The PbMDJ1 Gene Belongs to a Conserved MDJ1/LON Locus in Thermomorphimic Pathogenic Fungi and Encodes a Heat Shock Protein That Localizes to both the Mitochondria and Cell Wall of Paracoccidioides brasiliensis

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J-domain (DnaJ) proteins, of the Hsp40 family, are essential cofactors of their cognate Hsp70 chaperones, besides acting as independent chaperones. In the present study, we have demonstrated the presence of Mdj1, a mitochondrial DnaJ member, not only in the mitochondria, where it is apparently sorted, but also in the cell wall of Paracoccidioides brasiliensis, a thermomorphimic pathogenic fungus. The molecule (PbMdj1) was localized to fungal yeast cells using both confocal and electron microscopy and also flow cytometry. The anti-recombinant PbMdj1 antibodies used in the reactions specifically recognized a single 55-kDa mitochondrial and cell wall (alkaline β-mercaptoethanol extract) component, compatible with the predicted size of the protein devoid of its matrix peptide-targeting signal. Labeling was abundant throughout the cell wall and especially in the budding regions; however, anti-PbMdj1 did not affect fungal growth in the concentrations tested in vitro, possibly due to the poor access of the antibodies to their target in growing cells. Labeled mitochondria stood preferentially close to the plasma membrane, and gold particles were detected in the thin space between them, toward the cell surface. We show that Mdj1 and the mitochondrial protease Lon homologues are heat shock proteins in P. brasiliensis and that their gene organizations are conserved among thermomorphimic fungi and Aspergillus, where the genes are adjacent and have a common 5′ region. This is the first time a DnaJ member has been observed on the cell surface, where its function is speculative.

Paracoccidioides brasiliensis is the fungal species responsible for paracoccidioidomycosis (PCM), which is endemic in certain areas of Latin America. PCM is one of the four deep-seated granulomatous mycoses caused by thermomorphimic fungi that also include Histoplasma capsulatum, Blastomyces dermatitidis, and Coccidioides immitis. These fungal species are phylogenetically related, according to sequence analysis of the ribosomal RNA gene locus (3, 36), and have been classified as Ascomycetes of the Oniginemaceae family. P. brasiliensis grows as a multibudding yeast when in parasitism or cultivated at 37°C and as a mycelium when incubated at temperatures below 28°C. The fungus is multinucleated, and its sexual form is still unknown. Genetic manipulation in this species is only starting to be attempted (23).

The human host most frequently acquires the fungus by inhalation of mycelial particles, but infection can be established only after transition to the yeast parasitic phase takes place (28). A number of biochemical processes are associated with fungal adaptation to the host’s environment and temperature, and many of them are probably responsible for phase transition and/or phase maintenance. Different groups have recently addressed the overall scenario of gene expression in P. brasiliensis yeast versus mycelium, or undergoing phase transition in vitro, making use of microarrays, suppression subtraction hybridization, real-time reverse transcriptase (RT) PCR, and in silico analyses (14, 15, 27, 33). Considering that these analyses have been undertaken with P. brasiliensis transforming to the yeast phase upon temperature increase, differentially regulated heat shock protein homologues have indeed been detected, especially in the initial hours of temperature change (15, 33). Some of them, however, seem to be necessary for growth in the yeast phase, e.g., the HSP70 and HSP60 genes in P. brasiliensis that have been previously characterized (18, 41).

We have characterized a LON gene homologue from P. brasiliensis (PbLon) (2). Lon proteins are conserved ATP-binding, heat-inducible serine proteinases. They form high-molecular-mass complexes of homo-oligomers, which in Saccharomyces cerevisiae contain the stoichiometry of seven subunits of 117 kDa (46). The yeast Lon, also called PIM1, is synthesized as a pre-proenzyme precursor in the cytosol and then is sorted to the mitochondrial matrix where it is processed (54). It controls proteinolysis by mediating cleavage of misfolded or unassembled matrix proteins and has an essential role in the maintenance of mitochondrial DNA integrity and mitochondrial homeostasis (51). Lon is also a DNA-binding protein (26) and is essential for cell survival in humans (4).

In P. brasiliensis, PbLon consists of 1,063 residues containing a mitochondrial import signal, a conserved ATP-binding site, and a serine catalytic motif (2). Its open reading frame (ORF) is within a 3,369-bp fragment interrupted by two introns lo-
cated in the 3′ segment; an MDJ1-like gene was partially sequenced in the opposite direction but sharing with PbLOH a common 5′ untranslated region (2). This chromosomal organization might be functionally relevant, since Mdj1p is a type I DnaJ molecule located in the yeast mitochondrial matrix and is essential for substrate degradation by Lon and other stress-inducible ATP-dependent proteases (39, 53). In bacteria, DnaJ alone and/or the DnaJ/DnaK complex determine the fate of nonnative substrates to be cleaved by Lon (17).

DnaJ members (more recently referred to as J-domain proteins) belong to the Hsp40 family of molecular chaperones and localize to various cellular compartments, where their primary role is to regulate the activity of their cognate Hsp70 homologues (55). In the bacterial DnaK (Hsp70)/DnaJ/GrpE complex, a transient association of DnaJ stimulates the ATPase activity of DnaK, prompting substrate binding, while GrpE effects substrate dissociation (12). This complex prevents aggregation of functional mitochondria, but it is not involved in protein translocation into the matrix (reviewed in reference 52). Typically, DnaJ members are constituted of a structurally conserved J domain located near the N-terminal end, followed by a glycine/phenylalanine-rich segment (G/F domain) and four repetitions of a zinc-binding CXXCXXGXG motif (55). The J domain and the G/F segment are essential for interaction with DnaK, while the zinc finger-like motif contains elements for binding non-native substrates (49) and also displays thiol-disulfide oxidoreductase activity (50). In S. cerevisiae, 22 J-domain proteins (types I, II, and III) have been found in the genome; their subcellular localizations are attributed to the cytoplasm, nucleoplasm, mitochondria, and endoplasmic reticulum (55).

We have presently characterized the P. brasiliensis PbMDJ1 gene and verified that the PbLOH/PbMDJ1 locus is conserved among the dimorphic pathogenic fungi besides Aspergillus nidulans and A. fumigatus. We show that PbLOH and PbMDJ1 encode mitochondrial heat shock proteins; however, PbMdj1 has also been found in the cell wall of P. brasiliensis. The finding of a DnaJ member on the cell surface is a novel observation.

MATERIALS AND METHODS

Fungal strains and culture conditions. We used P. brasiliensis isolates B-339 and 18, provided by Angela Restrepo (Colombia) and Zoilo P. Camargo (Brazil), respectively. More details about the fungal isolates can be found elsewhere (29). Cultures were maintained at 36°C (yeast phase) in solid modified YPD (0.5% casein peptone, 0.5% yeast extract, 1.5% glucose, pH 6.3).

Isolation of total DNA and RNA. For DNA extraction, strain B-339 was grown for 10 days at 36°C in liquid modified YPD under agitation. Total DNA was extracted from a 10-ml pellet of nitrogen-free cells that were disrupted in a mortar and then briefly in a pestle, as described previously (11). Total RNA was isolated from fresh cells mechanically disrupted by vortexing with glass beads for 10 min in the presence of TRIzol reagent (Invitrogen), according to the instructions provided by the manufacturer. Contaminating DNA in these preparations was digested with RNase-free DNase I (Promega), as described previously (11). The finding of a DnaJ member on the cell surface is a novel observation.

Cloning of the 3′ end of PbMDJ1. We followed the strategy of 3′ rapid amplification of cDNA ends to clone the 3′ end of the PbMDJ1 cDNA, using the SuperScript II RNase H RT kit (Invitrogen), because at that time the PbMDJ1 expressed sequence tag (EST) had not yet been found in the P. brasiliensis EST bank (15). The positions of the primers can be seen in Fig. 1A. The first strand of the total cDNA pool was obtained from total RNA (2.5 μg) extracted from strain B-339 yeast cells previously heat shocked for 60 min at 42°C. The template was incubated with 1 μl of random hexamers as reverse primer (reverse primer: 5′-AACCCCTTGAGTTTCT-3′), in a 10-μl PCR mixture containing 1× PCR buffer from Promega, 0.2 μM of each primer, 0.2 μM of MnCl2, 0.1 μl of bovine serum albumin (BSA). A β-mercaptoethanol cell wall extract was obtained from intact cells as described previously (9). P. brasiliensis yeast cells grown under shaking for 10 days

Northern blotting. Standard conditions were used for electrophoresis and Northern blotting (38), carried out with 25 μg of total RNA per lane. Hybridizations with [α-32P]dCTP-labeled probes were done in nylon hybridization membrane, using the method described by Suzuki et al. (48) for S. cerevisiae. In order to accomplish cell lysis under mild conditions, nitrogen-frozen yeast cells were sonicated for 5 min, alternating 15-s pulses with 15 s in ice. Cell debris were pelleted for 10 min to precipitate the mitochondrial fraction (brownish pellet), which was then resuspended in 0.2 ml of BB, and the supernatant was used as template in a second round of PCR primed with antisense primer 5′-TGCTTTCGCGGCGGTTT TTC-3′. The final product was used as a template in a second round of PCR primed with antisense primer 5′-GGCGGAACCGAGGTTTC-3′. This complex prevents aggregation of functional mitochondria, but it is not involved in protein translocation into the matrix (reviewed in reference 52). Typically, DnaJ members are constituted of a structurally conserved J domain located near the N-terminal end, followed by a glycine/phenylalanine-rich segment (G/F domain) and four repetitions of a zinc-binding CXXCXXGXG motif (55). The J domain and the G/F segment are essential for interaction with DnaK, while the zinc finger-like motif contains elements for binding non-native substrates (49) and also displays thiol-disulfide oxidoreductase activity (50). In S. cerevisiae, 22 J-domain proteins (types I, II, and III) have been found in the genome; their subcellular localizations are attributed to the cytoplasm, nucleoplasm, mitochondria, and endoplasmic reticulum (55).

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FIG. 1. (A) Schematic representation of PbMDJ1, highlighting the localization of the primers and probes used in this work. Intronons were localized from nt 205 to 313 and nt 1552 to 1683. (B) Schematic representation of PbMdj1 showing the localization of conserved domains. Kyte-Doolittle hydrophilicity plots (Protein module; DNASTar Inc.) of fungal Mdj1-like sequences are as follows: Pb, P. brasiliensis (AF334811); Bd, B. dermatitidis (http://genome.wustl.edu/BLAST/histo_client.cgi); Hc, H. capsulatum (http://genome.wustl.edu/BLAST/histo_client.cgi); Ci, C. immitis (http://www.broad.mit.edu/annotation/fungi/coccidioides_immitis); An, A. nidulans (EAA57980); Cn, C. neoformans (EAL21819); Ca, C. albicans (EAK92195); Sc, S. cerevisiae (CAA82189); Ec, Escherichia coli (BA000007). The boxes indicate the predicted mitochondrial target sequence (MT) and the J domain. The dots localize the Zn$^{2+}$ finger domains. Horizontal bars indicate the location of mouse T-cell epitopes with a minimum of 12 amino acids, as predicted by the Sette major histocompatibility complex motif (Protein module; DNASTar Inc.). Percentages of identity with PbMdj1, as calculated with the MegAlign module of the DNASTar program, are given at right.
thoroughly rinsed with PBS–0.5 M NaCl, and the bound antibodies were eluted with 50 mM glycine, pH 3.0 (500 μl), which was immediately neutralized with 1 M Tris-HCl, pH 9.0. The protein profile of the eluted material in silver-stained SDS-PAGE gels indicated the presence of a major IgG band. Preimmune sera and/or IgG fractions were processed similarly. IgG and affinity purified aliquots were kept at –20°C.

Western immunoblot reactions with sera from hyperimmunized rabbits or patients with PCM were carried out for 3 h at room temperature under shaking, after which the membranes were washed six times with 0.1% Tween 20 in PBS and incubated for 1 h at room temperature with goat anti-rabbit or horse anti-human Ig conjugated to peroxidase (Sigma). The reactions were developed with an enhanced chemiluminescence kit (Amersham Pharmacia).

Confocal analysis. P. brasiliensis yeast cells growing in aerated liquid cultures in logarithmic phase (viability, >90%) were collected, washed twice in modified YPD, and adjusted to a concentration of 2 × 10^6 to 3 × 10^6 viable cells/ml. Washed cells were initially incubated with a 20 nM concentration of MitoTracker Red CMXRsos probe (Molecular Probes) for 20 min at 36°C, in order to stain for active mitochondria, and then rinsed three times with PBS and fixed in cold methanol for 30 min in the dark. The MitoTracker Red-coded labeled cells were incubated with 5% (wt/vol) BSA in PBS (blocking buffer) for 1 h at 4°C, washed three times with PBS, and then incubated with either 100 μg/ml of IgG or 60 μg/ml of affinity-purified antibodies from polyclonal rabbit anti-rPbMdj1 and anti-rPbLon for 4 h at room temperature. Control reactions used preimmune IgG. The cells were then incubated for 1 h in the dark with 7.5 μg/ml of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) in blocking buffer. Between each incubation step with antibodies, the fungal cells were washed six times with PBS. A drop of 100 μl of cell suspension on glass slides was grown on Vectashield mounting medium (VECTOR Laboratories, Inc., Burlingame, CA), and the slides were sealed with nail polish. Labeling was analyzed using a laser scanning confocal microscope, LSM-510 NLO (Carl Zeiss, Jena, Germany).

Electron microscopy. P. brasiliensis yeast cells growing in logarithmic phase (viability, >90%) under aerated conditions were collected by centrifugation, washed twice with 0.1 M sodium cacodylate buffer, pH 7.2, and fixed with 2% (wt/vol) paraformaldehyde and 2.5% (vol/vol) glutaraldehyde in cacodylate buffer for 3.5 h at room temperature under agitation (47). Yeast cells were stored in fixative (wt/vol) formaldehyde and 2.5% (vol/vol) glutaraldehyde in cacodylate buffer for 10 min. The grids were washed five times with ultrapure water and dried. The grids were incubated with 1% (wt/vol) acetylated BSA (cBSA) (Aurion) in PBS for 1 h and incubated for 30 min with saturated sodium periodate, freshly prepared, and washed five times in PBS and fixed for 1 h at room temperature with an equal volume of 4% (vol/vol) paraformaldehyde and 2.5% (vol/vol) glutaraldehyde in cacodylate buffer for 3.5 h at room temperature under agitation (47). Yeast cells were stored in fixative in a moist chamber at 4°C. After five washes with buffer A, the grids were incubated with 3% (wt/vol) BSA in PBS (blocking buffer) for 16 h at 4°C, washed three times with PBS, and then incubated with either 100 μg/ml of IgG or 60 μg/ml of affinity-purified antibodies from polyclonal rabbit anti-rPbMdj1 and anti-rPbLon for 4 h at room temperature.

RESULTS

The PbMDJ1 ORF lies within a 1,897-bp fragment (GenBank accession number AF334811), where two introns were localized (Fig. 1A). The deduced PbMdj1 protein contains 551 amino acids and has a deduced molecular mass of 58.7 kDa and a basic isoelectric point of 8.9. In the sequence we can recognize the J domain and the glycine/phenylalanine-rich and zinc finger domains (Fig. 1B), which are characteristic of type I J-domain proteins. Although we found six potential glycosylation sites (Asp-X-Ser/Thr) in PbMdj1, apparently none is substituted with endoglycosidase H-sensitive oligosaccharide chains (not shown), suggesting that the molecule does not go through the secretory pathway. The computer program TargetP (http://www.cbs.dtu.dk/services/TargetP/) predicted a mitochondrial targeting peptide within the 28 first amino acid residues (2.94 kDa). A comparison among fungal Mdj1 homologues indicated a high percentage of identity (85%) with sequences from the dimorphs B. dermatitidis and H. capsulatum, which can be visualized by the similarities in the hydrophilicity profiles (Fig. 1B). The J domain is the most conserved region among the Mdj1 homologues analyzed. It contains four characteristic helices (reviewed in reference 20) and a highly conserved HPD tripeptide between helixes II and III, which is essential for the DnaJ/Hsp interaction (35). In the C-terminal half of PbMdj1 there is one predicted T-cell mouse epitope with a minimum of 12 residues (LYTAQIPLTTALL) between amino acids 379 and 391 (Fig. 1B), which is conserved in the homologues from B. dermatitidis and H. capsulatum.

We observed an extremely conserved gene organization of MDJ1 and LON among members of the Eurotiomycetes family, i.e., P. brasiliensis, B. dermatitidis, H. capsulatum, C. immitis, A. nidulans, and A. fumigatus. The number and position of the introns are conserved, although their sizes and sequences may differ (Fig. 2). MDJ1 from Neurospora crassa and Fusarium graminearum have the same intron numbers and positions as in P. brasiliensis, while their LON gene has only one 3′ intron. In Cryptococcus neoformans, both genes bear several small introns. The most interesting finding of our comparative analysis was the fact that the whole MDJ1/LON locus is conserved in Eurotiomycetes species, where the genes are adjacent, inversely oriented, and separated by a common 5′ region ranging between 400 and 485 nt (Fig. 2). In these species, a Broa (Bro1) gene homologue is found adjacent to MDJ1, in the same di-
In S. cerevisiae, BroA encodes a cytoplasmic protein involved in the sorting of membrane proteins into the luminal vesicles of endosomal multivesicular bodies (34).

We expressed a His-tagged N-terminal region of PbMdj1 (252 amino acids, 25.8 kDa), which included the entire J domain, the Gly/Phe-rich region, and two zinc finger domains. This fragment contains a number of hydrophilic regions (Fig. 1B) and antibody epitopes, as predicted by the Jameson-Wolf antigenic index of the Protean module of the Lasergene program (DNastar Inc.). We additionally expressed a 282-amino-acid His-tagged N-terminal fragment of PbLon (31.7 kDa, between residues 179 and 463), which also contains several peaks of predicted antibody epitopes. Both truncated recombinant proteins (rPbMdj1 and rPbLon) were expressed as major insoluble cytoplasmic proteins of calculated molecular masses of approximately 31 kDa for rPbMdj1 and 36 kDa for PbLon, including their vector sequences. These values are compatible with their SDS-PAGE mobilities (Fig. 3A). The correspondent pH 4.5-eluted SDS-PAGE bands were cut off the gels to immunize rabbits. Anti-rPbMdj1 and anti-rPbLon rabbit immune sera had high immunoblot titer, of 1:8,000, when tested with small amounts of the correspondent recombinant proteins, as seen in Fig. 3B. We therefore used these antibodies in subcellular localization experiments.

Immunoblot reactions were carried out with both cell lysates and total mitochondrial proteins from P. brasilensis yeast cells growing at 36°C or heat shocked for 30 min at 42°C (Fig. 3C). Anti-rPbMdj1 immune serum specifically reacted with a single 55-kDa band when 20 mg of total cell extracts was tested, possibly because of their small relative concentration in the cytoplasm. The heat shock nature of the mitochondrial proteins was confirmed at the transcriptional level. PbMdj1 and anti-rPbLon antibodies reacted with the correspondent proteins in the mitochondria, as suggested by colocalization with MitoTracker Red (Fig. 4). Both antibodies (green) and MitoTracker (red) stained the cytoplasmic compartment with a
punctuated pattern, which merged in tones of yellow and orange. Surprisingly, however, anti-rPbMdj1 antibodies also labeled the whole cell surface, where patches of stronger green staining could be seen at the budding sites (Fig. 4A). This is particularly evident in the images of Fig. 4D, where the bud neck is intensely bright. The longitudinal slice of this image facilitated a view of a large fluorescent green surface on the daughter cell. Control images obtained with preimmune antibodies showed only background fluorescence (Fig. 4C). We tried to label *S. cerevisiae*, *C. albicans*, and *C. neoformans* with anti-PbMdj1 immune serum, but the reactions were negative, as were immunoblotting reactions with *S. cerevisiae* cell extracts (not shown).

Details of the cellular localization of PbMdj1 were further exploited by transmission electron microscopy using immunogold labeling (Fig. 5). Our preparations had preserved cell surface and organelle structures (Fig. 5A), and the images show intense labeling with anti-PbMdj1 antibodies of the double-layered cell wall. A large number of mitochondria are seen surrounding the cell membrane (Fig. 5A) yet are not in contact with it (Fig. 5B and C). The immunogold particles seem to be clustered inside the mitochondria, but in Fig. 5C they can also
be seen near/at the mitochondrial surface and within the thin space between the mitochondrial surface and the cell membrane. Mitochondria that were not near the cell wall were less labeled, and the gold particles in the cytoplasm (far from the cell wall) were scarce. The bud neck in Fig. 5D has a great concentration of gold particles, in accordance with the intense fluorescence of this region seen in Fig. 4D. Nonspecific labeling with preimmune rabbit antibodies was reduced to scattered particles in the cytoplasm and the cell wall (Fig. 5E).

Surface labeling of *P. brasiliensis* yeast cells was also suggested by FACS. We observed a typical dose-response curve upon incubation of paraformaldehyde-fixed cells with 50, 100, or 200 μg/ml of anti-rPbMdj1 IgG. At a concentration of 200 μg/ml, over 50% of the cells were labeled, against 7% of preimmune IgG (Fig. 6A). In order to characterize the cell wall component that was reacting with anti-rPbMdj1 antibodies, cell wall components were released under mild conditions with β-mercaptoethanol at an alkaline pH (Fig. 6B). When this extract was assayed by immunoblotting with anti-rPbMdj1, a 55-kDa component was revealed, which was comparable in size to the mitochondrial band recognized by the same serum (Fig. 6B). This result adds evidence to the dual mitochondrial and cell wall localization of PbMdj1 in *P. brasiliensis*. It is of note that we have not detected measurable amounts of PbMdj1 in culture supernatants.

Since the structure of PbMdj1 predicts several antibody epitopes and one potential T-cell epitope, we questioned whether paracoccidioidomycosis patients produced antibodies recognizing the molecule and whether anti-rPbMdj1 would be able to interfere with fungal growth. We addressed the first question by testing the reactivity of rPbMdj1 with three patients’ sera by immunoblotting. As seen in Fig. 7, two of them recognized rPbMdj1 at a 1:200 dilution. The effect of polyclonal anti-rPbMdj1 IgG on in vitro growth of *P. brasiliensis* yeasts was tested in strain 18 cells, as described in Materials and Methods. Under these conditions, there was no inhibitory effect on the growth pattern or CFU counts compared with controls (Table 1).
DISCUSSION

The present work demonstrates the presence of a mitochondrial member of the J-domain protein family, Mdj1, not only in the mitochondria but also in the cell wall of the pathogenic dimorphic fungus *P. brasiliensis*. To our knowledge, this is the first description of a DnaJ member on the cell surface. The unexpected cell wall localization of PbMdj1 was visualized in the fungal yeast cells using both confocal and electron microscopy and was confirmed by FACS analyses. The protein was labeled with rabbit polyclonal antibodies (IgG and affinity-purified fractions) raised against a recombinant PbMdj1 containing the N-terminal half of the protein. Evidence for the specificity of the immunological reactions with PbMdj1 comes from the following observations: (i) the anti-rPbMdj1 antibodies specifically recognized in immunoblotting, before or after antibody capture of soluble proteins, a single 55-kDa component from total *P. brasiliensis* mitochondrial extracts; (ii) the anti-rPbMdj1 antibodies specifically recognized a single 55-kDa immunoblotted component from a cell wall extract obtained from yeast cells of *P. brasiliensis* with mildly alkaline β-mercaptoethanol treatment; and (iii) confocal reactions carried out with affinity-purified antibodies resulted in a labeling pattern of both the cell wall and mitochondria.

Although the presence of surface J-domain proteins has not been reported before, the occurrence of extramitochondrial DnaJ homologues had been predicted by Soltys and Gupta.

FIG. 5. Ultrastructural localization of PbMdj1 in *P. brasiliensis* yeast cells incubated with IgG fractions (60 μg/ml) of either anti-rPbMdj1 (A through D) or preimmune control (E). Each panel shows a different cell. (A) Panoramic photomicrograph. (B and C) Details. Note labeling inside the mitochondria (M, arrows) and in the cell wall (CW). In panel C, labeling is also visible in the region between the cell membrane and the mitochondrion. (D) The bud neck is intensely labeled.
Hsp60 has been detected in small clusters at discrete points on the *H. capsulatum* cell wall, and it has been shown to mediate attachment of the fungus to macrophages via CD11/CD18 receptors (24). Both Hsp70 and Hsp60 need specific cochaperones for optimum chaperone function, which is thus likely to occur extramitochondrially. Therefore, outside the mitochondria Mdj1 could be assisting Ssc1 (mitochondrial Hsp70), but it could also be functioning as an independent chaperone. Although an Ssc1 homologue has not been described in fungal cell walls, an Hsp70-like protein has been detected at the outer surface of the plasma membrane, throughout the cell wall, and at the cell surface of *C. albicans* (25). It will be interesting to find out if the *P. brasiliensis* Ssc1, whose gene homologue has been identified in the database, will also be found in the cell wall and if it colocalizes with Mdj1.

The fungal cell wall is a dynamic compartment that determines and maintains cell shape but is also actively involved in many other biological events. Although glucans and chitin are the main scaffold components of the cell wall, it has a fascinating, complex structure with a number of other constituents (glycoproteins, proteins, and lipids), such as enzymes, adhesins, and structural and heat shock proteins, whose features depend on multiple intrinsic and external factors (reviewed for *C. albicans* in reference 10). Some of these components from pathogenic fungi are promising targets for drugs and immunotherapy (31). The interactions among these molecules seem to involve not only hydrogen and hydrophobic bonds but also covalent and phosphodiester (mediated by glycosyl phosphatidylinositol) linkages with polylsaccharides. In the present study, we detected abundant labeling of the cell wall with anti-rPbMdj1 antibodies; however, low yields of the reactive 55-kDa protein were obtained upon alkaline extraction with β-mercaptoethanol, suggesting that the molecule is not loosely bound to this compartment. An additional observation was that when yeast cells were first treated with β-mercaptoethanol and then labeled for FACS analysis, the percentage of fluorescent cells increased instead of going down to background lev-

| Culture medium | No. of CFU |
|----------------|-----------|
| Anti-rPbMdj1 |         |
| 200            | 38.33 ± 4.7 |
| 100            | 46.75 ± 9.0 |
| 50             | 37.75 ± 2.7 |
| 25             | 38 ± 0.8   |
| 12.5           | 39 ± 0.8   |
| Preimmune serum |         |
| 200            | 46.5 ± 3.5 |
| 100            | 41.75 ± 4.9 |
| 50             | 42.25 ± 5.7 |
| 25             | 43.25 ± 3.1 |
| 12.5           | 41.6 ± 5.8 |

* Yeast cells (10⁵) were cultivated in the presence of 200, 100, 50, 25, or 12.5 μg/ml of IgG. After incubation for 48 or 72 h at 36°C, the cultures were supplemented with the same amount of IgG.

* Numbers of CFU were determined on day 6 of culture. Differences in numbers of CFU were not statistically significant (P > 0.05).
els, as would be expected if PbMdj1 had been totally extracted (not shown). Therefore, it is likely that the initial peeling caused by mild alkaline and β-mercaptoethanol treatment improved the access of anti-rPbMdj1 antibodies to formerly inaccessible PbMdj1 target molecules. That might also explain why, although PbMdj1 seems to be actively participating in yeast cell budding, anti-rPbMdj1 antibodies could not interfere with fungal growth in vitro within the range of concentrations tested: other cell components might be blocking antibody access to their target molecules. An example of such a blockage mechanism has been recently detected in Fonsecaea pedrosoi, for which anti-monohexosylceramide can react with the cell wall only when melanin is absent (32). Another possible explanation is that the antibodies cannot recognize PbMdj1 well in growing cells. On the other hand, genetic manipulation in P. brasiliensis is still an obstacle to research of P. brasiliensis; until we have mutants for both Lon and Mdj1, one can only speculate about their role in the fungal cell wall. Another aspect to be investigated is the functionality of the potential T-cell receptor that is conserved among dimorphs.

A Southern blot of P. brasiliensis DNA restricted with endonucleases and assayed with an E5/E4 PbMDJ1 probe (Fig. 1A) resulted in a pattern of single bands (not shown), which suggests the presence of a single copy of the PbMDJ1 gene in the genome, as seen before with the adjacent PbLON gene (2). This is supported by the previous observations that PbMDH1 and PbLON were mapped to a unique chromosomal band in 12 individual P. brasiliensis isolates (13). In other fungi analyzed here for which the genome is completely sequenced (Aspergillus and Candida), both LON and MDJ1 are present in single copies, reinforcing those observations. We have not found any evidence for the occurrence of alternative splicing in PbMDJ1: we visualized only one PbMDJ1 RNA band in Northern blots. Besides, RT-PCRs using primers designed to elongate the full-length ORF consistently resulted in a single band of the same size when we tested different RNA preparations of yeast cells either growing at 36°C or heat shocked at 42°C (not shown). Therefore, the components recognized by anti-rPbMDJ1 both in mitochondria and in the cell wall apparently derive from the same gene and are not a product of differentially sorted molecules bearing particular targeting sequences due to alternative splicing. The gene encoding alanine-glyoxylate aminotransferase from amphibian livers has two alternative transcription start sites at either side of the 24-nt-long mitochondrial targeting signal, so that the protein encoded by the long transcript localizes to the mitochondria, while the product of the shorter transcript is cytosolic (16). We have not been able to determine the transcription initiation site of PbMDJ1; however, computer analysis does not predict any internal translation start site that would result in an ORF lacking the mitochondrial matrix-targeting signal. Moreover, the similar SDS-PAGE gel migration of the protein labeled in mitochondria and cell wall extracts with anti-rPbMdj1 suggests that the molecule was first sorted to that organelle, where it was probably processed by a matrix-processing peptidase into a mature 55-kDa form and then migrated to the cell wall. A matrix-processing peptidase homologue has been found in the P. brasiliensis database.

Recent years have seen a growing number of examples of proteins that are sorted to the mitochondrial matrix and then apparently exported to an additional compartment(s) of the cell (extensively reviewed in reference 45). The extramitochondrial destination varies with the molecules, which include antigens, enzymes, receptors, hormones, and chaperones, and with their specific roles in the destination sites. The mechanism(s) involved in mitochondrial export and its control are so far speculative, but the endosymbiotic origin of mitochondria suggests that some ancient bacterial secretory pathways must have been retained and modified by the organelle (45). It is noticeable that in a recent proteomics study of plasma membrane lipid rafts, 24% of the 196 identified proteins were mitochondrial; however, rafts have not been found in that compartment (1). In our electron microscopic images, mitochondria formed a ring near the cell surface. This pattern has been mentioned previously for P. brasiliensis yeast cells (19), and they can be seen in electron microscopic pictures of recently transformed yeasts (7), although in hyphae that pattern was not evident (6). The image shown in Fig. 5C suggests that some gold particles, in small clusters, are leaving the mitochondrion and moving toward the cell surface, but it is not clear whether these clusters are inside vesicles. Thus, the peripheral localization of mitochondria might not be fortuitous but rather may suggest that active trafficking of molecules to the cell surface might be taking place.

We have previously characterized the P. brasiliensis LON homologue (2). Here we show that PbLON is a heat shock gene that encodes a 110-kDa mitochondrial heat shock protein. In our confocal analyses, PbLon localized to the mitochondria, showing that the presence of PbMdj1 in the cell wall does not include helping protein degradation by PbLon. We observed that the deduced Lon and Mdj1 sequences are remarkably homologous among dimorphic fungi, especially P. brasiliensis, H. capsulatum, and B. dermatitidis. It will be interesting to find out if Mdj1 localizes to the cell wall of these microorganisms. We found that the LON/MDJ1 locus organization is comparable among Eurotiomycetes species. The entire homologous locus probably also includes Bro1, CreA, a little farther from BroA, and a hypothetical protein adjacent to LON so far found in A. nidulans, A. fumigatus, and H. capsulatum. In terms of comparative genomics, this is a relevant finding that might have evolutionary significance. This is the first conserved chromosomal organization reported for Ascosporomycetes, for which genome sequencing of several species will soon be finished. We are presently studying the regulatory elements contained in the 5′ untranslated region shared by LON and MDJ1, and we hope to find conserved mechanisms of regulation among fungal heat shock proteins.

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REFERENCES

1. Bae, T. J., M. S. Kim, J. W. Kim, B. W. Kim, H. J. Choo, J. W. Lee, K. B. Kim, C. S. Lee, J. H. Kim, S. Y. Chang, C. Y. Kang, S. W. Lee, and Y. G. Ko. 2004.
46. Stahlberg, H., E. Kutejova, K. Suda, B. Wolpensinger, A. Lustig, G. Schatz, A. Engel, and C. K. Suzuki. 1999. Mitochondrial Lon of Saccharomyces cerevisiae is a ring-shaped protease with seven flexible subunits. Proc. Natl. Acad. Sci. USA 96:6787–6790.

47. Straus, A. H., E. Freymuller, L. R. Travassos, and H. K. Takahashi. 1996. Immunological and subcellular localization of the 43 kDa glycoprotein antigen of Paracoccidioides brasiliensis with monoclonal antibodies. J. Med. Vet. Mycol. 34:181–186.

48. Suzuki, C. K., E. Kutejova, and K. Suda. 1995. Analysis and purification of ATP-dependent mitochondrial lon protease of Saccharomyces cerevisiae. Methods Enzymol. 260:486–494.

49. Szabo, A., R. Korszun, F. U. Hartl, and J. Flanagan. 1996. A zinc finger-like domain of the molecular chaperone DnaJ is involved in binding to denatured protein substrates. EMBO J. 15:408–417.

50. Tang, W., and C. C. Wang. 2001. Zinc fingers and thiol-disulfide oxidoreductase activities of chaperone DnaJ. Biochemistry 40:14985–14994.

51. van Dyck, L., and T. Langer. 1999. ATP-dependent proteases controlling mitochondrial function in the yeast Saccharomyces cerevisiae. Cell. Mol. Life Sci. 56:825–842.

52. Voos, W., and K. Rottgers. 2002. Molecular chaperones as essential mediators of mitochondrial biogenesis. Biochim. Biophys. Acta. 1592:51–62.

53. Wagner, I., H. Arlt, L. van Dyck, T. Langer, and W. Neupert. 1994. Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. EMBO J. 13:5135–5145.

54. Wagner, I., L. van Dyck, A. S. Savel’ev, W. Neupert, and T. Langer. 1997. Autocatalytic processing of the ATP-dependent PIM1 protease: crucial function of a pro-region for sorting to mitochondria. EMBO J. 16:7317–7325.

55. Walsh, P., D. Bursac, Y. C. Law, D. Cyr, and T. Lithgow. 2004. The J-protein family: modulating protein assembly, disassembly and translocation. EMBO J. 5:567–571.

56. Wright, R., M. Basson, L. D’Ari, and J. Rine. 1988. Increased amounts of HMG-CoA reductase induce “karmellae”: a proliferation of stacked membrane pairs surrounding the yeast nucleus. J. Cell Biol. 107:101–114.