, with essentially the same intensity (positive reactions were obtained at 1:1000–1:5000 dilutions). The IF assay showed that both pAbs specifically labelled the surface of the hyphal extensions of the organism regardless of the culture medium in which the cells were grown, which confirmed the cross-reactivity detected by ELISA. Fig. 3(b) shows a representative IF field of cells grown in YPD medium and probed with pAb anti- β MEYPD. Conidia were not stained following reaction with the pAb preparations (Fig. 3b, arrows).

Fig. 4. Characterization of proteinaceous components released by β ME from the cell surface of mycelia grown in YPD medium (a) or Vogel's N medium (b) that bind to polystyrene microspheres. Crude material (lanes 1, 4 and 7), polystyrene-adsorbed material (lanes 2, 5 and 8) and unadsorbed material (lanes 3, 6 and 9) were analysed by SDS-PAGE and revealed by silver nitrate staining (lanes 1–3; 75 μ g total protein per well) or blotted onto nitrocellulose paper and revealed by indirect ConA-mediated peroxidase staining (lanes 4–6; 150 μ g total sugars per well) and immunodetection with pAb anti- β MEYPD (a, lanes 7–9; 150 μ g total protein per well) or with pAb anti- β MEV (b, lanes 7–9; 150 μ g total protein per well). Molecular masses (in kDa) of standard proteins are shown on the left.

Characterization of wall proteins adsorbed on polystyrene microspheres

Ability of β ME-solubilized mycelial surface proteins and glycoproteins to be specifically adsorbed on polystyrene microspheres was assayed, since affinity for this plastic material may be considered as indirect evidence of the hydrophobic nature of polypeptide moieties (López-Ribot *et al.*, 1991, 1995).

The polystyrene-adsorbed protein profile from β MEYPD extract revealed by silver staining of the gels (Fig. 4a, lane 2) appeared to be quite similar to that from β MEV extract (Fig. 4b, lane 2). About 21 polypeptide species exhibiting affinity for the surface of polystyrene microspheres were detected, with molecular masses ranging from 20 to > 112 kDa.

Analysis with ConA-peroxidase staining revealed, as previously stated (see Fig. 2, lanes 3 and 4), a complex array of medium- to high-molecular-mass glycoprotein species (from 50 to > 112 kDa) in crude β MEYPD (Fig. 4a, lane 4) and β MEV (Fig. 4b, lane 4). Adsorption of the crude material on polystyrene microspheres showed the affinity for polystyrene of two proteins with apparent molecular masses of 115 and 210 kDa (Fig. 4a, b, lanes 5). The polystyrene-adsorbed glycoprotein profile of β MEYPD extracts appeared to be substantially identical of that from β MEV extracts (compare Fig. 4a, lane 5 versus Fig. 4b, lane 5). However, the two high-molecular-mass (115 and 210 kDa) polystyrene-adsorbed glycoproteins from β MEYPD extract were still present in the unadsorbed material, whereas in the case of β MEV extract these species were almost completely adsorbed to polystyrene particles (compare lanes 6 and 9 in Fig. 4a versus 4b). Increasing the concentration of polystyrene microspheres in the adsorption assay did not cause any detectable reduction in the intensity of these two bands in the unadsorbed β MEYPD extract (data not shown). Most of the polystyrene-adsorbed polypeptides detected by silver staining (Fig. 4a, b, lanes 2), particularly those in the medium- to low-molecular-mass range (from 70 kDa down to about 20 kDa), lack reactivity towards ConA (Fig. 4a, b, lanes 2) and, most likely, are minor cell-wall-bound protein moieties.

Western immunoblot analysis of β MEYPD and β MEV extracts using the pAbs raised against the corresponding materials (pAbs anti- β MEYPD and anti- β MEV, respectively) as probes, is shown in lane 7 of Fig. 4a and 4b. The pAbs also recognized a complex array of medium- to high-molecular-mass-species (from 40 to > 112 kDa), most of them glycoproteins, as can be concluded from their reactivity against ConA (Fig. 4a, b, lanes 4). In addition, both pAb preparations also recognized several bands that were not stained with ConA-peroxidase. Immunodetection of material released from cells grown in YPD medium and adsorbed on the polystyrene microspheres (Fig. 4a, lane 8) revealed one other major protein with an apparent molecular mass of 55 kDa. This band (Fig. 4a, lane 8) did not exhibit reactivity toward ConA (Fig. 4a, lane 5). The pAbs also recognized the 115 and 210 kDa glycoprotein species mentioned above that

exhibited affinity for the surface of the polystyrene microspheres (Fig. 4a, b, lanes 8).

DISCUSSION

The outermost cell wall layer of *Aspergillus* conidia is characterized by the presence of interwoven fascicles of clustered proteinaceous microfibrils called rodlets, the formation of which is encoded by the recently cloned gene *rodA* (Thau *et al.*, 1994). This layer is insoluble and is responsible for the conidial hydrophobicity of *Aspergillus* spp. (Parta *et al.*, 1994). Inhaled resting conidia lose their hydrophobic outer surface layers, initially swell and then germinate to form mycelial filaments that may invade host tissues (Diamond, 1988). In this work, we determined CSH of *A. fumigatus* by different methods and showed that the strong CSH exhibited by resting conidia disappeared after 3 h of incubation in YPD medium, and that swollen conidia and short germ tubes appeared to be essentially non-hydrophobic, yet mature mycelial filaments were highly hydrophobic. Similar results have been reported in *C. albicans*, where mycelial filaments were invariably highly hydrophobic regardless of the CSH displayed by the mother blastoconidia (López-Ribot *et al.*, 1991). The fact that treatment of cells with β ME or coating of the polystyrene particles with β ME extracts abolished attachment of the microspheres to the mycelial surface suggests that the CSH of *A. fumigatus* mycelium can be ascribed to cell surface components susceptible to release by treatment with reducing agents such as β ME.

Extracts obtained by treatment of *A. fumigatus* cells with β ME, which releases fungal cell wall components without substantially altering their native characteristics (Calderone & Braun, 1991), were analysed by SDS-PAGE and Western blotting. As reported for *C. albicans* (Casanova & Chaffin, 1991; Casanova *et al.*, 1992a; Chaffin & Stocco, 1983; López-Ribot *et al.*, 1991; Ponton & Jones, 1986), β ME also solubilized from the walls of *A. fumigatus* hyphae a complex array of protein and glycoprotein species distributed within a wide molecular mass range (from 10 to > 112 kDa). Silver staining of polyacrylamide gels revealed similar polypeptide patterns in β ME extracts obtained from cells grown in complex (YPD) or minimal (Vogel's N) media. However, *in vivo* labelling of mycelial filaments with biotin demonstrated qualitative and quantitative differences between wall proteins released by β ME from cells grown in the two media. This suggests that growth in different culture media may affect the topological localization of particular protein components within the cell wall structure (and in turn accessibility of biotin to target molecules), rather than modifying the qualitative pattern of proteins released by β ME from the cell wall of *A. fumigatus* mycelium. Some qualitative differences between silver-stained and biotin-labelled polypeptide patterns (compare lanes 5 and 6 versus lanes 1 and 2 in Fig. 2), may reflect the existence of (i) minor cell wall components with a fast metabolic turnover, (ii) proteins with low affinity for biotin, or (iii) proteins located deep within the wall structure or masked by other wall components (both possibilities would hamper accessibility of biotin to target molecules).

IF assays revealed that antigens recognized by the different pAb preparations were only detectable at the surface of mycelial filaments and, interestingly, either long mature hyphae or short young mycelial filaments exhibited basically the same homogeneous confluent fluorescent pattern, yet only mature hyphal elements displayed strong CSH. This observation suggests that cell-wall-bound components responsible for CSH are expressed late during the germination process or, alternatively, they could be masked by hydrophilic molecules in short germ tubes.

In order to establish the particular cell wall moieties to which the hydrophobic character exhibited by the surface of *A. fumigatus* hyphae could be ascribed, we analysed the capacity of the protein and glycoprotein species released by β ME to be adsorbed onto the surface of polystyrene microspheres, which might be an indication of the possible hydrophobic character of such molecules (López-Ribot *et al.*, 1991, 1995). Patterns of β ME-released, polystyrene-adsorbed (hydrophobic?) proteins from cells grown in complex (YPD) or minimal (Vogel's N) media were similar, which is in agreement with the fact that mycelial filaments consistently exhibited strong CSH regardless of the culture medium in which the cells were grown, although some qualitative and/or quantitative differences were noted depending on the medium used. Thus, β MEYPD extracts may contain specific high-molecular-mass hydrophilic glycoproteins (as revealed by both ConA-mediated staining and immunodetection with pAbs) that can be masked by other hydrophobic species with similar electrophoretic mobility (between 115 and 210 kDa) present in both β MEYPD and β MEV extracts. This may account for the presence of non-adsorbed high-molecular-mass proteins only in the residual material after adsorption of β MEYPD extracts onto polystyrene. In addition, the walls of cells grown in YPD could contain more hydrophobic cell surface species (for instance, a 55 kDa polystyrene-adsorbed polypeptide) than the cells grown in Vogel's N medium.

In summary, we report here for the first time that *A. fumigatus* mycelia displayed strong CSH, a property that may have a potential role as a virulence determinant in medically important fungi (Hazen, 1990), and that CSH may be conferred by cell-wall-bound proteins and/or glycoproteins that are released by treatment of intact cells with β ME. This knowledge may help to establish the role (if any) that CSH plays in the virulence and pathogenicity of this fungal species. In addition, hydrophobic interactions may be of great importance in promoting further invasion of tissues by the filamentous forms of the fungus, once the initial colonization of host cells and/or mucosal surfaces by conidia, the infectious air-borne propagules, has occurred. It has to be stressed that *A. fumigatus* conidia are able to adhere to fibrinogen, laminin, complement and fibronectin via proteins (receptors?) of the outer cell wall (Annaix *et al.*, 1992; Coulot *et al.*, 1994; Peñalver *et al.*, 1996; Sturtevant & Latgé, 1992; Tronchin *et al.*, 1993) but binding sites are not present on the surface of mycelial elements. In this context, CSH could represent an indirect mechanism allowing attachment of *A.*

fumigatus to host tissues leading to further development of infection.

ACKNOWLEDGEMENTS

The support of grants from the DGICYT (PM92-0246) and CICyT (SAF95-0595), Ministerio de Educación y Ciencia, Spain, is acknowledged.

REFERENCES

- Annaix, V., Bouchara, J. P., Larcher, G., Chabasse, D. & Tronchin, G. (1992). Specific binding of fibrinogen fragment D to *Aspergillus fumigatus* conidia. *Infect Immun* **60**, 1747–1755.
- Antley, P. P. & Hazen, K. C. (1988). Role of yeast cell growth temperature on *Candida albicans* virulence in mice. *Infect Immun* **56**, 2884–2890.
- Beachey, E. H. (1981). Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J Infect Dis* **143**, 325–345.
- Bodey, G. P. & Vartivarian, S. (1989). Aspergillosis. *Eur J Clin Microbiol Infect Dis* **8**, 413–437.
- Bouchara, J. P., Tronchin, G., Larcher, G. & Chabasse, D. (1995). The search for virulence determinants in *Aspergillus fumigatus*. *Trends Microbiol* **3**, 327–330.
- Calderone, R. A. & Braun, P. C. (1991). Adherence and receptor relationships in *Candida albicans*. *Microbiol Rev* **55**, 1–20.
- Casanova, M. & Chaffin, W. L. (1991). Cell wall glycoproteins of *Candida albicans* as released by different methods. *J Gen Microbiol* **137**, 1045–1051.
- Casanova, M., Gil, M. L., Cardeñosa, L., Martínez, J. P. & Sentandreu, R. (1989). Identification of wall-specific antigens synthesized during germ tube formation by *Candida albicans*. *Infect Immun* **57**, 262–271.
- Casanova, M., López-Ribot, J. L., Martínez, J. P. & Sentandreu, R. (1992a). Characterization of cell wall proteins from yeast and mycelial cells of *Candida albicans* by labelling with biotin: comparison with other techniques. *Infect Immun* **60**, 4898–4906.
- Casanova, M., Lopez-Ribot, J. L., Monteagudo, C., Llobart-Bosch, A., Sentandreu, R. & Martínez, J. P. (1992b). Identification of a 58-kilodalton cell surface fibrinogen-binding mannoprotein from *Candida albicans*. *Infect Immun* **60**, 4221–4229.
- Chaffin, W. L. & Stocco, D. M. (1983). Cell wall proteins of *Candida albicans*. *Can J Microbiol* **29**, 1438–1444.
- Cohen, J. (1991). Clinical manifestations and management of aspergillosis in the compromised patient. In *Fungal Infection in the Compromised Patient*, 2nd edn, pp. 117–152. Edited by D. W. Warnock & M. D. Richardson. Chichester: John Wiley.
- Coulot, P., Bouchara, J. P., Renier, G., Annaix, V., Planchenault, C., Tronchin, G. & Chabasse, D. (1994). Specific interaction of *Aspergillus fumigatus* with fibrinogen and its role in cell adhesion. *Infect Immun* **62**, 2169–2177.
- Diamond, R. D. (1988). Fungal surfaces: effects of interactions with phagocytic cells. *Rev Infect Dis* **10**, S428–S431.
- Drouhet, E., Camay, L. & Segretain, G. (1972). Valeur de l'immunoprecipitation et de l'immunofluorescence indirecte dans les aspergilloses broncho-pulmonaires. *Ann Inst Pasteur* **123**, 379–395.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**, 350–356.
- Glee, P. M., Sundstrom, P. & Hazen, K. C. (1995). Expression of

- surface hydrophobic proteins by *Candida albicans* in vivo. *Infect Immun* **63**, 1373–1379.
- Goldstein, I. J. & Hayes, C. E. (1978).** The lectins: carbohydrate-binding proteins of plants and animals. In *Advances in Carbohydrate Chemistry and Biochemistry*, vol. 35, pp. 127–340. Edited by R. S. Tipson & D. Horton. New York: Academic Press.
- Hazen, K. C. (1990).** Cell surface hydrophobicity of medically important fungi, especially *Candida* species. In *Microbial Cell Surface Hydrophobicity*, pp. 249–295. Edited by R. J. Doyle and M. Rosenberg. Washington, DC: American Society for Microbiology.
- Hazen, K. C. & Hazen, B. W. (1987).** A polystyrene microsphere assay for detecting surface hydrophobicity variations within *Candida albicans* populations. *J Microbiol Methods* **6**, 289–299.
- Hazen, K. C., Lay, J. G., Hazen, B. W., Fu, R. C. & Murthy, S. (1990).** Partial biochemical characterization of cell surface hydrophobicity and hydrophilicity of *Candida albicans*. *Infect Immun* **58**, 3469–3476.
- Hearn, V. M. (1984).** Surface antigens of intact *Aspergillus fumigatus* mycelium: their localization using radiolabelled protein A as marker. *J Gen Microbiol* **130**, 907–917.
- Hearn, V. M. (1992).** Antigenicity of *Aspergillus* species. *J Med Vet Mycol* **30**, 11–25.
- Hearn, V. M. & Sietsma, J. H. (1994).** Chemical and immunological analysis of the *Aspergillus fumigatus* cell wall. *Microbiology* **140**, 789–795.
- Hearn, V. M., Wilson, E. V., Latgé, J.-P. & Mackenzie, D. W. R. (1990).** Immunochemical studies of *Aspergillus fumigatus* mycelial antigens by polyacrylamide gel electrophoresis and Western blotting techniques. *J Gen Microbiol* **136**, 1525–1535.
- Hearn, V. M., Latgé, J. P. & Prévost, M. C. (1991).** Immunolocalization of *Aspergillus fumigatus* mycelial antigens. *J Med Vet Mycol* **29**, 73–81.
- Kwon-Chung, K. J. & Bennett, J. E. (1991).** Aspergillosis. In *Medical Mycology*, pp. 201–247. Edited by K. J. Kwon-Chung & J. E. Bennett. Philadelphia: Lea & Febiger.
- Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Latgé, J.-P., Kobayashi, H., Debeaupuis, J. P., Diaquin, M., Sarfati, J., Wieruszkeski, J. M., Parra, E., Bouchara, J. P. & Fournet, B. (1994).** Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. *Infect Immun* **62**, 5424–5433.
- López-Ribot, J. L., Casanova, M., Martínez, J. P. & Sentandreu, R. (1991).** Characterization of cell wall proteins of yeast and hydrophobic mycelial cells of *Candida albicans*. *Infect Immun* **59**, 2324–2332.
- López-Ribot, J. L., Gozalbo, D., Sepúlveda, P., Casanova, M. & Martínez, J. P. (1995).** Preliminary characterization of the material released to the culture medium by *Candida albicans* yeast and mycelial cells. *Antonie Leeuwenhoek* **68**, 195–201.
- Martínez, J. P., Casanova, M., Gil, M. L. & Sentandreu, R. (1991).** Analysis of the polypeptide composition of the cell walls of *Neurospora crassa*. Similarities with the proteinaceous material secreted by the slime variant. *Mycol Res* **95**, 315–319.
- Morrissey, J. H. (1981).** Silver stain for protein in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal Biochem* **117**, 307–310.
- Parta, M., Chang, Y., Rulong, S., Pinto-DaSilva, P. & Kwong-Chung, K. J. (1994).** HYP1, a hydrophobin gene from *Aspergillus fumigatus*, complements the *rodletless* phenotype in *Aspergillus nidulans*. *Infect Immun* **62**, 4389–4395.
- Peñalver, M. C., O'Connor, J. E., Martínez, J. P. & Gil, M. L. (1996).** Binding of human fibronectin to *Aspergillus fumigatus* conidia. *Infect Immun* **64**, 1146–1153.
- Ponton, J. & Jones, J. M. (1986).** Analysis of cell wall extracts of *Candida albicans* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot techniques. *Infect Immun* **53**, 565–572.
- Reijula, K. E., Kurup, V. P., Kumar, A. & Fink, J. N. (1992).** Monoclonal antibodies bind identically to both spores and hyphae of *Aspergillus fumigatus*. *Clin Exp Allergy* **22**, 547–553.
- Rosenberg, M., Gutnick, D. & Rosenberg, E. (1980).** Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* **9**, 29–33.
- Rotrosen, D., Calderone, R. A. & Edwards, J. E. (1986).** Adherence of *Candida* species to host tissues and plastic surfaces. *Rev Infect Dis* **8**, 73–85.
- Silva, T. M. J., Glee, P. M. & Hazen, K. C. (1995).** Influence of cell surface hydrophobicity on attachment of *Candida albicans* to extracellular matrix proteins. *J Med Vet Mycol* **33**, 117–122.
- Ste-Marie, L., Senechal, S., Boushira, M., Garzon, S., Strykowski, H., Pedneault, L. & de Repentigny, L. (1990).** Production and characterization of monoclonal antibodies to cell wall antigens of *Aspergillus fumigatus*. *Infect Immun* **58**, 2105–2114.
- Sturtevant, J. E. & Latgé, J.-P. (1992).** Interactions between conidia of *Aspergillus fumigatus* and human complement C3. *Infect Immun* **60**, 1913–1918.
- Stynen, D., Sarfati, J., Goris, A., Prévost, M. C., Lesourd, M., Kamphuis, H., Darras, V. & Latgé, J.-P. (1992).** Rat monoclonal antibodies against *Aspergillus* galactomannan. *Infect Immun* **60**, 2237–2245.
- Thau, N., Monod, M., Crestani, B., Rolland, C., Tronchin, G., Latgé, J.-P. & Paris, S. (1994).** *rodletless* mutants of *Aspergillus fumigatus*. *Infect Immun* **62**, 4380–4388.
- Tronchin, G., Bouchara, J. P., Larcher, G., Lissitsky, J. C. & Chabasse, D. (1993).** Interaction between *Aspergillus fumigatus* and basement membrane laminin: binding and substrate degradation. *Biol Cell* **77**, 201–208.
- Wilson, E. V. & Hearn, V. M. (1982).** A comparison of surface and cytoplasmic antigens of *Aspergillus fumigatus* in an enzyme-linked immunosorbent assay (ELISA). *Mykosen* **25**, 653–661.

Received 19 December 1995; revised 31 January 1996; accepted 8 February 1996.