Ectophosphatase activity in conidial forms of *Fonsecaea pedrosoi* is modulated by exogenous phosphate and influences fungal adhesion to mammalian cells

Lucimar F. Kneipp,^{1,4} Marcio L. Rodrigues,¹ Carla Holandino,² Fabiano F. Esteves,³ Thais Souto-Padrón,¹ Celuta S. Alviano,¹ Luiz R. Travassos⁴ and José R. Meyer-Fernandes³

Correspondence

José R. Meyer-Fernandes
meyer@bioqmed.ufrj.br

^{1,2,3}Instituto de Microbiologia Professor Paulo de Góes¹, Departamento de Medicamentos, Faculdade de Farmácia² and Departamento de Bioquímica Médica³, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-590, Brazil

⁴Disciplina de Biologia Celular, Universidade Federal de São Paulo, São Paulo, SP 04023-062, Brazil

A cell-wall-associated phosphatase in hyphae of *Fonsecaea pedrosoi*, a fungal pathogen causing chromoblastomycosis, was previously characterized by the authors. In the present work, the expression of an acidic ectophosphatase activity in *F. pedrosoi* conidial forms was investigated. The surface phosphatase activity in *F. pedrosoi* is associated with the cell wall, as demonstrated by transmission electron microscopy. This enzyme activity was strongly inhibited by exogenous inorganic phosphate (P_i). Accordingly, removal of P_i from the culture medium of *F. pedrosoi* resulted in a marked (130-fold) increase of ectophosphatase activity. With the artificial phosphatase substrate *p*-nitrophenyl phosphate, a *K_m* value of 0.63 ± 0.04 mM was estimated for the phosphatase activity of fungal cells strongly expressing the enzyme activity. This enzyme activity was not modulated by cations. Conidia with greater ectophosphatase activity showed greater adherence to mammalian cells than did fungi cultivated in the presence of P_i (low phosphatase activity). Surface phosphatase activity was apparently involved in the adhesion to host cells, since the enhanced attachment of *F. pedrosoi* to host cells was reversed by pre-treatment of conidia with phosphatase inhibitor. Since conidial forms are the putative infectious propagules in chromoblastomycosis, the expression and activity of acidic surface phosphatases in these cells may contribute to the early mechanisms required for disease establishment.

Received 16 June 2004

Accepted 6 July 2004

INTRODUCTION

Fonsecaea pedrosoi, the primary causative agent of chromoblastomycosis (CBM) in man and animals, is a dematiaceous filamentous fungus with worldwide distribution (Rippon, 1988; Kwon-Chung & Bennett, 1992). The chronic subcutaneous fungal infection occurs most frequently in tropical and subtropical regions, especially South America and Japan (Fabra *et al.*, 1994; Silva *et al.*, 1999; Tanuma *et al.*, 2000). The infection generally affects individuals who are engaged in farming (Silva *et al.*, 1999), resulting from the traumatic implantation of fungal propagules. Males are more commonly affected, and the lesions are usually in the lower limbs (Rippon, 1988), which are more likely to be in

contact with infected materials, soil, plants or rotting wood (Kwon-Chung & Bennett, 1992). Current therapies against CBM involve use of antifungal agents and/or surgical excision but, as with other subcutaneous mycoses, treatment of CBM is poorly effective, producing relapses during therapy and problems with lack of tolerance to antifungal drugs (Esterre *et al.*, 2000; Koga *et al.*, 2003).

A number of fundamental processes in fungi such as the cell cycle, gene transcription and mating have been shown to require protein phosphorylation (Madhani & Fink, 1998; Dickman & Yarden, 1999; Zhan *et al.*, 2000). Levels of cellular phosphorylation are controlled by the coordinated actions of protein kinases and protein phosphatases (Hunter, 1995). Acid phosphatases (EC 3.1.3.2) have been described in many yeasts (Vogel & Hinnen, 1990; Vasileva-Tonkova *et al.*, 1996). These enzymes may exist as soluble

Abbreviations: CBM, chromoblastomycosis; FBS, fetal bovine serum; *p*-NP, *p*-nitrophenol; *p*-NPP, *p*-nitrophenyl phosphate.

or secreted forms (Jolivet *et al.*, 1998), or remain attached to the outer surface of the inner membrane (Arnold *et al.*, 1988) or cell wall (González *et al.*, 1993; Bernard *et al.*, 2002; Kneipp *et al.*, 2003).

The presence of surface-located acid phosphatases, called ecto- or extracytoplasmic phosphatases, has been reported in many micro-organisms (Fernandes *et al.*, 1997; Dutra *et al.*, 1998; Meyer-Fernandes *et al.*, 1999; Braibant & Content, 2001), including the fungi *Saccharomyces cerevisiae* (Mildner *et al.*, 1975), *Candida parapsilosis* (Fernando *et al.*, 1999), *Sporothrix schenckii* (Arnold *et al.*, 1986) and *Aspergillus fumigatus* (Bernard *et al.*, 2002). The specific functions of these enzymes are not fully known, but they probably participate in cell wall biosynthesis in yeast cells (Novick *et al.*, 1981). They may also have a role as 'safeguard' enzymes to protect the cells from acidic conditions, by buffering the periplasmic space with phosphate released from polyphosphates (Touati *et al.*, 1987). These enzymes may also provide fungal cells with a source of orthophosphate by hydrolysing organic phosphates (Toh-e, 1989). In *C. parapsilosis*, a surface phosphatase activity was correlated with fungal adhesion to mammalian cells (Fernando *et al.*, 1999), indicating that ectophosphatases can also influence the interaction of fungal cells with the host.

We demonstrated recently that mycelial forms of *F. pedrosoi* express a Zn^{2+} -activated acid ectophosphatase in their walls, and studied it biochemically and by ultrastructural examination (Kneipp *et al.*, 2003). In the present work we characterized an ectophosphatase activity in conidial forms of *F. pedrosoi*. The enzyme activity was shown to be negatively modulated by exogenous phosphate (P_i). Conidial cells expressing high ectophosphatase activity were significantly more capable of adhering to epithelial cells than fungi expressing basal levels of enzyme activity, indicating that surface phosphatases may have a role in the interaction of *F. pedrosoi* with host cells, an essential step in the infectious process.

METHODS

Micro-organisms and growth conditions. The pathogenic strain of *F. pedrosoi* (5VPL) used in this study was isolated from a human patient with CBM (Oliveira *et al.*, 1973). Stock cultures were maintained in Sabouraud-dextrose (glucose) agar, at 4 °C. Transfers were made every 6 months. The conidial cells were cultivated in a chemically defined medium (complete medium) containing, per litre: 30 g sucrose; 2 g NaNO_3 ; 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5 g KCl ; 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1 g KH_2PO_4 ; 2.88 g citric acid; 7.29 g sodium citrate (Czapek medium, modified from Alviano *et al.*, 1992). For preparation of conidia with differential phosphatase activities, cells were sometimes cultivated in the same medium, except for the absence of KH_2PO_4 (P_i -depleted medium). Conidial forms were grown at room temperature for 3 days, with shaking. To obtain conidia free of hyphae, the culture was filtered through gauze and the conidia were collected by centrifugation (13 000 g, 20 min, 4 °C). For experiments, conidial forms were washed three times in 0.9% saline.

Mammalian cells. MA 104 epithelial cell line, from monkey's kidney, and 3T3-L1 mouse fibroblasts were purchased from a Rio de

Janeiro cell culture collection (BCRJ, registration numbers CR053 and CR089, respectively). The cells were grown at 37 °C in 25 cm² culture flasks containing DMEM medium (Gibco), supplemented with 10% fetal bovine serum (FBS). The pH was maintained at 7.2 by the addition of HEPES (3 g l⁻¹) and NaHCO_3 (0.2 g l⁻¹) to the medium (Freshney, 1994). The initial inoculum was 5×10^4 cells ml⁻¹; cultures were subcultured every 2 days and the cells maintained in exponential-phase growth as described by Freshney (1994).

Measurement of phosphatase activity. Phosphatase activity was determined by measuring the rate of *p*-nitrophenol (*p*-NP) production from the hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP). Intact cells (10^7 conidia) of *F. pedrosoi* were incubated at room temperature for 60 min in a reaction mixture (0.5 ml) containing 15 mM MES/HEPES buffer at pH 5.5 and 5 mM *p*-NPP as substrate. For determining the concentration of *p*-NP formed through phosphatase activity, the tubes were centrifuged at 1500 g for 10 min (4 °C). The reaction was terminated by taking 0.2 ml of the supernatant and mixing it with 0.4 ml 1 M NaOH. The addition of NaOH after centrifugation was done to avoid extraction of melanin present in *F. pedrosoi* (Alviano *et al.*, 1991), which interferes with the colorimetric method. This mixture was measured spectrophotometrically at 425 nm, using *p*-NP as standard (Fernandes *et al.*, 1997). The phosphatase activity was calculated by subtracting the nonspecific *p*-NPP hydrolysis measured in the absence of cells. As detailed in Results and figure legends, several experiments were performed in the presence of different cations, substrates or phosphatase inhibitors, at standardized concentrations (Kneipp *et al.*, 2003). Cell viability was assessed before and after incubations by the Trypan blue method. The viability of the cells was not affected by the conditions used in this work.

Cytochemical detection of acid phosphatase. The cytochemical assay was carried out as previously described (Kneipp *et al.*, 2003). Briefly, the conidial forms were fixed for 30 min at 4 °C with 1% glutaraldehyde in 5% sucrose, 0.1 M cacodylate buffer at pH 7.2, washed in the same buffer, followed by 0.1 M Tris/maleate buffer pH 5.5, and incubated for 2 h at room temperature in 2 mM cerium chloride, 5% sucrose, 0.1 M Tris/maleate buffer pH 5.5, with 2 mM sodium β -glycerophosphate as substrate. The cells were then washed in Tris/maleate and cacodylate buffers, fixed again with 4% paraformaldehyde, 2.5% glutaraldehyde, 5 mM CaCl_2 , postfixed in 1% osmium tetroxide, dehydrated in a graded acetone series, and embedded in Epon. As a control, cells were incubated in the absence of substrate. Ultrathin sections were observed unstained in a transmission electron microscope (Zeiss 900 EM; Carl Zeiss) operated at 80 kV.

Adhesion of *F. pedrosoi* to host cells. Animal cells were plated onto 24-well multidishes at a density of 10^5 cells per well. They were then incubated at 37 °C for 24 h in the presence of DMEM medium supplemented with 10% FBS. Before interaction with animal cells, conidia of *F. pedrosoi* (10^6) were incubated for 30 min at room temperature in 0.9% NaCl (control cells) or in the same solution containing 1 mM sodium orthovanadate. Conidia were then washed twice with 0.9% saline and finally rinsed in DMEM. Fungal cells were suspended in the same medium to a ratio of 10 *F. pedrosoi* conidia per animal cell on monolayers. After the addition of *F. pedrosoi*, the cells were incubated at 37 °C for 2 h, washed three times in PBS to remove nonadherent conidia, fixed in Bouin's solution and stained with Giemsa. Adhesion indices were determined with a microscope at a magnification of 1000 (Zeiss Axioplan 2, Germany). The adhesion index is the ratio of attached and internalized conidia to the number of host cells per field. For each experiment, 400 animal cells were counted.

Statistical analysis. All experiments were performed in triplicate, with similar results obtained from at least three separate cell suspensions. Data were analysed statistically using Student's *t* test. The

Fig. 1. Cytochemical assay for localization of acid phosphatase activity of *F. pedrosoi*. Conidial cells were incubated for 2 h in a buffer containing Tris/maleate, pH 5.5, in the presence (a) or in the absence (b) of β -glycerophosphate substrate. Cerium phosphate deposits are clearly visible on the cell wall in (a). Scale bars, 1 μ m.

maximal velocity ($V_{\max}$) and Michaelis constant (K_m) for *p*-NPP were calculated using a computerized nonlinear regression program (Sigma Plot 4.0; Jandel Scientific Software).

RESULTS

Conidial forms of *F. pedrosoi* express cell-wall-associated phosphatase activities

Intact *F. pedrosoi* conidia converted the artificial substrate *p*-NPP to its hydrolysed form *p*-NP at pH 5.5, as previously established for mycelial cells (Kneipp *et al.*, 2003). After 60 min incubation in the presence of the phosphorylated substrate, the enzyme activity reached 4.5 ± 0.5 nmol *p*-NP h^{-1} per 10^7 cells. Since cell viability in these assays was around 95 % and phosphatase activity was not detected in culture supernatants, the possibility of hydrolysis of *p*-NPP by intracellular or secretory phosphatases was discarded. To confirm the surface distribution of phosphatases in *F. pedrosoi* conidia, fungal cells were incubated with cerium chloride and β -glycerophosphate and prepared for transmission electron microscopy, which clearly showed the occurrence of electron-dense cerium phosphate deposits on the fungal cell wall (Fig. 1).

Effect of phosphatase inhibitors on the hydrolysis of *p*-NPP by intact conidia

The effects of several well-known inhibitors of phosphatases on the hydrolysis of *p*-NPP by intact conidia are shown in Fig. 2 (black bars). The highest indices of enzyme inhibition were observed when sodium molybdate, sodium fluoride, sodium orthovanadate and P_i were used. Among these compounds, P_i was considered as a highly specific inhibitor of phosphatase activity, since it is the natural product of reactions catalysed by phosphoprotein phosphatases. We therefore investigated the effect of P_i removal on the ectophosphatase activity of growing *F. pedrosoi* conidia.

Conidial cells that were cultivated in a P_i -depleted medium had an ectophosphatase activity significantly higher than that of fungal cells grown in the complete medium. As described above, conidia from the complete medium had an activity of around 4.5 ± 0.5 nmol *p*-NP h^{-1} per 10^7 cells, while those cultivated in the absence of P_i had an activity of 584.3 ± 77.7 nmol *p*-NP h^{-1} per 10^7 cells. Although the

Fig. 2. Effect of inhibitors on the phosphatase activity of intact conidia of *F. pedrosoi*. Phosphatase activities of fungal cells grown for 3 days in complete (black bars) or P_i -depleted (white bars) were statistically compared; significant values of *P* are shown at the top of the bars. The uninhibited phosphatase activity [4.5 ± 0.5 nmol *p*-NP h^{-1} per 10^7 cells (complete medium) and 584.3 ± 77.7 nmol *p*-NP h^{-1} per 10^7 cells (P_i -depleted medium)] were taken as 100%. The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentages of the control values. NS, Not significant. Inset: Effect of P_i on the phosphatase activity of *F. pedrosoi* grown in complete medium.

latter cells had a lower growth rate, they remained viable, thus validating the measurements of ectophosphatase activity (data not shown). The possibility that the discrepancies in enzyme activity could be due to differences in the kinetics of differentiation was considered, since the morphological stages of *F. pedrosoi* differ in ectophosphatase activity (Kneipp *et al.*, 2003). However, in our current experimental model, formation of hyphae from conidia after 3 days of culture is mostly absent in both complete and P_i -depleted media (not shown). In addition, the indices of germinating conidia after prolonged periods of cultivation in both complete and P_i -depleted media are very similar (data not shown), ruling out a possible influence of germination in the modulation of phosphatase activity in *F. pedrosoi* conidia.

Conidial cells expressing different levels of phosphatase activity were also compared in their sensitivity to phosphatase inhibitors (Fig. 2, white bars). Different profiles were observed; for instance, conidia cultivated in P_i -depleted medium had a surface phosphatase activity significantly more susceptible to inhibition by sodium orthovanadate, sodium fluoride and sodium molybdate than those cultivated in the complete medium. In addition, the ectophosphatase from conidia grown in P_i -depleted medium was partially inhibited by levamisole.

Conidia from both culture conditions had their surface phosphatase activity strongly inhibited by P_i (Fig. 2). The inhibition of the surface phosphatase activity in *F. pedrosoi* conidia by P_i followed a dose-dependent pattern, as demonstrated by K_i determination. Enzyme activity was measured in the absence of P_i (control) or in a reaction medium containing increasing concentrations of KH_2PO_4 . Phosphatase activity was almost completely inhibited at

10 mM KH_2PO_4 ; a K_i corresponding to 2.05 ± 0.28 mM was estimated.

Potential substrates for the ectophosphatase activity such as β -glycerophosphate and α -naphthyl phosphate seemed to compete with *p*-NPP, inhibiting its hydrolysis (Table 1). However, α -naphthyl phosphate and β -glycerophosphate were more efficient in inhibiting the activity of conidia cultivated in P_i -depleted medium. The possible existence of phosphotyrosin phosphatases, initially suggested by the decrease of *p*-NPP hydrolysis by orthovanadate (Gordon, 1991), was confirmed by the fact that P-Tyr, but not P-Ser and P-Thr, inhibited the hydrolysis of *p*-NPP by conidial cells cultivated in both complete and P_i -depleted medium (Table 1). This observation was further confirmed by the fact that intact *F. pedrosoi* conidial cells preferentially liberated P_i from P-Tyr (data not shown).

Conidia from complete and P_i -depleted medium express different surface phosphatase activities

The significantly different profiles of enzyme inhibition in conidia cultivated in complete and P_i -depleted media indicated that these cells express different phosphatases. This hypothesis was further supported by the analysis of the influence of various cations (Ca^{2+} , Sr^{2+} , Zn^{2+} , Mg^{2+} and Fe^{3+}) on the hydrolysis of *p*-NPP by *F. pedrosoi* conidia. Ectophosphatase activity was stimulated when conidia cultivated in the complete medium were assayed in the presence of 1 mM Fe^{3+} (Fig. 3), while other cations were ineffective (not shown). The Fe^{3+} -induced stimulation followed a dose-dependent pattern and was not reversed by the addition of EDTA or removal of ions by washing. Phosphatase activity from conidia cultivated in P_i -depleted

Table 1. Effect of competing substrates on the *p*-NPP phosphatase activity of intact conidia of *F. pedrosoi*

Conidia, cultivated for 3 days in complete or P_i -depleted medium, were suspended in 15 mM MES/HEPES buffer, pH 5.5, with 5 mM *p*-NPP for 60 min at room temperature, in the absence or presence of different substrates. The phosphatase activity determined using *p*-NPP in the absence of competing substrates was taken as 100%; this activity was 4.5 ± 0.5 nmol *p*-NP h⁻¹ per 10⁷ cells (complete medium) and 584 ± 77.7 nmol *p*-NP h⁻¹ per 10⁷ cells (P_i -depleted medium). The standard errors were calculated from the absolute activity values of three experiments with different cell suspensions and converted to percentages of the control values. The *P* values indicate Student's *t*-test significance of differences between enzyme activity from fungal cells cultivated in P_i -depleted versus complete medium (NS, not significant).

Substrate (10 mM)	Relative activity (%)		<i>P</i>
	Complete medium	P_i -depleted medium	
β -Glycerophosphate	48.9 ± 2.4	40.7 ± 2.6	<0.05
α -Naphthyl phosphate	53.1 ± 1.9	23.3 ± 1.7	<0.001
P-Tyr	56.0 ± 5.4	53.4 ± 1.3	NS
P-Thr	88.6 ± 4.3	98.9 ± 4.1	NS
P-Ser	82.3 ± 4.3	92.7 ± 4.7	NS

Fig. 3. (a) Effect of Fe³⁺ on the phosphatase activity of conidial forms of *F. pedrosoi*. Intact cells were incubated for 60 min at room temperature in 15 mM MES/HEPES buffer, pH 5.5, supplemented with increasing concentrations of FeCl₃. (b) The basal levels of enzyme activity (no treatment) are enhanced in the presence of 1 mM Fe³⁺. Removal of the cation by washing or treatment with 1 mM EDTA treatment did not reverse the Fe³⁺-induced stimulation. Values are means ± SE of three determinations with different cell suspensions. Asterisks denote significant differences ($P < 0.001$) in comparison with control cells (no treatment).

medium remained unaltered after the addition of the cations cited above (data not shown).

Kinetic determinations demonstrated that the ectophosphatases from conidia cultivated in complete and *P_i*-depleted media had different affinities for the artificial substrate *p*-NPP. K_m and V_{max} values of 15.12 ± 3.55 mM *p*-NPP and 18.07 ± 2.30 nmol *p*-NP h⁻¹ per 10⁷ cells, respectively, were estimated for fungal cells cultivated in the presence of exogenous phosphate, while cultivation of cells in the absence of *P_i* gave K_m and V_{max} values of 0.63 ± 0.04 mM *p*-NPP and 649.30 ± 11.37 nmol *p*-NP h⁻¹ per 10⁷ cells, respectively. These results confirmed that exogenous *P_i* modulates the expression of different surface phosphatases during fungal growth.

Expression of ectophosphatase influences the adhesion of *F. pedrosoi* to animal epithelial cells and fibroblasts

The differential expression of ectophosphatase activity in fungal cells cultivated in the absence or presence of exogenous phosphate provided the basis for new experiments to investigate the possible participation of fungal ectophosphatases in the interaction of *F. pedrosoi* with animal cells. Conidia with greater ectophosphatase activity had indices of adhesion to host cells higher than those of fungi cultivated in the presence of *P_i* (i.e. expressing low ectophosphatase activity) (Fig. 4). When the epithelial cell lineage MA 104 was used, conidia from *P_i*-depleted medium were 7-fold more adherent than fungi from the complete medium. Conidia expressing higher indices of ectophosphatases were also more efficient in attaching to mouse fibroblasts; they were 3.7-fold more adherent than fungal cells cultivated in

Fig. 4. Influence of phosphatase activity on the interaction of *F. pedrosoi* with mammalian cells. Conidia cultivated in *P_i*-depleted medium (black bars), expressing high levels of surface phosphatase activity, showed greater adherence to cultured fibroblasts (a) or renal epithelial cells (b) than conidia with basal levels of ectophosphatase, grown in complete medium (white bars). Pre-treatment of fungi with sodium orthovanadate inhibited adhesion of conidia expressing high ectophosphatase activity ($P < 0.0001$). Adhesion of conidia cultivated in complete medium was not significantly (NS) affected by sodium orthovanadate.

the complete medium. Surface phosphatases seemed to be involved in adhesion of fungal cells cultivated in P_i -depleted medium, since the enhanced conidial attachment to both cell lines was reversed by pre-treating fungi with sodium orthovanadate.

DISCUSSION

Infection by *F. pedrosoi* begins with the traumatic implantation of conidia or fragments of hyphae on subcutaneous tissues, producing initial lesions consisting of papules or nodules that become verrucous (De Hoog *et al.*, 2000). Inside the host, conidial forms differentiate into mycelium, which finally produce sclerotic bodies, the parasitic forms of CBM (Rippon, 1988). Sclerotic bodies are melanized cells that are extremely resistant to destruction by immune effector cells (Esterre *et al.*, 2000; Hamza *et al.*, 2003). A successful infection depends on the attachment of infectious propagules to host epithelial cells and their subsequent morphological transition. In this context, the elucidation of the mechanisms by which *F. pedrosoi* conidia attach to host cells and differentiate into sclerotic bodies is of crucial significance.

Phosphatases are fundamental components in cellular events regulated by phosphorylation-dephosphorylation systems, which are coordinately controlled through the action of protein kinases and phosphoprotein phosphatases (Hunter, 1995). The ectophosphatase activity in mycelial cells of *F. pedrosoi* (Kneipp *et al.*, 2003), and that shown in *F. pedrosoi* conidia in the present study, are strongly inhibited by exogenously added P_i , suggesting that this reaction product may regulate the fungal ectophosphatase activity.

The presence of phosphatases regulated by phosphate in the culture medium was reported in several prokaryotic organisms (Torriani-Gorini *et al.*, 1994; Hulett, 1996) and also in fungi, including *Neurospora crassa* (Jacob *et al.*, 1971), *Aspergillus niger* (MacRae *et al.*, 1988), *Yarrowia lipolytica* (González *et al.*, 1993), *Pichia pastoris* (Payne *et al.*, 1995) and *S. cerevisiae* (Ogawa *et al.*, 2000). In *S. cerevisiae*, transcription of genes encoding acid and alkaline phosphatases and the P_i transporter are coordinately repressed and derepressed depending on the P_i concentration in the culture medium (Oshima *et al.*, 1996). The regulation of this adaptive response is very complex, involving several genes that signal P_i starvation (Ogawa *et al.*, 2000). Most of the phosphatases synthesized under P_i -limiting conditions are either located extracellularly or are associated with the plasma membrane or cell wall (Metzenberg, 1979).

In the present study, the presence of cell wall phosphatases in conidial forms of *F. pedrosoi* was demonstrated by transmission electron microscopy. The phosphatase activity was strongly inhibited by P_i , and cultivation of *F. pedrosoi* conidia in the absence of exogenous P_i resulted in the generation of fungal cells expressing an ectophosphatase activity 130-fold higher than that expressed by fungi

cultivated in the presence of P_i , which agreed with our previous biochemical observations (Kneipp *et al.*, 2003). The depletion of phosphate from the culture medium apparently induced the expression of a different ectophosphatase, as suggested by the differences in the affinity for the artificial substrate *p*-NPP and by the fact that Fe^{3+} did not enhance the ectophosphatase activity from conidia cultivated in P_i -depleted medium. On the other hand, the expression of other surface structures was apparently not affected by exogenous phosphate; for instance, the ecto-ATPase activities of *F. pedrosoi* conidia after growth in the two different media described above were quite similar (data not shown).

The generation of conidial cells with clearly distinct levels of surface phosphatase activity allowed the design of experiments to investigate the putative participation of the ectophosphatase activity in the interaction of *F. pedrosoi* conidia with host cells. Conidia expressing higher levels of surface phosphatase activity showed 7- and 3.7-fold greater adhesion to epithelial cells and fibroblasts, respectively. To evaluate whether phosphatases were in fact involved in adhesion, experiments were performed in the presence of enzyme inhibitors. P_i , the natural inhibitor of the *F. pedrosoi* ectophosphatases, produces a reversible pattern of inhibition. Therefore, experimental models in which fungal cells are pre-incubated with P_i were impaired, since it is efficiently removed by washing. When P_i is added as a phosphatase inhibitor during the interaction of *F. pedrosoi* with host cells, surface structures of fibroblasts and epithelial cells seemed to be modified also (data not shown), making the interpretation of data very difficult. Sodium orthovanadate (irreversible inhibition) was therefore chosen as the most suitable inhibitor to control the influence of ectophosphatases in fungal adherence. The pre-treatment of fungal cells with sodium orthovanadate inhibited the adhesive ability of conidia, confirming the involvement of ectophosphatases in the adhesion of *F. pedrosoi* to mammalian cells.

Our results indicate that ectophosphatases, besides their possible functions in the biology of fungal cells, may play a role in the interaction of *F. pedrosoi* with host tissues. The reasons for increased adherence of fungal propagules with enhanced ectophosphatase activity remain unclear, but the removal of phosphate groups from surface structures of host cells could result in conformational changes resulting in the exposure of additional sites for interaction with infectious agents. Alternatively, ectophosphatases may contain adhesive domains that could directly promote the attachment of fungal cells to their hosts, therefore functioning similarly to the well-characterized microbial adhesins. Finally, they could regulate the functional activation of surface adhesins, which would be the key structures mediating fungal attachment.

The knowledge of the functions of ectophosphatases in fungal pathogens is still very preliminary. We currently report on the involvement of these enzymes in fungal

adhesion, but their cell wall distribution suggests that they could also play important roles in other biological processes occurring at the fungal surface. Ectophosphatases could, for instance, regulate the functions of chitin synthase, which is activated by calmodulin-mediated phosphorylation as in *N. crassa* (Suresh & Subramanyam, 1997). Alternatively, ectophosphatases may regulate morphological transitions in fungal pathogens. Recent results from our laboratory demonstrated that the addition of propranolol or platelet-activating factor, which are inducers of differentiation in *F. pedrosoi*, stimulates the ectophosphatase activity concomitantly with morphogenetic transition (Alviano *et al.*, 2003). These observations are in agreement with our previous results demonstrating that the three morphological stages of *F. pedrosoi*, i.e. sclerotic, mycelial and conidial cells, show different levels of ectophosphatase activity (Kneipp *et al.*, 2003). In the same study, we demonstrated that a fungal strain recently isolated from a human case of CBM had an ectophosphatase activity significantly higher than that of a laboratory-adapted strain. In addition, sclerotic cells, which are the parasitic forms of *F. pedrosoi*, had a surface phosphatase activity higher than that of mycelia and conidia. Taken together, these observations suggest a link between ectophosphatase activity and fungal parasitism. Our current results indicate that the activity of surface phosphatases indeed influences the interaction of *F. pedrosoi* with host cells *in vitro*. If the same occurs *in vivo*, ectophosphatases could contribute to the establishment of CBM, possibly acting in the early steps of fungal adhesion.

ACKNOWLEDGEMENTS

We thank Venício F. Veiga for assistance with microscopy. This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Financiadora de Estudos e Projetos (FINEP), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Fundação Universitária José Bonifácio (FUJB).

REFERENCES

- Alviano, C. S., Farbiarz, S. R., De Souza, W., Angluster, J. & Travassos, L. R. (1991). Characterization of *Fonsecaea pedrosoi* melanin. *J Gen Microbiol* **137**, 837–844.
- Alviano, C. S., Farbiarz, S. R., Travassos, L. R., Angluster, J. & De Souza, W. (1992). Effect of environmental factors on *Fonsecaea pedrosoi* morphogenesis with emphasis on sclerotic cells induced by propranolol. *Mycopathologia* **119**, 17–23.
- Alviano, D. S., Kneipp, L. F., Lopes, A. H., Travassos, L. R., Meyer-Fernandes, J. R., Rodrigues, M. L. & Alviano, C. S. (2003). Differentiation of *Fonsecaea pedrosoi* mycelial forms into sclerotic cells is induced by platelet-activating factor. *Res Microbiol* **154**, 689–695.
- Arnold, W. N., Mann, L. C., Sakai, K. H., Garrison, R. G. & Coleman, P. D. (1986). The acid phosphatases of *Sporothrix schenckii*. *J Gen Microbiol* **132**, 3421–3432.
- Arnold, W. N., Garrison, R. G., Mann, L. C. & Wallace, D. P. (1988). The acid phosphatases of *Thermoascus crustaceus*, a thermophilic fungus. *Microbios* **54**, 102–112.
- Bernard, M., Mouyna, I., Dubreucq, G., Debeaupuis, J.-P., Fontaine, T., Vorgias, C., Fuglsang, C. & Latgé, J.-P. (2002). Characterization of a cell-wall acid phosphatase (PhoAp) in *Aspergillus fumigatus*. *Microbiology* **148**, 2819–2829.
- Braibant, M. & Content, J. (2001). The cell surface associated phosphatase activity of *Mycobacterium bovis* BCG is not regulated by environmental inorganic phosphate. *FEMS Microbiol Lett* **195**, 121–126.
- De Hoog, G. S., Queiroz-Telles, F., Haase, G. & 10 other authors (2000). Black fungi: clinical and pathogenic approaches. *Med Mycol* **38** (Suppl 1), 243–250.
- Dickman, M. B. & Yarden, O. (1999). Serine/threonine protein kinases and phosphatases in filamentous fungi. *Fungal Genet Biol* **26**, 99–117.
- Dutra, P. M. L., Rodrigues, C. O., Jesus, J. B., Lopes, A. H. C. S., Souto-Pradón, T. & Meyer-Fernandes, J. R. (1998). A novel ecto-phosphatase activity of *Herpetomonas muscarum muscarum* inhibited by platelet-activating factor. *Biochem Biophys Res Commun* **253**, 164–169.
- Esterre, P., Jahevitra, M. & Andriantsimahavandy, A. (2000). Humoral immune response in chromoblastomycosis during and after therapy. *Clin Diagn Lab Immunol* **7**, 497–500.
- Fabra, A. R., Restrepo, A. M. & Istúriz, R. E. (1994). Fungal infection in Latin American countries. *Infect Dis Clin North Am* **8**, 129–154.
- Fernandes, E. C., Meyer-Fernandes, J. R., Silva-Neto, M. A. C. & Vercesi, A. E. (1997). *Trypanosoma brucei*: ecto-phosphatase activity on the surface of intact procyclic forms. *Z Naturforsch* **52c**, 351–358.
- Fernando, P. H. P., Panagoda, G. J. & Samaranayare, L. P. (1999). The relation between the acid and alkaline phosphatase activity and the adherence of clinical isolates of *Candida parapsilosis* to human buccal epithelial cells. *APMIS* **107**, 1034–1042.
- Freshney, R. I. (1994). *Culture of Animal Cells: a Manual of Basic Technique*. New York: Wiley-Liss.
- González, F. J., Fauste, C., Burguillos, F. J. & Domínguez, A. (1993). Kinetic behaviour of a repressible acid phosphatase from yeast *Yarrowia lipolytica*: a comparative study between the solubilized enzyme, the enzyme bound to cell-wall fragments and the enzyme bound to intact cells. *Biochim Biophys Acta* **1162**, 17–27.
- Gordon, J. A. (1991). Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol* **201**, 447–482.
- Hamza, S. H., Mercado, P. G., Skelton, H. G. & Smith, K. J. (2003). An unusual dematiaceous fungal infection of the skin caused by *Fonsecaea pedrosoi*: a case report and review of the literature. *J Cutan Pathol* **30**, 340–343.
- Hulett, F. M. (1996). The signal-transduction network for *pho* regulation in *Bacillus subtilis*. *Mol Microbiol* **19**, 933–939.
- Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80**, 225–236.
- Jacob, M. M., Nyc, J. F. & Brown, D. M. (1971). Isolation and chemical properties of a repressible acid phosphatase in *Neurospora crassa*. *J Biol Chem* **246**, 1419–1425.
- Jolivet, P., Queiroz-Claret, C., Bergeron, E. & Meunier, J.-C. (1998). Characterization of an exocellular protein phosphatase with dual substrate specificity from the yeast *Yarrowia lipolytica*. *Int J Biochem Cell Biol* **30**, 783–796.
- Kneipp, L. F., Palmeira, V. F., Pinheiro, A. A. S., Alviano, C. S., Rozental, S., Travassos, L. R. & Meyer-Fernandes, J. R. (2003).

Phosphatase activity on the cell wall of *Fonsecaea pedrosoi*. *Med Mycol* **41**, 469–477.

Koga, T., Matsuda, T., Matsumoto, T. & Furue, M. (2003). Therapeutic approaches to subcutaneous mycoses. *Am J Clin Dermatol* **4**, 537–543.

Kwon-Chung, K. J. & Bennett, J. E. (1992). Chromoblastomycosis. In *Medical Mycology*, pp. 337–355. Edited by K. J. Kwon-Chung & J. E. Bennett. Philadelphia: Lea & Febiger.

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

Madhani, H. D. & Fink, G. R. (1998). The control of filamentous differentiation and virulence in fungi. *Trends Cell Biol* **8**, 348–353.

Metzenberg, R. (1979). Implications of some genetic control mechanisms in *Neurospora*. *Microbiol Rev* **43**, 361–383.

Meyer-Fernandes, J. R., Silva-Neto, M. A. C., Soares, M. S., Fernandes, E., Vercesi, A. E. & Oliveira, M. M. (1999). Ecto-phosphatase activities on the cell surface of the amastigote forms of *Trypanosoma cruzi*. *Z Naturforsch* **54c**, 977–984.

Mildner, P., Ries, B. & Barbaric, S. (1975). Acid phosphatase and adenosine triphosphatase activities in the cell wall of baker's yeast. *Biochim Biophys Acta* **391**, 67–74.

Novick, P., Ferro, S. & Schekman, R. (1981). Order of events in the yeast secretory pathway. *Cell* **25**, 461–469.

Ogawa, N., DeRisi, J. & Brown, P. O. (2000). New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. *Mol Biol Cell* **11**, 4309–4321.

Oliveira, L. G., Resende, M. A., Lopes, C. F. & Cisalpino, E. D. (1973). Isolamento e identificação dos agentes da cromomicose em Belo Horizonte. *Rev Soc Brasil Med Trop* **7**, 7–10.

Oshima, Y., Ogawa, N. & Harashima, S. (1996). Regulation of phosphatase synthesis in *Saccharomyces cerevisiae* – a review. *Gene* **179**, 171–177.

Payne, W. E., Gannon, P. M. & Kaiser, C. A. (1995). An inducible acid phosphatase from the yeast *Pichia pastoris*: characterization of the gene and its product. *Gene* **163**, 19–26.

Rippon, J. W. (1988). Chromoblastomycosis. In *Medical Mycology*, pp. 276–296. Edited by J. W. Rippon. Philadelphia: W. B. Saunders.

Silva, J. P., De Souza, W. & Rozental, S. (1999). Chromoblastomycosis: a retrospective study of 325 cases on Amazonian Region (Brazil). *Mycopathologia* **143**, 171–175.

Suresh, K. & Subramanyam, C. (1997). A putative role for calmodulin in the activation of *Neurospora crassa* chitin synthase. *FEMS Microbiol Lett* **150**, 95–100.

Tanuma, H., Hiramatsu, M., Mukai, H., Abe, M., Kume, H., Nishiyama, S. & Katsuoka, K. (2000). Case report. A case of chromoblastomycosis effectively treated with terbinafine. Characteristics of chromoblastomycosis in the Kitasato region, Japan. *Mycoses* **43**, 79–83.

Toh-e, A. (1989). In *Genetic Engineering*, pp. 41–52. Edited by P. J. Barr, A. J. Brake & P. Valenzuela. Boston: Butterworths.

Torriani-Gorini, A., Silver, S. & Yagil, E. (1994). *Phosphate in Microorganisms: Cellular and Molecular Biology*. Washington DC: American Society for Microbiology.

Touati, E., Dassa, E., Dassa, J. & Boquet, P. L. (1987). Acid phosphatase (pH 2.5) of *Escherichia coli*: regulatory characteristics. In *Microorganisms*, pp. 31–40. Edited by A. Torriani-Gorini, F. G. Rothman, S. Silver, A. Wright & E. Yagil. Washington, DC: American Society for Microbiology.

Vasileva-Tonkova, E., Balasheva, D. M. & Galabova, D. (1996). Influence of growth temperature on the acid phosphatase activity in the yeast *Yarrowia lipolytica*. *FEMS Microbiol Lett* **145**, 267–271.

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

Zhan, X.-L., Hong, Y., Zhu, T., Mitchel, A. P., Deschenes, R. J. & Guan, K.-L. (2000). Essential functions of protein tyrosine phosphatases Ptp2 and Ptp3 and Rim11 tyrosine phosphorylation in *Saccharomyces cerevisiae* meiosis and sporulation. *Mol Biol Cell* **11**, 663–676.