Cloning, Characterization, and Epitope Expression of the Major Diagnostic Antigen of Paracoccidioides brasiliensis*

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The 43,000-Da glycoprotein (gp43) of Paracoccidioides brasiliensis is an immunodominant antigen for antibody-dependent and immune cellular responses in patients with paracoccidioidomycosis. In order to identify the peptide epitopes involved in the immunological activities of the gp43 and to obtain highly specific recombinant molecules for diagnosis of the infection, genomic and cDNA clones representing the entire coding region of the antigen were sequenced. The gp43 open reading frame was found in a 1,329-base pair fragment with 2 exons interrupted by an intron of 78 nucleotides. The gene is present in very few copies per genome, as indicated by Southern blotting and chromosomal mega-restriction analysis. A single transcript of 1.5 kilobase pairs was verified in the yeast phase. The gene encodes a polypeptide of 416 amino acids (M, 45,947) with a leader peptide of 35 residues; the mature protein has a single N-glycosylation site. The deduced amino acid sequence showed similarities of 56–58% with exo-1,3-β-glucanases from Saccharomyces cerevisiae and Candida albicans. However, the gp43 is devoid of hydrolase activity and does not cross-react immunologically with the fungal glucanases. Internal and COOH-terminal gene fragments of the gp43 were expressed as recombinant fusion proteins, which reacted with antibodies elicited against the native antigen.

Paracoccidioides brasiliensis is a dimorphic fungus that causes paracoccidioidomycosis, a deep-seated infection, prevalent in rural workers in several Latin American countries. The yeast phase is the infective form of the fungus which synthesizes antigens heteropolysaccharides, glycoproteins, and glycolipids that may have a role in pathogenicity and interact with the immune system (1).

The main diagnostic antigen of paracoccidioidomycosis is an exacellularly secreted glycoprotein of 43,000 Da (gp43)1 which reacts with 100% of sera from patients with this mycosis in double immunodiffusion and immunoprecipitation reactions (2, 3). This molecule has been purified by immunofinity chromatography with an anti-gp43 monoconal antibody (4). It contains immunodominant peptide epitopes that (a) react with human antibodies and are not affected by N-deglycosylation (5) and (b) elicit T-cell dependent delayed hypersensitivity reactions (6). It also induced the proliferation of T-CD4 lymphocytes in mice primed with the antigen and of human peripheral lymphoid cells from a sensitized individual (7). The role of the gp43 in the pathogenicity of P. brasiliensis was suggested based on some of its properties. Thus, the gp43 is the main secreted component of the fungus that binds to murine laminin. It has been shown that laminin-coated yeast forms of this fungus show a marked increase in their ability to invade and destroy the infected tissues (8). As a high-mannose glycoprotein, detectable in the serum of patients with acute and chronic paracoccidioidomycosis (9), the gp43 and other fungal components binding to concanavalin A may act as metabolic inhibitors or cause a negative regulation of natural killer lymphocyte cytotoxicity (10). Finally, the gp43 has been associated with a proteolytic activity (4) not necessarily a property of the glycoprotein itself but that of an aggregated protease of very high specific activity (11). Early attempts to clone and express the gene encoding the gp43 (12) aimed at isolating a recombinant molecule that could be used in the immunodiagnosis, be sequenced for peptide epitope identification, and be tested as a virulence factor. The isolated clone, however, was unstable and could not be used in subsequent studies.

In the present work we report on the characterization of the complete sequence of the gene encoding the gp43 antigen from P. brasiliensis. Determination of the sequence of peptide fragments derived from the native molecule by enzymatic proteolysis permitted PCR amplification of a genomic fragment which was used as a probe to isolate the entire gene from a genomic library of the fungus. A genomic fragment corresponding to the COOH-terminal portion of the protein was cloned into pGEX plasmid, and the expression product reacted in immunoblots with anti-gp43 polyclonal rabbit and human patient antibodies.

EXPERIMENTAL PROCEDURES

Purification of the Gp43—Gp43 was purified by affinity chromatography in columns of Affi-Gel 10 (Bio-Rad) coupled with rabbit anti-gp43 immunoglobulin G, followed by gel filtration in Sephacryl S-200, from supernatant fluids of P. brasiliensis yeast cells (strain B339) grown in TOM medium, as described previously (3, 4, 13).

Amino Acid Sequence Determination—Purified gp43 was dialyzed

1 The abbreviations used are: gp43, 43-kilodalton glycoprotein; GST, glutathione S-transferase; nt, nucleotide; kb, kilobases; PCR, polymerase chain reaction; GP43G, the gp43 gene; RT, reverse transcribed.
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against Milli-Q grade deionized water and a 829-pmol sample was used for enzymatic digestion and peptide sequencing at the Harvard Microchemistry Facility, Harvard University, Cambridge, MA. Accordingly, peptides obtained by digestion with endoproteinase Lys-C were fractionated on Hewlett-Packard 1090 HPLC equipped with a 1040 diode array detector, using a Vydac 2.1 × 150 mm C-18 column, as described (14). The peaks were selected based on size and purity by matrix-assisted laser desorption ionization time of flight performed on a Finnigan Lasermat™ mass spectrometer. Sequencing of the NH2-terminal and of the internal peptides was carried out by automated Edman degradation on an ABI 477A protein sequencer with 120A on-line phenylthiohydantoin amino acid analyzer.

DNA Sequencing. Construction of the Genomic DNA Libraries—Nucleic acids of P. brasiliensis strain B339 were prepared from powdered yeast cells, frozen in liquid nitrogen, as described previously (12, 15). Genomic DNA fragments of 0.5–2.0 and 2.0–7.0 kb were used as primers. The sequences were analyzed using the PC/ sequence deduced from the 987-bp fragment encompassed all positions, degenerate, inosine-containing oligonucleotides (30-mer); by matrix-assisted laser desorption ionization time of flight performed on a Finnigan Lasermat™ mass spectrometer. Sequencing of the NH2-terminal and of the internal peptides was carried out by automated Edman degradation on an ABI 477A protein sequencer with 120A on-line phenylthiohydantoin amino acid analyzer.

The specific oligomers were synthesized and used as restriction enzymes were introduced in the oligonucleotides.

Each reaction (25 µl) was carried out in 25 cycles. Optimized conditions included denaturing at 94 °C (1 min), annealing at 50 °C (1 min), and extension at 72 °C (1.5 min). The PCR fragments of interest were recovered from agarose gel by using the Sephaglass™ kit (Pharmacia). The data were processed by an Automated Laser Fluorescent DNA

PCR product (1 µl) was used to amplify cDNA fragments of 0.5–2.0 and 2.0–7.0 kb using a random-primer labeling kit (Life Technologies, Inc./BRL). Cloning was carried out using the Sureclone™ kit (Pharmacia) and the 987-bp fragment suggesting the existence of an intron, as described previously (12, 15). The genomic library in S. cerevisiae Y1090 and screened by plaque hybridization (16, 17). The data were processed by an Automated Laser Fluorescent DNA

Preparation and Reactivity of Antisem Against the Recombinant Molecule—Exogucanase activity was investigated in dialyzed culture supernatants using 50 mM acetate buffer, pH 5.5 (250 µl), and laminarin (Sigma), p-nitrophenyl-β-glucoside (Sigma) and α-glucan from P. brasiliensis yeast phase as substrates (0.2% final concentration) at 37 °C, for 2.5–4 h. Control exoglucanase activity was obtained from culture supernatants of S. cerevisiae 5288 and Canavalia alba in a 1 cm dialysis tube at 30 °C for 24 h in YPD medium (1% yeast extract, 2% casein peptone, 2% glucose). The reactions with laminarin and α-glucan were terminated by boiling for 10 min and the liberation of glucose measured by the glucose oxidase method (Enzymatic Glicemy kit, Wiener Laboratory, Argentina). The reactions with p-nitrophenyl-β-glucoside were interrupted by addition of 2.25 ml of 0.3 M Na2CO3, and the A405 measured.

RESULTS

Gp43 Peptide Microsequencing—Peptides obtained by cleavage of purified gp43 with endoproteinase Lys-C were purified on high pressure liquid chromatography and subjected to Edman degradation. The amino acid sequences of the amino terminal (AGSAIYGVNIG) and of three distinct internal peptides (Fig. 1 B, lanes 2 and 3) of the gp43 (Figs. 1 and 3) showed 54–60% identity with sequences present in the NH2- and COOH-terminal domains of the exo-1,3-β-D-glucanase of S. cerevisiae (24).

Cloning and Sequencing of the Gp43 Gene—The significant degree of homology of all gp43 peptides to the S. cerevisiae exogucanase suggested a certain arrangement of the sequenced peptides in the protein (Fig. 1A). Based on their assumed positions, degenerate, inosine-containing oligonucleotides were designed from the peptides 1 (forward primer, OLCK53), 2 and 3 (reverse primers, OLCK59, OLCK33) and were used for PCR amplification of the genomic DNA from P. brasiliensis. The PCR amplified products from the genomic DNA template of P. brasiliensis using as primers the OLCK53/OLCK33 and the OLCK53/OLCK59 pairs were of 987 and 570 bp, respectively (Fig. 1B, lanes 3 and 2). The amino acid sequence deduced from the 987-bp fragment encompassed all three native internal peptides obtained before, confirming that the PCR product encoded the expected region of the gene. This was considered as evidence for the authenticity of the isolated sequence. A stop codon (TAG) was noticed at position nt 199 of the 987-bp fragment suggesting the existence of an intron, as we have demonstrated in a later stage.
The gp43 protein has 41.8% nonpolar, 34.5% polar non-charged, 12.8% positively charged, and 9.7% negatively charged residues. The codon usage in the gp43 gene showed a strong preference for triplex codons (73.2%) over pentamers (25.2%) and heptamers (1.6%). The gp43 gene is transcribed using the ATG (positions 1601 to 1603) as the initiation codon. The 3'-untranslated region contains motifs thought to be necessary for termination of transcription, processing, and addition of poly(A) at the 3' terminus. Although a perfect match to the described eukaryotic polyadenylation consensus sequence (27), 5'-AATAAA-3', is not found, a similar pentanucleotide (5'-AATAA-3') is observed at position 1616, 276 nt downstream from the termination codon. In addition, a tripartite sequence (TAG...TATTT...TTT) was identified between nucleotides 1354 and 1388. This sequence presents a high degree of homology to a tripartite consensus sequence (TAG...TATTT...TTT) that has been postulated to be a signal for termination and/or polyadenylation in S. cerevisiae (28, 29). The size of the gp43 transcript deduced from the nucleotide sequence analysis is in agreement with the estimated size of transcript (approximately 1.5 kb) detected in the Northern blot hybridization (see Fig. 7).

The coding region of the gp43 gene is interrupted by an intron of 78 nt (Fig. 3), with 5' and 3' extremities at positions +464 and +541, respectively. The 5' and 3' extremities of the intron presented the GT/AG consensus (25). It is interesting to note the presence in the intron of a 11-nt motif (TA-GAATATCTC) which was also found perfectly repeated in the 3'-untranslated region of the gp43 gene (positions 1601 to 1612). Several repetitions of the motif (TA) were also detected in the intron. The coding region of the gp43 gene showed A + T content of 48.2%, whereas its 5'- and 3'-flanking regions gave higher A + T contents of 54% and 63.8%, respectively.

Analysis of the 5' 326-bp flanking sequence of the gp43 gene revealed structural feature characteristics of the promoter regions of eukaryotic genes. A TATA element (TATAAAA) is at position 71 to 75 and a T + C-rich pyrimidine block is found immediately downstream (nt 71 to 40). The CAAG motif (26) is found once, 114 nt downstream of the TC block (nt 25), and twice upstream, at positions 195 and 259.

The 3' region immediately downstream from the gp43 open reading frame contains motifs thought to be necessary for termination of transcription, processing, and addition of poly(A) at the 3' terminus. Although a perfect match to the described eukaryotic polyadenylation consensus sequence (27), 5'-AATAAA-3', is not found, a similar pentanucleotide (5'-AATAA-3') is observed at position 1616, 276 nt downstream from the termination codon. In addition, a tripartite sequence (TAG...TATTT...TTT) was identified between nucleotides 1354 and 1388. This sequence presents a high degree of homology to a tripartite consensus sequence (TAG...TATTT...TTT) that has been postulated to be a signal for termination and/or polyadenylation in S. cerevisiae (28, 29). The size of the gp43 transcript deduced from the nucleotide sequence analysis is in agreement with the estimated size of transcript (approximately 1.5 kb) detected in the Northern blot hybridization (see Fig. 7).

The gp43 protein has 41.8% nonpolar, 34.5% polar non-charged, 12.8% positively charged, and 9.7% negatively charged residues. The codon usage in the gp43 gene showed a relaxed bias toward preferential codons for the amino acids Ala, Ser, Thr, Val, Ile, Arg, Leu, and Pro. Of the 61 possible codon triplets, all are used in the gp43 gene. From the calculations described by Benetzen and Hall (30) we obtained a codon bias index of 0.476 for the gp43 gene, which for yeasts would represent a gene of moderate level of expression.
Protein Structure and Processing—Sequencing of the NH₂-terminal segment of the native protein (see above) indicated that the first residue of the mature protein is the alanine residue at position 36 (Ala36). This gives rise to a mature peptide of 381 amino acids with a predicted size of 42,281 Da.

Thus, the predicted gp43 gene product has a leader peptide (residues 1–35). The initiator methionine is followed by a block of hydrophobic amino acid residues typical of signal sequences (residues 1–23, Fig. 3). The deduced amino acid composition of the gp43, without the leader peptide, is quite similar to that obtained by conventional chemical methods using the native protein (7).

There were two potential N-glycosylation sites at residues 2 and 195 (residues 2–4, NFS, and 195–197, NRT, Fig. 3). Since amino acid 2 is within the signal sequence the mature gp43 should contain a single N-glycosylation signal. The net charge of the gp43 gives a pI of 7.43. The polypeptide is composed of alternating hydrophobic and hydrophilic regions, which is consistent with the water soluble character of the gp43; there is no hydrophobic sequence in the mature protein long enough to span the membrane.

Immunological Reactivity of gp43 Recombinant Proteins—Subfragments of the gp43 gene encoding parts of the internal and COOH-terminal domains of the protein were inserted in frame with the GST gene of plasmid pGEX (21) and were expressed as GST fusion proteins in E. coli. The recombinant fusion proteins were recognized in immunoblots by a polyclonal rabbit anti-gp43 antiserum, as well as by sera of patients with paracoccidioidomycosis (Fig. 4A). The reaction with rabbit antiserum raised against GST demonstrated that the products were expressed as fusion proteins (Fig. 4A). Moreover, antibodies generated in rabbits against the recombinant antigen carrying the COOH-terminal domain of the gp43 recognized the native antigen in immunoblots of P. brasiliensis culture filtrate (Fig. 4B).

Comparison of the Amino Acid Sequence of gp43 with Known Sequences—Previous database search had shown that peptide sequences (Fig. 3) shared homology with regions of the S. cerevisiae exo-1,3- b-D-glucanase (EXG1) (24). Comparison of the gp43 deduced amino acid sequence with those of known cloned fungal exoglucanases from S. cerevisiae (vegetative, EXG1, and spore specific, SPR1) (24, 31) and C. albicans (CAXOG) (32) showed that they shared, respectively, 49.8, 48.3, and 50.7% identity at the amino acid level, and if conservative substitutions were included, 56.3, 56.0, and 57.9% similarity. Fig. 5 shows the multiple alignment of the amino acid sequences encoded by the GP43G and those of S. cerevisiae and C. albicans exoglucanases. The entire gp43 sequence can be divided into parts of high and low homology, that could suggest functional domains. There are several blocks of 2 to 9 amino acids of 100% identity among the known glucanases separated by divergent regions. Some bacterial glucanase and/or cellulase sequences of the GenBank or EMBL data bases showed minor homology to the gp43 deduced amino acid sequence. The comparison of the secondary structures of the four aligned proteins (Fig. 5) showed a similar pattern of folding, especially at those regions indicated as extended in each individual molecule.

Exoglucanase Activity—Culture supernatants (50 µl) of
S. cerevisiae and C. albicans dialyzed against 50 mM acetate buffer, pH 5.5, showed exoglucanase activity when either laminarin or p-nitrophenyl-β-glucoside were used as substrates, whereas purified gp43 at 840 μg/ml or P. brasiliensis culture filtrates were negative. No hydrolysis of P. brasiliensis α-glucan was detectable with purified gp43 in the same conditions.

**Fig. 4.** Immunological reactivity of recombinant proteins and anti-recombinant protein antiserum. The subfragments of the internal (residues 110–272) and COOH-terminal (residues 288–411) domains of the gp43 were fused with GST. In A the reaction of an anti-GST rabbit antiserum with the recombinant proteins (lanes 1 and 2; GST alone, lane 3) confirms that the products are expressed as fusion proteins; a rabbit polyclonal monospecific anti-gp43 antiserum (anti-gp43R) and sera (pool) from patients (anti-gp43H) recognize the recombinant proteins in immunoblots (lanes 5, 6, and 9, 10, respectively). Lanes 4, 8, and 12, plasmid-less bacterial extracts. Lanes 7 and 11, GST. In B antibodies generated in rabbits against the recombinant antigen carrying the gp43 COOH-terminal sequence recognized the native gp43 in immunoblots of P. brasiliensis culture filtrate (lane 3) as shown by [125I]-protein A binding; lanes 1 and 2, reactions with rabbit preimmune serum and with the monospecific rabbit antiserum (anti-gp43R), respectively.

**Fig. 5.** Alignment of the amino acid sequence of the gp43 with known sequences. Alignment of the gp43 deduced amino acid sequence with known sequences of cloned fungal exoglucanases: S. cerevisiae, vegetative (EXG1), and spore specific (SPR1) exoglucanases, and C. albicans exoglucanase (CAXOG). The alignment was performed by the J. Hein method. Asterisks and dots indicate identical and similar residues, respectively.
Genomic Organization and Transcription of GP43G—Southern blot of P. braziliensis genomic DNA digested with several restriction enzymes was probed with the 987-bp fragment derived from the coding region of the GP43G. The Southern blot autoradiogram showed a single hybridized band of 3.8 kb with the EcoRI-digested genomic DNA, which corresponds to the size of the insert of the recombinant pgt11 clone. The fungal homologous genes are determined by the presence of introns. The presence of an intron was demonstrated in the GP43G coding region (Fig. 6A). The chromosomal blot hybridization of NotI-digested genomic DNA showed a 210-kb NotI fragment and two, 300- and 440-kb SfiI fragments (Fig. 6B).

The Northern blot hybridization of total RNA using the cloned DNA as a probe identified a transcript of approximately 1.5 kb which is large enough to encode a protein of the size predicted for native gp43.

The deduced gp43 open reading frame encodes a polypeptide of 416 amino acids (M, 45,947), with a leader peptide of 35 amino acids which was defined by peptide sequencing of the NH$_2$ terminus of the mature protein. The leader peptide is a hydrophobic sequence and a putative signal peptide cleavage site after residue 23 (Ala$^{32}$-Ser$^{24}$) (33, 34). If the signal peptidase cleavage observes these rules there could be a second post-translational processing of residues 24–35, which are not present in the secreted antigen. The specificity of the second proteolytic step would differ from that of the Kex2-like proteases which process the homologous fungal exoglycanases at a cleavage site consisting of basic amino acid residues, KR (24, 32, 35). This basic pair is absent in the gp43 leader peptide (Fig. 3).

Analysis of the sequences upstream and downstream from the gp43 open reading frame showed several motifs similar to the consensus elements important for the control of transcription in eukaryotic organisms. The CAAG motif, closely associated with the TC block, is correlated with a high level of expression of certain yeast genes (26, 36).

The presence of an intron was demonstrated in the GP43G determining two exons, 1 and 2. The fungal homologous genes of exo-1,3-glucanases of S. cerevisiae and C. albicans have no introns. The presence of the intron in the P. braziliensis DNA may implicate a transcriptional regulation. In fact, P. brasiliensis morphological transition is dependent on temperature, and therefore adaptation to temperature shifts and environmental stress is essential for the pathogen to survive in mammalian tissues. It is well known that heat shock affects RNA metabolism, including RNA processing as well as mRNA deg-

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So far, this is the first gene of the fungus to be cloned, entirely sequenced, and expressed in bacteria as recombinant fusion proteins. Several lines of evidence indicate that the sequenced clones encode the gp43 antigen. First, the deduced amino acid sequence encodes all the previously characterized partial peptide sequences of the native protein. Furthermore, it is remarkably consistent with previous analyses of amino acid composition in different preparations of the native gp43 (7). Second, different regions of the gp43 protein, expressed as fusion proteins in E. coli, reacted strongly with rabbit antiserum against the gp43 native protein and with sera from patients with paracoccidioidomycosis. On the other hand, the antiserum elicited against the gp43 recombinant fusion protein specifically recognized the native gp43 by immunoblotting of the P. brasiliensis culture filtrate. Third, Northern hybridization using the cloned DNA as a probe identified a transcript of 1.5 kb, which is large enough to encode a protein of the size predicted for native gp43.

**DISCUSSION**

In this report we describe the cloning and characterization of genomic and cDNA recombinant clones representing the entire coding region of the secreted glycoprotein of 43,000 Da previously described as the main diagnostic antigen of the dimorphic pathogenic fungus P. brasiliensis (2).

**Fig. 6. Genomic organization of the gp43 gene.** A, autoradiogram of a Southern blot of P. brasiliensis genomic DNA digested with restriction enzymes and probed with the 987-bp fragment: 1, EcoRI; a single fragment of 3.8 kb, which corresponds to the size of the insert of the recombinant pgt11 clone; 2, BamHI; 3, HindIII; 4, Smal; 5, EcoRI and BamHI, double digestion; 6, EcoRI and HindIII; 7, EcoRI and Smal; 8, BamHI and HindIII; 9, BamHI and Smal; 10, HindIII and Smal; 11, EcoRI, BamHI, and HindIII, triple digestion; 12, EcoRI, BamHI, and Smal. B, an ethidium bromide stained pulsed field gel of chromosomal megarestriction fragments from P. brasiliensis mycelium (M) (lanes 2 and 4) and yeast phase (Y) (lanes 3 and 5). Digestions employed the rare cutting enzymes NotI (lanes 2 and 3) and SfiI (lanes 4 and 5). S. cerevisiae intact chromosomes were used as molecular weight markers (lane 1). The corresponding autoradiogram shows that the NotI-digested M and Y chromosomes hybridized with a single 210-kb fragment (lanes 7 and 8). The M and Y SfiI digests hybridized with two fragments of approximately 330 and 440 kb (lanes 9 and 10). Lane 6, S. cerevisiae DNA.

**Fig. 7. Northern blot hybridization.** P. brasiliensis total RNA (lane 2) and poly(A)$^+$ RNA (lane 3) were isolated from 5-day-old yeast cells and probed with the radiolabeled 987-bp PCR fragment. Lane 1, Trypanosoma cruzi total RNA (specificity control and M, marker) and lane 4, a RNA ladder. A single transcript of approximately 1.5 kb was hybridized.
radiation. In dimorphic fungi, those changes require responses at the gene level and DNA sequences containing introns could play a vital role in adaptation to the new environment (37).

In spite of the significant identity shared at the amino acid level with glucanases of the vegetative forms of S. cerevisiae and C. albicans, and with S. cerevisiae spore-specific glucanase, we could not demonstrate any glucanase activity in the native gp43 molecule using different substrates. It is also noteworthy that no immunological cross-reactivity could be detected between gp43 and the glucanases of the other fungi.

Although the homologies among blocks of amino acid residues of the aligned glucanases and the gp43 might suggest functional domains, identification of the amino acid sequences and conformation of the catalytic and binding domains of the fungal exo-1,3-glucanases is not possible at present. In many highly unrelated bacterial 1,4-β-endoglucanases and in two fungal endoglucanases, however, the amino acid sequence NEP flanked by hydrophobic amino acid sequences is a conserved structure. Another similar sequence, LEP, was noted in bacterial and fungal glucanases. The NEP sequence is essential for the catalytic activity, as demonstrated by site-directed mutagenesis of this conserved motif in two highly unrelated bacterial endo-β-glucanases (38). It has been suggested that the E residue of the NEP sequence could be the proton donor in the hydrolysis process. In fact, the NEP sequence is similar to the MNEP sequence that exists in human lysosomal α-glucosidase and human and rabbit isomaltase and sucrase and reacts with the sugar unit of the substrate in the pyranose configuration (38, 39). The LEP and NEP sequences are conserved in C. albicans (residues 64–66 and 229–231, respectively) and S. cerevisiae EXG1 (residues 64–66 and 231–233) and SPR1 (residues 65–67 and 232–234) exo-1,3-glucanases (24, 31, 32). The gp43 has the LEP sequence (residues 51–53), but the NEP is altered to NKP (residues 207–209). Such difference in the amino acid sequence at the catalytic site with the introduction of a basic amino acid replacing an acidic one can itself account for the absence of glucanase activity in the gp43 protein. With the present evidence, one can suggest that the fungal glucosidases as well as the gp43 may have had a common ancestral gene and that a divergent evolutionary processing of these molecules has occurred. In the absence of a functional glucosidase activity, the gp43 may have been conserved mainly as an immunomodulating antigen and a virulence factor.

The two GST-gp43 fusion proteins represent different parts of the whole antigen and were both recognized by rabbit monoclonal polyclonal antibodies and by human sera from patients with paracoccidioidomycosis, indicating that the recombinant proteins shared epitopes with native gp43 including those recognized by antibodies from human patients. Therefore, at least two different B cell epitopes must be present in the gp43 molecule. The highest peak of hydrophilicity, computed using an average group length of 6 amino acids, which might be considered as a third epitope, corresponds to the sequence GRDAKR (residues 78–83). This sequence is 1 amino acid shorter than the homologous sequences of C. albicans and S. cerevisiae vegetative glucanases (Fig. 5) which are in turn more hydrophobic. Such variations and others detected by comparing the hydropathic profiles of the three amino acid sequences justify the absence of immunological cross-reactivity of the glucanases with antibodies elicited against the gp43. Recombinant molecules containing peptide epitopes of the gp43 can, on the other hand, be helpful to increase the specificity of the diagnostic antigen since all reported cross-reactivities depended on the N-linked carbohydrate chain of the glycoprotein (3).

Genomic Southern blot hybridization indicated that the gp43 is encoded by a gene with very few copies, similarly to the homologous fungal exo-1,3-glucanase genes (24, 31, 32). P. brasiliensis is a multinucleate organism both in the mycelial and yeast phases (40). The intensity of the hybridization signals obtained with the chromosome 5fl megarestriction fragments was very similar (Fig. 6B) indicating that they may represent two alleles of the GP43G and therefore, suggesting that both the yeast and mycelial phases of P. brasiliensis could be at least diploid. These results are of interest as there is no information about the fungus ploidy.

We have previously described the gp43 from P. brasiliensis as the most specific antigen in the paracoccidioidomycosis-P. brasiliensis system (2). It is presently being used in a variety of serological tests for diagnostic purposes (41, 42). The present study opens the perspective of analyzing the individual peptides and epitopes that play a role in the interaction of the gp43 with cells of the immune system, antibodies, and elements of the extracellular matrix. Recognition of individual epitopes that elicit a favorable immunological response can contribute for the immunotherapy of this systemic mycosis. The recombinant molecule and/or the selected epitopes cloned and amplified will be extremely helpful to define both the specificities and their functional role in the biology of this pathogenic fungus. The primary sequence of the gp43 will also permit evolutionary studies involving related molecules, including the glucanases of bacteria and fungi.

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