Identification of a gene encoding adaptin-like protein in the *Paracoccidioides brasiliensis* genome by random amplified polymorphic DNA analysis

Patrícia Ferrari Andreotti,¹ Juliana Leal Monteiro da Silva,¹ Elaine Cristina Teixeira,² Maria Céla Bertolini,² Christiane Pienna Soares,¹ Gil Benard³ and Maria José Soares Mendes-Giannini¹

Correspondence

Maria José Soares Mendes-Giannini
giannini@fcfar.unesp.br

¹Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas, UNESP, Araraquara, SP, Brazil

²Departamento de Bioquímica e Tecnologia Química, Instituto de Química, UNESP, Araraquara, SP, Brazil

³Laboratório de Dermatologia e Imunodeficiências, Departamento de Dermatologia, Faculdade de Medicina, Universidade de São Paulo, Brazil

Paracoccidioides brasiliensis isolates are not homogeneous in their patterns of pathogenicity in animals and adhesion to epithelial cells. During this investigation, genotypic differences were observed between two samples of *P. brasiliensis* strain 18 yeast phase (Pb18) previously cultured many times, one taken before (Pb18a) and the other after (Pb18b) animal inoculation. Random amplified polymorphic DNA analysis using the primer OPJ4 distinguished Pb18b from Pb18a by one 308 bp DNA fragment, which after cloning and sequencing was shown to encode a polypeptide sequence homologous to the protein β -adaptin. It is suggested, by comparison to other micro-organisms, that this protein might play an important role in the virulence of *P. brasiliensis*. This result demonstrates the influence of *in vitro* subculturing on the genotype of this organism.

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INTRODUCTION

The virulence of *Paracoccidioides brasiliensis* can be attenuated or lost during consecutive cycles of subculturing over long periods (Brummer *et al.*, 1990), but re-established after inoculating into animals (Castaneda *et al.*, 1987). Newly isolated samples of *P. brasiliensis* strain 18 (Pb18), either from infected animals or from cell cultures, recovered the capacity to adhere to and to invade epithelial cells (Andreotti *et al.*, 2005).

Molecular biology techniques are a powerful tool to explore the diversity of pathogen strains. Random primers have been refined so that they can be used to distinguish and characterize genetic diversity among isolates of *P. brasiliensis* (Molinari-Madlum *et al.*, 1999; Soares *et al.*, 1995; Motta *et al.*, 2002) and could differentiate the fungus based on virulence (Kurokawa *et al.*, 2005).

Several coat proteins, such as heterotetrameric adaptor protein (AP) complexes, have been described that are

involved in the formation of carrier vesicles and consist of two large adaptins (γ and α are found together with β 1 and β 2), one medium-sized adaptin (μ 1 or μ 2) and one small adaptin (σ 1 or σ 2) (Boehm & Bonifacio, 2001).

The aim of this study was to demonstrate differences in the random amplified polymorphic DNA (RAPD) profiles of two samples of Pb18, one subcultured *in vitro* 72 times and the other recently recovered from an inoculated animal. A polymorphic DNA band that was found in the sample recovered from an inoculated animal was cloned and sequenced. The DNA fragment was part of a gene encoding an adaptin-like protein.

METHODS

Pb18 was obtained from a patient with paracoccidioidomycosis and maintained at Faculdade de Medicina, Universidade de São Paulo. During the current work, *P. brasiliensis* was cultivated in solid YEPD medium (2 % peptone, 1 % yeast extract, 2 % glucose) at 35 °C and subcultured 72 times, for 3–4 days each, to produce the Pb18a sample. The Pb18b sample was obtained after inoculation into hamster by an intratesticular route as described by Andreotti *et al.* (2005).

Abbreviation: RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DBJ accession no. for the adaptin-like protein sequence of *Paracoccidioides brasiliensis* is AY675222.

The Pb18a and Pb18b samples were cultivated in YEPD broth at 35 °C, overnight, under agitation, and DNA extraction for both samples was carried out based on the method of Lasker *et al.* (1992). The DNA was resuspended and stored in TE buffer (40 mM Tris, 2 mM EDTA) at –20 °C.

The molecular typing of the samples was carried out by RAPD-PCR, using the Ready-To-Go/RAPD analysis beads kit (GE Healthcare). For PCR amplification of the two DNA samples, the following six primers from the kit were used: 1 (5'-GGTGC GGGAA-3'), 2 (5'-GTTTCGCTCC-3'), 3 (5'-GTAGACCCGT-3'), 4 (5'-AAGAGCCCGT-3'), 5 (5'-AACGCGCAAC-3'), 6 (5'-CCCGTCAGCA-3'). The PCR amplification products were separated by electrophoresis in a 2 % agarose gel in TBE buffer and visualized with ethidium bromide.

The DNA fragment (approximately 300 bp) amplified by RAPD-PCR with primer 4 was removed from the agarose gel and purified with a S.N.A.P. gel purification kit (Invitrogen). The purified DNA fragment was cloned into vector pMOS (GE Healthcare) according to the manufacturer's instructions and amplified in *Escherichia coli* strain DH10B. For DNA sequencing, plasmids were extracted from overnight cultures and sequenced, on both strands, using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems), and the universal T7 and U-19 primers, in an automatic DNA sequencer (ABI 377; Perkin Elmer). The nucleotide sequence of the fragment (308 bp) was translated by the Gene Runner program, analysed by BLAST at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and Broad Institute (www.broad.mit.edu/annotation/cgi) databases, and the protein sequence alignment performed by ClustalX program.

Approximately 30 µg genomic DNA was digested with the restriction enzymes *Sall*, *EcoRI* and *NdeI*, electrophoresed on a 0.8 % agarose gel and transferred to nylon membrane (GE Healthcare). The blot was probed with the 308 bp DNA fragment labelled with fluorescein using the Gene Images random prime labelling module kit (GE Healthcare) and the results revealed with the Gene Images CDP-Star detection module kit (GE Healthcare) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

In our study we have evaluated two samples of *P. brasiliensis* strain 18, correlating their virulence with RAPD profiles. RAPD-PCR was used for the PCR amplification and no differences in the band profiles between the two DNA samples were observed using the primers 1, 2, 3, 5 and 6. However, primer 4 was able to differentiate Pb18b from Pb18a samples by one band of approximately 300 bp that was visualized in the Pb18b DNA sample (Fig. 1). The RAPD profile was related to the *P. brasiliensis* sample presenting a high epithelial adhesion pattern (Andreotti *et al.*, 2005). This result contrasts with the data of Motta *et al.* (2002), which showed that different samples of *P. brasiliensis* were not homogeneous in their ability to infect mice, but had identical RAPD profiles, showing 100 % similarity between their genotypes. Calcagno *et al.* (1998) demonstrated that genetic variations could be associated with geographical regions but not with different clinical manifestations of paracoccidioidomycosis. However, Molinari-Madlum *et al.* (1999) showed that RAPD profiles could be correlated with the degree of virulence of the *P. brasiliensis* isolates. Our results support

Fig. 1. RAPD-PCR analysis of *P. brasiliensis* 18 (a) before and (b) after animal inoculation. 1, 2, 3, 4, 5, 6 indicate the primers used from the Ready-To-Go/RAPD analysis beads kit (GE Healthcare). The DNA molecular mass marker is indicated on the left. The arrow indicates the polymorphic band of 308 bp.

the hypothesis that there is an influence of *in vitro* subculturing on the genotype of *P. brasiliensis*.

During this investigation, a 308 bp polymorphic band from genomic DNA of *P. brasiliensis* with high virulence was sequenced on both strands, and the nucleotide sequence was translated by Gene Runner program. One putative intron (66 bp) was identified in the nucleotide sequence, showing the consensus 5' splice-donor site (GT) and the consensus 3' splice-acceptor site (AG) for splice donors and acceptors (Fig. 2a). The polypeptide sequence deduced from the nucleotide sequence (308 bp) was analysed by BLAST. It encodes a polypeptide homologous to proteins from the adaptin family from *Aspergillus terreus* (GenBank accession no. XM_001213650), *Aspergillus fumigatus* (GenBank accession no. XM_750274), *Histoplasma capsulatum* (Broad Institute accession no. HCAG_08087.1) and *Aspergillus nidulans* (Broad Institute accession no. AN5950.3). The polypeptide sequence corresponds to part of the N-terminal region of the protein. We classified the DNA as a sequence encoding *P. brasiliensis* adaptin-like protein and it was deposited in GenBank under the accession no. AY675222 (Fig. 2b). Four AP complexes, named AP-1, AP-2, AP-3 and AP-4, have been described in eukaryotic organisms. Biochemical and morphological evidence indicates that AP complexes play a role in the formation of vesicular transport intermediates and in the selection of cargo molecules for inclusion into these intermediates (Boehm & Bonifacino, 2001).

The role of the adaptins in pathogenic fungus has not been studied so far. In *Saccharomyces cerevisiae*, the complex AP-2 is not essential for endocytosis or cellular viability

genetic diversity between both samples is still unclear, but it is possible that the fungal population changes and adapts in order to escape pressure or eradication by the immune system, as demonstrated in *Cryptococcus neoformans* by Fusco-Almeida *et al.* (2007).

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