The highly expressed yeast gene pby20 from Paracoccidioides brasiliensis encodes a flavodoxin-like protein

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Abstract

A gene encoding the entire highly expressed protein previously identified in the proteome of Paracoccidioides brasiliensis yeast cells as PbY20 has been isolated. The pby20 sequence reveals an open reading frame of 1364 bp and a deduced amino acid sequence of 203 residues, which shows high identity to benzoquinone reductase from Phanerochaete chrysosporium (72.0%), Saccharomyces cerevisiae Ycp4 (65%), and Schizosaccharomyces pombe p25 (59%), and to allergens from Alternaria alternata Alt a7 (70%) and from Cladosporium herbarum, Cla h5 (68%). Low levels of the pby20 transcript in the mycelium and highly induced ones in infective yeast cells during the transition of this dimorphic fungus indicate transcriptional control of its expression. PbY20 was immunologically detected only in yeast cell extract, suggesting an important role in cell differentiation or even in the maintenance of the yeast form. Immunoelectron microscopy showed that PbY20 is found inside large granules and vacuoles, in the nucleus, and also in the cytoplasm. Through sequence comparisons analysis and fluorescence emission assay, PbY20 was recognized as a member of the flavin mononucleotide flavodoxin-like WrbA family, which are involved in heat shock and oxidative stress in biological systems. Assuming that PbY20 belongs to this family, a similar role could be attributed to this protein.

Keywords: Dimorphic fungus; Overexpressed yeast protein; WrbA family; FMN-binding; Flavodoxin; Quinone reductase

1. Introduction

The fungus Paracoccidioides brasiliensis, the ethiologic agent of Paracoccidioidomycosis (PCM), is one of the most important human systemic mycosis in Latin America (Restrepo, 1985). PCM usually affects rural male workers and immunocompromised

patients. Over 10 million people are estimated to be infected with P. brasiliensis but only up to 2% develop the disease (McEwen et al., 1995). Infection occurs by inhalation of airbone mycelial structures of the saprophytic phase or propagules of the fungus that can differentiate to yeast form. The shift from 26 °C to host body temperature (37 °C) triggers the transition from mycelium to yeast cells. This process can be induced in vitro by changing the temperature.

Mycelium to yeast dimorphic transition is an important condition to the establishment of P. brasiliensis in the host. The identification and characterization of genes/proteins differentially expressed in cell types, yeast and mycelium, is essential to understanding the fungus dimorphism, its life cycle and to the development of new strategies for PCM treatment. To characterize P. brasiliensis differentially expressed genes, our group has employed several methodological approaches such as differential display reverse transcriptase PCR (DDRT-PCR), 1 Northern blot as well as proteome analysis. Among the differentially expressed genes previously described, we could mentioned several phase-specific cDNA fragments corresponding to genes differentially expressed in P. brasiliensis mycelium form (Venancio et al., 2002), two mycelium specific hydrophobin genes (Albuquerque et al., 2004) as well as the yeast-specific pby20 and hsp70 genes (Cunha et al., 1999 and Silva et al., 1999). Cunha et al. (1999), comparing proteome patterns from both forms of P. brasiliensis on 2D-gel electrophoresis, have identified the PbY20. The 34 N-terminal amino acid region showed high degree of identity compared to two allergen proteins, Alt a7 from A. alternata (88%) and Cla h5 from C. herbarum (82%). Moreover, the protein presents identity to Ycp4 (76%) and a hypothetical gene (71%), both from Saccharomyces cerevisiae and to p25 protein from Schizosaccharomyces pombe (71%).

More recently, Felipe et al. (2003) have developed the functional and differential genome project of P. brasiliensis (https://www.biomol.unb.br/Pb) to map the yeast and mycelium transcriptomes of this microorganism, based on the generation of expressed sequence tags (ESTs). Transcriptome analysis confirms the differential character of the previously described genes, including the differential and highly yeast expressed gene pby20.

The WrbA is a 21 kDa multimeric protein identified by Yang et al. (1993) as a tryptophan (W) repressor-binding protein (WrbA). The WrbA protein from Escherichia coli is a member of multimeric flavodoxin-like family (Grandori and Carey, 1994). Flavodoxins are small proteins that bind to FMN coenzyme and are able to transfer electrons at low oxidation–reduction potential. WrbA binds specifically and reversibly to FMN with 1:1 stoichiometry. However, unlike other flavodoxins, WrbA presents lower FMN-binding constant values (Grandori et al., 1998).

A WrbA protein from Phanerochaete chrysosporium with quinone reductase (QR) activity has been isolated and is stimulated by a wide variety of compounds as quinones, hydroquinones, and aromatic acids (Akileswaran et al., 1999). Flavodoxin-like QRs from Gloeophyllum trabeum (Jensen et al., 2002) and P. chrysosporium present high similarity to p25/orb1 from Sc. pombe, a protein related resistance against brefeldin A (Turi et al., 1994). All these proteins, including Ycp4, are overexpressed on oxidative stress and heat shock conditions (Gasch et al., 2000 and Kudo et al., 1999).

The complete pby20 gene and the comparative analysis of its deduced amino acid sequence with proteins from WrbA family are described in this paper. Differential expression of the pby20 gene in both mycelium and yeast cells and in the course of the transition process was investigated. Subcellular localization of this protein was assessed by immunoelectronic microscopy. Finally, PbY20 was characterized as a flavodoxin-like protein by fluorescence analysis.

2. Materials and methods

2.1. Paracoccidioides brasiliensis strain and growth conditions

Paracoccidioides brasiliensis Pb01 strain (ATCC-MYA-826) was grown as mycelium at 22 °C and sub-cultured every 15 days or as yeast at 36 °C and sub-cultured each 10 days. All cultures were maintained on solid medium (Fava-Neto, 1955). For RNA extractions, cells were grown at 22 °C in liquid medium (Negroni, 1966) for 48 h, shifted to 36 °C, and collected after 0, 0.5, 1, 2, 6, 12, and 24 h and 15 days.

2.2. RNA and genomic DNA extractions

Total RNA was isolated using Trizol reagent Gibco-BRL (Silva et al., 1999). The RNA was precipitated twice with LiCl (Sambrook et al., 1989), followed by RNase-free-DNaseI treatment (Mello et al., 1997) for DNA decontamination. Total genomic DNA was isolated from yeast cells with minor modifications to the protocol of Felipe et al. (1993). To obtain high-quality DNA, the protocol was performed in non-RNase-free conditions.

2.3. pby20 gene sequence and deduced protein analysis

The PCR, using P. brasiliensis genomic DNA and two degenerate primers (5'-CARAARAARGGIATYGAR-3'-forward and 5'-GAGAAGGTACCAGCRCCCCA-3'-reverse), resulted in a fragment of 555 bp which was sequenced. The PCR was performed in a total of 25 µL containing 100 ng of P. brasiliensis genomic DNA, 250 mM dNTP, 2.5 mM MgCl2, 1× Taq buffer, 0.4 µM of primers, and 2 U Taq DNA polymerase. Reaction conditions were: (1) 94 °C/3 min; (2) 94 °C/2 min; (3) 56 °C/1 min; (4) 72 °C/1.5 min; (5) 30 times from step 2; (8) 72 °C/10 min; (9) holding 4 °C. Based on the obtained genomic sequence of 555 bp, we designed six primers that were used in the 3' RACE and TAIL-PCR strategies to obtain the complete sequence of the pby20 gene (Fig. 1A). The first strand cDNA was synthesized from 1 µg of total RNA primed with 10 pmol of AP universal primer using 200 U of SuperScript II reverse transcriptase, at 42 °C for 50 min, and the second strand, using the gene specific primers L20/44 and AUAP. The 5' end TAIL-PCR strategy consists of consecutive PCRs performed with nested sequence-specific primers (L20/42, L20/43, and L20/13) and shorter arbitrary degenerated primers AD1–AD4 (Liu and Whittier, 1995). The 600 bp DNA fragment was sequenced and confirmed the 3' and 5' UTR regions. The complete pby20 gene was finally obtained using the same above described PCR protocol using genomic DNA, and Y20/5 and Y20/3 primers (Fig. 1A).

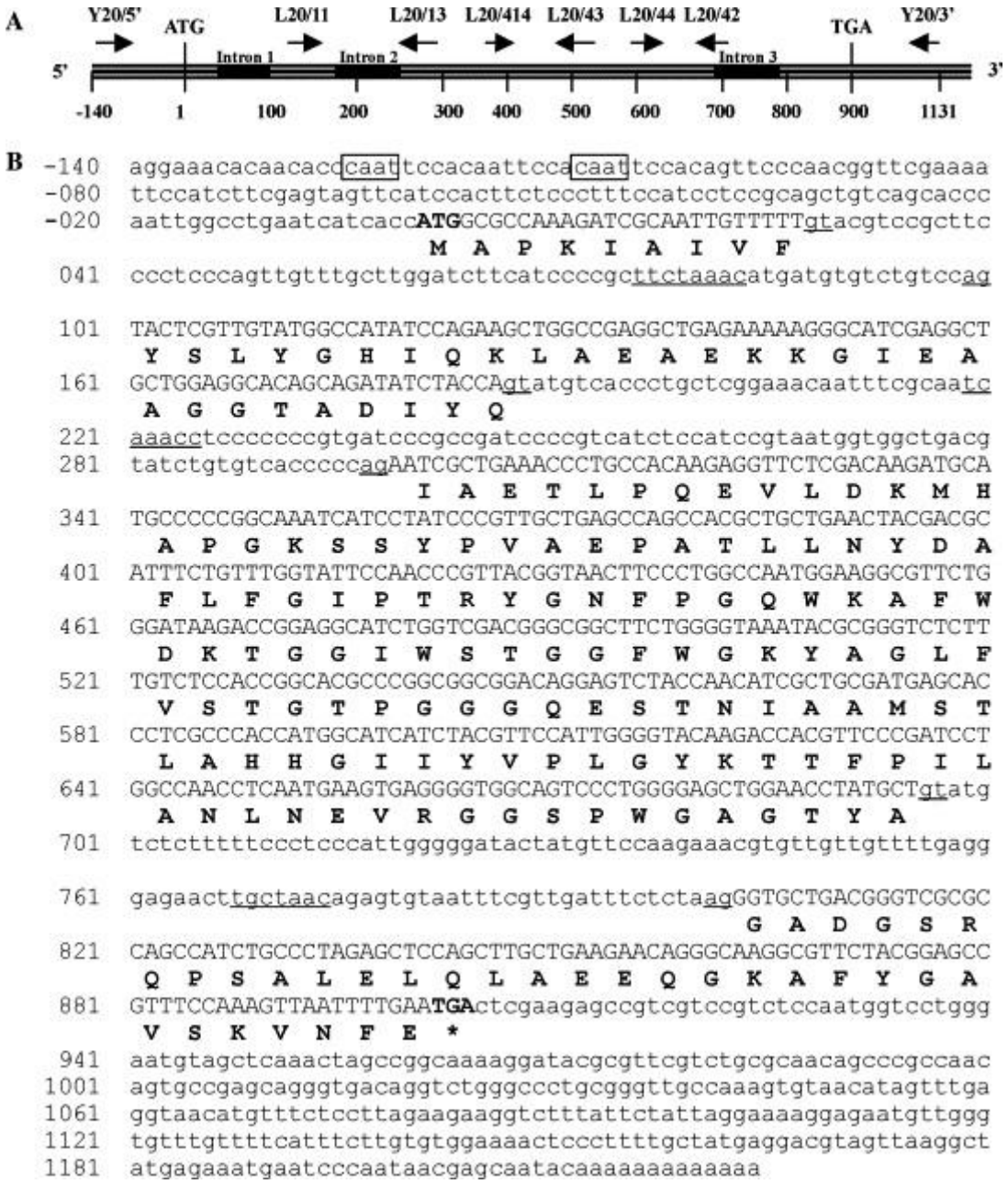

Fig. 1. (A) Schematic representation of the pby20 gene with primer annealing regions and introns positions. (L20/11, 5'-GAAGGGGATTGAGGCTGCTGGAGG-3'; L20/13, 5'-GCATCTTGTCGAGAACCTCTTGTGG-3'; L20/414, 5'-CTGAGCCAGCCACGCTGCTGAAC-3'; L20/42, 5'-GAGAAGGTACCAGCGCCCCAGGGAC-3'; L20/43, 5'-CGTATTTACCCCAGAAGCCGCCC-3'; L20/44, 5'-GGTACAAGACCAAGTTCCCGATCCTGGC-3'; Y20/5', 5'-CAACACCCAATTCCACAATTC-3'; Y20/3', 5'-AAACAAACACCCAACATTCTCC-3'). Introns are indicated by a black box and both start (ATG) and stop (TGA) codons are shown in bold. (B) pby20 complete nucleotide and deduced amino acid sequences. Exons sequences are indicated by upper case letters; the introns and 5'/3' non-coding regions are indicated by lower case letters. Initiation and termination codons (ATG and TGA, respectively) are in bold. The two putative CAAT motifs are boxed. The intron flanking sequences GT/AG and the lariat consensus motif (TACTAAC) are underlined. The deduced amino acid sequence is indicated in bold uppercase letters.

The obtained nucleotide sequence was analyzed by the computational programs PHRED (Ewing et al., 1998), PHRAP and CONSED (Gordon et al., 1998). Alignments were performed using BLAST (http://www.ncbi.nlm.nih.gov) and Clustal programs (http://www.ebi.ax.uk/clustaw). The signal peptide was predicted by SOSUI program (http://sosui.proteome.bio.tuat.ac.jp).

2.4. Western and Northern blots

Mycelium and yeast protein crude extracts were obtained by disruption of frozen cells in the presence of protease inhibitor cocktail (50 μg/ml TLCK, 50 μg/ml TPCK, 4 mM PMSF, 5 mM iodoacetamide, 1 mM EDTA, 20 μM leupeptin, and 1 mM PCMB). After debris removal, proteins precipitated with 10% (w/v) TCA were washed with 100% cold acetone. About 30 μg of mycelium and yeast total protein extracts in lysis buffer was submitted to SDS–PAGE according to Laemmli (1970) and blotted onto nitrocellulose membranes (Sambrook et al., 1989). The membrane was incubated with a PbY20 polyclonal antibody obtained from PbY20 immunized rabbits. After reaction with alkaline phosphatase anti-rabbit IgG, the reaction was developed using 5-bromo-4-chloro-3-indolylphosphate/nitro-blue-tetrazolium (NBT/BCIP).

Total RNA (15 μg) was fractionated by electrophoresis on formaldehyde-containing 1% agarose gels, transferred onto nylon membrane Hybond N, and hybridization was performed under stringent conditions (Mello et al., 1997). The membrane was washed three times using 0.1× SSPE and 0.1% SDS at 65 °C for 30 min, followed by exposure to autoradiography film at −80 °C for 24 h.

2.5. Overexpression of the PbY20 protein

The pby20 cDNA, after amplification with primers which introduce BamHI and XhoI, was cloned into pGEX-4T-3 to produce the pGEX-PbY20 fusion in E. coli BL21. A single colony was used for protein expression in LB media plus 100 μg/ml of ampicillin. The induction was started with 0.1 mM IPTG addition to the culture under growth for 3 h at 37 °C. The cells were pelleted, resuspended in PBS, and sonicated. The recombinant fused protein was purified using glutathione–Sepharose 4B affinity resin and submitted to SDS–PAGE and Western blot analysis.

2.6. FMN-binding

Qualitative analysis of the PbY20 FMN-binding was performed using fluorescence emission assay at 25 °C with a Jasco FP-777 Spectrofluorimeter with thermostated cuvette by electrical Peltier temperature controller. FMN spectra were recorded over the range of 480–580 nm under excitation wavelength of 459 nm. The GST-PbY20 (10 µM) was previously incubated at room temperature for 5 min with FMN (10 µM) in 50 mM sodium citrate, pH 6.0. The controls were done using GST incubated with FMN (10 µM) and the isolated FMN at the same concentration. The qualitative PbY20 FMN-binding was observed from the fluorescence quenching emission at 522 nm.

2.7. Ultrastructure of the yeast cells from P. brasilliensis

Yeast cells were fixed overnight at 4 °C in 2% glutaraldehyde, 2% paraformaldehyde, and 3% of sucrose in 0.1 M sodium cacodylate buffer at pH 7.2. After rinsing in 0.1 M sodium cacodylate buffer, the yeast cells were postfixed for 1 h in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM CaCl2 in sodium cacodylate buffer, pH 7.2. The material was dehydrated in a series of ascending concentrations of acetone (30–100%) and embedded in Spurr's epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Observations were done using a Jeol 100C transmission electron microscope.

2.8. Immunocytochemistry of the PbY20 protein

Yeast cells were fixed for 24 h at 4 °C in a mixture containing 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 M sodium cacodilate buffer at pH 7.2. Specimens were rinsed several times using the same buffer, and free aldehyde groups were quenched with 50 mM ammonium chloride in 0.1 M sodium cacodilate buffer for 1 h. Specimens were dehydrated in a series of ascending concentrations of acetone (30–90%). Embedding was performed in LRGold resine. Ultrathin sections were collected on nickel grids, pre-incubated in phosphate-buffered saline (PBS) containing 1.5% bovine serum albumin (PBS–BSA) and 0.02% Tween 20, and subsequently incubated for 1 h with polyclonal antibody against PbY20 (dilutions of 1:2 and 1:5). After washing with PBS–BSA, grids were incubated for 1 h with labeled secondary antibody (mouse-IgG-Au-conjugated 10 nm) at a dilution of 1:20 and washed with PBS and distilled water. Grids were stained with uranyl acetate and lead

citrate, and observed in a transmission electronic microscope (Jeol JEM 100C 1011). The control was incubated only with labeled secondary antibody.

2.9. Nucleotide sequence accession number

GenBank accession number of the pby20 gene is AF452883 (EMBL).

3. Results

3.1. The pby20 gene from P. brasiliensis

The EKKGIE and WGAGTY conserved domains, located, respectively, at amino acid positions 25–30 and 169–174 (Fig. 2), were strategically used to design the oligonucleotides to isolate a DNA fragment corresponding to a part of the gene encoding the yeast highly expressed PbY20 protein. The entire nucleotide sequence of the pby20 gene, obtained by 3'RACE and TAIL-PCR, showed highest identity to quinone reductase genes and also to Alt a7, Cla h5, p25, and Ycp4 genes. The P. brasiliensis gene was named as pby20 (P. brasiliensis yeast 20 kDa).

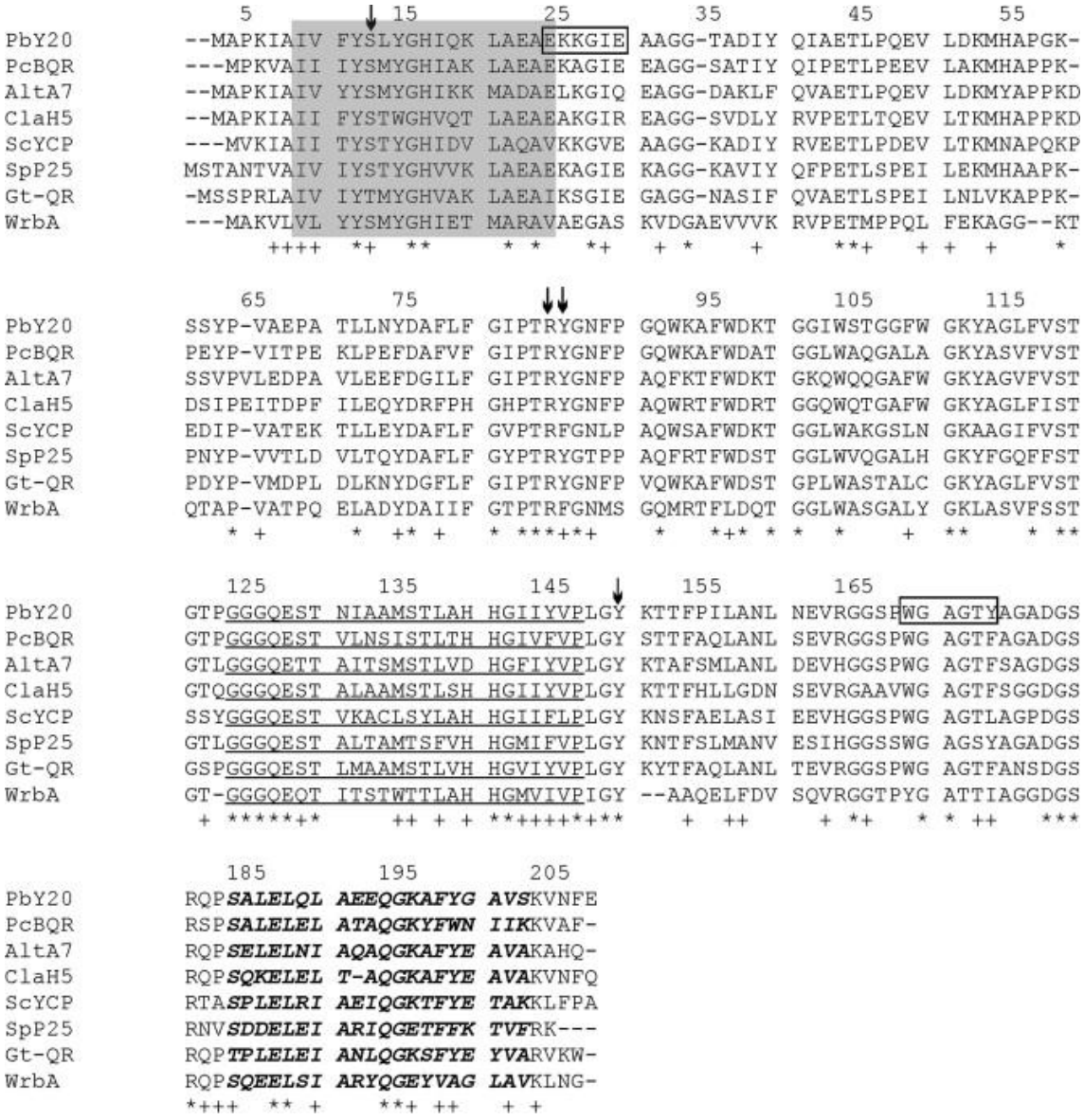

Fig. 2. Multiple sequence alignment (Clustal W) of deduced amino acid sequences of PbY20 protein from P. brasiliensis (PbY20) (Accession No. AAL50803), 1,4-benzoquinone reductase from P. chrysosporium (PcBQR) (Accession No. AAD21025), Alt A7 from A. alternata (AltA7) (Accession No. P42058), Cla H5 from C. herbarum (ClaH5) (Accession No. P42059), YCP4 from S. cerevisiae, (ScYCP) (Accession No. NP_009930), P25 from S. pombe (SpP25) (Accession No. P30821), NADH:Quinone oxidoreductase from G. trabeum (GT-Qr) (Accession No. AAL67860), and WrbA from E. coli (WrbA) (Accession No. M99166). Identical amino acids in all proteins are indicated by (∗). Conserved amino acids are indicated by (+) and amino acids S13, R85, Y86, and Y150 are indicated by the arrow (↓). The insertion of 24 amino acid residues, typical of the WrbA family, is underlined. The N-terminal region matching the flavodoxin signature is shaded in gray and HTH motifs are bolded and italic.

The pby20 gene, 5’ and 3’ flanking regions, and the deduced amino acid sequence are shown in Fig. 1B. It indicates an open reading frame (ORF) of 609 bp with four exons interrupted by three putative introns of 73, 112, and 107 bp at positions 28–100, 187–298, and 696–802, respectively. To precisely define the introns splicing sites, the yeast RNA extract was investigated by PCR using specific primers that amplify the entire pby20 ORF sequence. As

expected, the presence and localization of all three introns were confirmed by sequencing and alignment of genomic DNA and cDNA sequences (data not shown). Consistent with the predicted ORF, there are two putative CAAT boxes at positions −125 to −122 and −108 to −105. No TATA and transcriptional start sites element were found in the 5′ flanking region of the identified pby20. The 5′ and 3′ ends of the introns obey the GT/AG rule for donor and acceptor splicing sites (Mount, 1982) as shown in Fig. 1B. All three intron regions contain an internally conserved sequence upstream of the 3′ end that resembles the consensus TACTAAC claimed to be important for branch site formation.

3.2. The deduced amino acid sequence of PbY20

The pby20 ORF encodes a deduced primary sequence of 203 amino acids residues that extends from the ATG (position 1) to TGA (position 902). PbY20 has a predicted molecular mass of 21.7 kDa and pI of 6.02, similar to those previously identified by Cunha et al. (1999) and to other fungi quinone reductase proteins. Comparative analysis of the deduced protein (Table 1) revealed close identities to Alt a7 (70%) and Cla h5 (68%), in agreement with Cunha et al. (1999). PbY20 also showed close identities to NADH quinone oxidoreductase (67%), YCp4 (65%), p25 (59%), and flavoprotein WrbA (46%). In fact, a high identity was found to 1,4-benzoquinone reductase (72%).

Table 1.
Similarity analysis of PbY20 from P. brasiliensis with other fungi related proteins

Organism	Protein	Identity score	Length (aa)
Phanerochaete chrysosporium	1,4-Benzoquinone reductase	144/200 (72%)	201
Alternaria alternata	Minor allergen Alt a7	143/203 (70%)	204
Cladosporium herbarum	Minor allergen Cla h5	140/203 (68%)	204
Gloeophyllum trabeum	NADH quinone oxidoreductase	135/201 (67%)	257
Saccharomyces cerevisiae	Ycp4	129/197 (65%)	247
Schizosaccharomyces pombe	p25 protein (brefeldin A resistance protein)	117/196 (59%)	202
Escherichia coli	Flavoprotein wrbA	92/200 (46%)	198

The alignment of PbY20 sequence with WrbA family shows three conserved motifs in these proteins. At the N-terminal region a conserved signature motif of flavodoxins [I]-[V]-[F]-x-[S]-x-x-[G]-x-[I]-x-x-x-[A]-x-x-[E] was found. The C-terminal of the PbY20 shows a well-conserved insertion of 24 amino acid residues related to a predicted α/β segment and also the helix-turn-helix (HTH) motif, which are typical of WrbA family (Fig. 2). A putative pro-peptide leader sequence, which contains two potential KEX2 cleavage sites, is also present in QR from P. chrysosporium (Akileswaran et al., 1999) and G. trabeum (Jensen et al., 2002). However, such sequence was not identified in the PbY20 deduced amino acid sequence, suggesting that

the protein is not synthesized as a pro-protein. In addition, the putative signal peptide of the PbY20 was not found, in agreement with Cunha et al. (1999).

3.3. Differential expression of P. brasiliensis PbY20 protein

A significant increase in levels of the pby20 transcripts can be observed in the fully differentiated yeast pathogenic cells. In contrast, a very low basal level of the pby20 mRNA was observed in mycelium and also during the temperature-induced morphological transition (Fig. 3IB). A shift in the incubation temperature from 26 to 36 °C did not lead to an immediate increase in the pby20 mRNA level, at least until 24 h, but it was necessary that the fungus underwent the complete transition to yeast form before the maximal level was reached. The low level of mycelium expression clearly characterizes a highly differentially expressed pby20 in yeast cells of this pathogen. The size of the corresponding transcript was estimated at 1 kb, in agreement with the pby20 cDNA size.

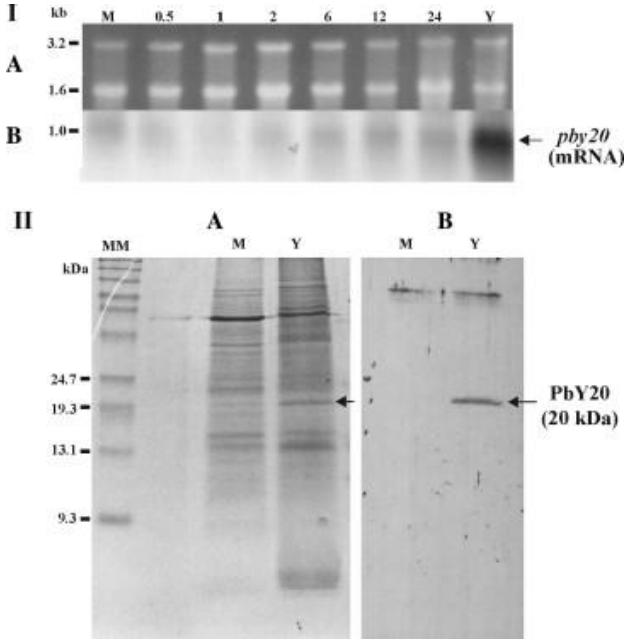

Fig. 3. Differential expression of pby20 gene during mycelium (M) to yeast (Y) transition: (I) Northern blot analysis, showing the differential expression of pby20 gene during mycelium to yeast transition. A probe corresponding to a 350 bp cDNA fragment of pby20 was prepared using MegaPrime labeling kit (Amersham Biosciences) and used to probe a membrane containing 15 μg of M and Y total RNA, and from M cells induced to dimorphic transition at different times after 26–36 °C temperature shift (0.5, 1, 2, 6, 12, and 24 h, Y-about 15 days). (A) Ethidium bromide stained 1% denaturing agarose gels, showing P. brasiliensis major and minor ribosomal RNAs, of about 3.2 and 1.6 kb, respectively. The same amount of loaded total RNA can be observed in all samples. (B) Resulting hybridization profile, showing the yeast-specific pby20 mRNA. (II) Western blot analysis of the differential expression of PbY20 during the M to Y transition. (A) About 30 μg of total protein extract from M and Y cells was loaded into 12% SDS–PAGE. The PbY20 observed in Y protein extract is indicated by an arrow. (B) Proteins from SDS–PAGE were blotted onto a nitrocellulose membrane and detected with a polyclonal anti-PbY20 antibody. The position of proteins in the molecular weight marker is indicated at left.

The Western blot indicates the differential expression of a 20 kDa protein in the yeast cell extract, strongly suggesting that it corresponds to a transcriptional induction level of the pby20 gene. The protein product in the mycelium phase was not detected (Figs. 3IIA and IIB), indicating that PbY20 is preferentially yeast-specific.

3.4. Immunolocalization of PbY20 protein in yeast cells

To define the cellular localization of PbY20 protein into the yeast cells of P. brasiliensis, we have performed immunocytochemistry experiments using ultrathin sections of LRGold embedded yeast P. brasiliensis. Electron microscopy of conventionally embedded cells revealed the ultrastructure of P. brasiliensis yeast form. A weakly electron dense cell wall and the plasma membrane also appear as a defined structure. The contours of the nucleus are relatively smooth and appear with condensed chromatin that is homogeneously distributed. Cytoplasm is occupied by a Golgi complex and electron lucent, well-developed vacuoles (Fig. 4A). Gold particles were detected mainly in the cytoplasm, inside large granules (Figs. 4B–C), and in the cell wall (Fig. 4C). PbY20 was also observed in the nucleus (Figs. 4B–C). No significant gold labels were detected in control experiments with the omission of primary antibody (Fig. 4D).

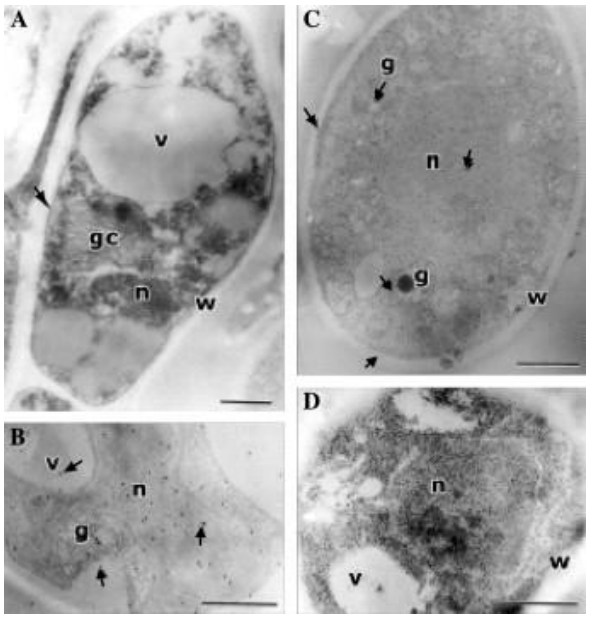

Fig. 4. Immunolocalization of PbY20 protein in yeast cells. (A) Yeast form of P. brasiliensis showing nucleus (n), intracytoplasmic vacuole (v), and Golgi complex (gc). Also, note the plasma membrane (arrowhead) and cell wall (w). (B–C) Gold particles (arrowheads) are observed at fungus cell wall (w), cytoplasmic granules (g), cytoplasmic vacuoles (v), and nucleus (n). (D) No label was observed in the control. The bars indicate 1 µm in (C), 0.5 µm in (A), (D), and 0.2 µm in (B).

3.5. FMN-binding assays

The purified GST-PbY20 fusion protein, showing by SDS–PAGE analysis a molecular mass of 46 kDa, was specifically recognized by a polyclonal antibody against PbY20, as confirmed by Western blot assay (data not shown). As observed for flavodoxins (Grandori et al., 1998, Munro and Noble, 1999 and Murray and Swenson, 2003) the fluorescence emission by FMN is partly quenched when the protein binds FMN. Therefore, measurement of the FMN fluorescence emissions can be used to monitor changes in the flavin–protein complex. Fluorescence spectra of free FMN, GST protein, and GST-PbY20 fusion protein incubated with FMN are shown in Fig. 5. The FMN emission in the presence of the GST-PbY20 is quenched compared to that of free FMN, indicating GST-PbY20–FMN interactions. Moreover, in the control experiment, the FMN emission upon adding GST is practically maintained constant, revealing that the FMN-binding is specific for the PbY20. These data strongly suggest that PbY20 belongs to the flavodoxin-like family.

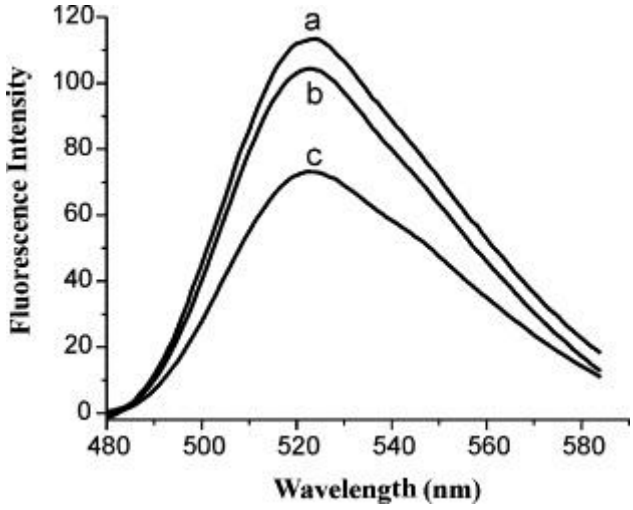

Fig. 5. Fluorescence spectra of free PbY20 and FMN bound PbY20 (A) Free FMN; (B) GST incubated with FMN; (C) GST-PbY20 fusion protein incubated with FMN. Equal volumes of FMN and proteins at the same concentration of 10 μM in sodium citrate 50 mM, pH 6.0, were incubated for 5 min and the emission spectra were generated using an excitation wavelength of 459 nm. FMN fluorescence quenching as a result of PbY20-FMN-binding was evident for the GST-PbY20 fusion protein as observed for flavodoxins (Grandori et al., 1998).

4. Discussion

Analysis of the PbY20 amino acid sequence alignment shows the high identity score with 1,4-benzoquinone reductase, Alt a7, Cla h5, NADH quinone oxireductase, Ycp4, p25, and flavoprotein WrbA. A multiple sequence alignment grouped all these proteins in the WrbA family. The results presented here strongly suggest that the PbY20 belongs to the WrbA family

according to the following five criteria: (i) The alignment of the PbY20 sequence with proteins from WrbA family shows a conserved N-terminal motif [I]-[V]-[F]-x-[S]-x-x-[G]-x-[I]-x-x-x-[A]-x-x-[E] corresponding to a signature of flavodoxin proteins. (ii) A conserved insertion of 24 amino acid residues in the C-terminal region (Fig. 2, G124–P147), forming an α/β motif exclusive to the WrbA family, was predicted by secondary structure analysis of PbY20 (data not shown). (iii) In addition, the secondary structure prediction reveals a HTH DNA-binding motif in the PbY20 (Fig. 2, S184–S203) C-terminal region, in agreement with WrbA proteins (Grandori and Carey, 1994). (iv) The typical FMN-binding site defined by three amino acid residues (S7; M56; W90), pdb code 4FXN, (Grandori and Carey, 1994) is highly conserved in the flavodoxins. According to the alignment analysis, we propose that the first two amino acids residues of the PbY20 involved in the FMN–binding are S13 and R85 or Y86. The third amino acid residue W90 (pdb code 4FXN) may correspond to Y150 of the PbY20 sequence, considering the conserved insertion of 24 amino acids residues for WrbA proteins. However, its involvement with FMN-binding site is yet unclear. (v) Fluorescence is one of the most powerful tools in the study of flavoproteins in several aspects, including structural properties and kinetics of protein-bound flavins (Munro and Noble, 1999). The intrinsic fluorescence of flavin cofactors, when binds to flavodoxins, is almost quenched, providing a convenient way to follow the FMN-binding kinetics (Murray and Swenson, 2003). The decrease of about 40% in the intrinsic FMN fluorescence in the presence of the PbY20-GST fusion protein (Fig. 5) is an indicative of the PbY20 FMN-binding, suggesting that PbY20 is a flavodoxin-WrbA-like.

Proteins from WrbA family have been characterized as a QR and are overexpressed under high levels of quinone redox cycling generating a non-toxic reduced quinone. These enzymes prevent the one-electron reduction of a quinone to a semiquinone that may react with oxygen generating highly destructive reactive oxygen species (Jensen et al., 2002). The transcription factors Yap1 and Pap1 play an important role in the expression of multiple genes involved in heat shock and oxidative stress responses, respectively (Kudo et al., 1999 and Kuge et al., 2001). Under these conditions, Yap1 and Pap1 are overexpressed resulting on increased transcription levels of the known WrbA-QR proteins, Ycp4 and p25 (Gasch et al., 2000 and Toda et al., 1992), which present high similarity to PbY20 protein from P. brasiliensis.

Northern blot analysis, using hydrogen peroxide and menadione as oxidative stress agents, showed no increase of pby20 transcript, when total RNA extracted from treated and control mycelium or yeast cells of P. brasiliensis were compared (data not shown). In contrast to Ycp4 and p25, which are up regulated by temperature and also by oxidative stress, our results revealed that pby20 gene is highly expressed in yeast cells only after the P. brasiliensis thermo-controlled cell differentiation, since under the oxidative stress conditions tested, we

could not observe further increase in the expression level. The lack of increased levels of pby20 transcript under these conditions does not exclude its possible participation in the mechanism against oxidative stress during the infection but suggests that probably the pby20 gene is pre-programmed to be overexpressed in yeast cells.

As recently pointed out, the transcriptome analysis from mycelium (23 °C) and yeast (36 °C) forms (Felipe et al., 2003) confirmed that pby20 is one of the main overexpressed transcript in P. brasiliensis pathogenic yeast cells, also confirmed in this work (Fig. 3). Therefore, given the high sequence identity of the PbY20 with Ycp4 and p25, and their similar overexpression on heat shock conditions in cell differentiation of this pathogen, a FMN-QR function can be attributed to the PbY20.

The Northern and Western blot analyses from mycelium (23 °C) and yeast (36 °C) cells showed a significant increase in the levels of pby20 mRNA and protein in the fully differentiated yeast form (Fig. 3). These data strongly suggest that pby20 is controlled at transcriptional level during in vitro cell differentiation of the thermo-regulated and pathogenic fungi P. brasiliensis and probably also during the natural human infection pathway process. The preferential expression of the PbY20 protein in the yeast form also suggests that it might be involved in P. brasiliensis survival in the host thermal conditions and/or that it could plays a role in the fungus–host interaction.

In this work, we have showed by immunoelectron microscopy, that PbY20 was detected mainly in the cytoplasm, inside large granules, in the cell wall as well as in the nucleus. The nuclear localization of PbY20 is reinforced with the predicted helix–turn–helix motif in the C-terminal region, which also suggests a possible DNA-binding function for PbY20. Furthermore, PbY20 is found in large cytoplasmatic vacuoles and is possibly exported to the cell wall. These results are in agreement with different cellular localization described for fungi flavodoxins. For example, the subcellular localization of the flavodoxin protein vanillyl-alcohol oxidase (VAO) from Penicillium simplicissimum is not restricted to a specific cell compartment, being localized on the peroxisomal matrix and also in the cytosol (Fraaije et al., 1998).

Furthermore, macrophages, neutrophils, and other phagocyte cells are key components of the antimicrobial immune responses and are capable to generate reactive oxygen intermediates that are toxic molecules which contribute to control of microbial agents (Bogdan et al., 2000). In P. brasiliensis, macrophages constitute one of the primary cellular mechanisms which prevents parasite invasion of host tissues. In fact, toxic and antimicrobial effects of reactive oxygen intermediates on P. brasiliensis have been reported (Brummer et al., 1988). In this context, and considering that QR activity is closely related with the oxidative stress defense system, a putative biological activity attributable to PbY20 could be its

involvement in the mechanism of the P. brasiliensis protection against macrophage functions. However, the in vivo function of this protein in protecting against oxidative stress remains still an open question. These features address further investigation regarding the role of the overexpressed PbY20 in the intracellular detoxification processes, in the infection pathway and in P. brasiliensis survival in the host.

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