Use of Recombinant gp43 Isoforms Expressed in Pichia pastoris for Diagnosis of Paracoccidioidomycosis

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gp43 is the main diagnostic antigen for paracoccidioidomycosis (PCM). In vitro, gp43 expression in supernatant fluids of Paracoccidioides brasiliensis cultures can be unstable, and its regulation is poorly understood. We have been able to express soluble recombinant gp43 (gp43r) isoforms as N-mannosylated proteins secreted in the supernatants of Pichia pastoris cultures induced with methanol. They were secreted as major components from day 2 of induction and could be purified with affinity columns containing anti-gp43 monoclonal antibodies. We have expressed P. brasiliensis GP43 (PbGP43) sequences from genotypes A, D, and E, and the correspondent gp43r isoforms (gp43r A, -B, and -C, respectively; 200 ng) were compared to native gp43 in immunodiffusion (ID) and dot blot assays. Among 90 PCM patient sera showing ID-positive reactions with purified native gp43, 100% were positive with gp43rD and gp43rE and 98% reacted with gp43rA. Of these sera, 78 were tested in dot blot assays at a 1:1,000 dilution, and 100% reacted with all recombinant isoforms. In ID assays, the specificity was 100%, since 40 sera from patients with related mycoses and 30 sera from healthy individuals did not react with any of the antigens. In dot blot assays, 100% specificity for PCM occurred when cross-reactive mannos epitopes were neutralized with 10 mM metaperiodate or eliminated through deglycosylation. However, a 1:1,000 serum dilution was already discriminatory for most sera. We suggest that P. pastoris recombinant gp43, especially isoforms D and E, may replace the native antigen in ID and dot blot assays for diagnosis and prognosis of PCM. Regulated expression of large amounts of antigen in nonpathogenic yeast would justify its preferred use.

gp43 is a secreted glycoprotein from Paracoccidioides brasiliensis. It is the best-studied fungal molecule and the main antigen for diagnosis and prognosis of paracoccidioidomycosis (PCM) described so far (7, 20, 25, 37). PCM is a granulomatous systemic mycosis that occurs as active disease in 1 to 2% of infected people, whose number is estimated to be 10 million throughout areas of endemicity in Latin America (32). Adult PCM is the commonest form and gradually affects the lungs, mainly of male adults; it can be unifocal or disseminate to any organ, generally involving mucous membranes and skin. Acute and subacute PCM progress rapidly and spread through the lymphatic system; they are characteristic of children and young adults of both sexes (14). Histoplasmosis, blastomycosis, and coccidioidomycosis are related endemic mycoses caused by dimorphic ascomycetes genetically close to P. brasiliensis (30). Cellular immunity is protective against these mycoses, whereas high antibody titers against fungal antigens suggest heavy fungal loads during severe stages of disease.

Apart from being a major P. brasiliensis antigen for the humoral response, gp43 also contains T-cell epitopes that elicit protective cellular immunity in experimentally infected animals (17, 36, 37) and displays adhesive capacity toward proteins associated with the extracellular matrix (15, 21). The open reading frame of the P. brasiliensis GP43 (PbGP43) gene lies within a 1,329-bp genomic fragment that has a single 78-bp intron (11). The full protein bears 416 amino acids, of which the first 35 correspond to a signal peptide. Mature or exocellular gp43 contains a single N-glycosylation site at sequon Asn-X-Ser between amino acids 18 and 19 (78) (27). Exocellular gp43 is composed of a mixture of isoforms that have close but distinct isoelectric points (pI), as originally observed in SDS-PAGE gels (31). Exocellular gp43 is composed of a mixture of isoforms that have close but distinct isoelectric points (pI), as originally observed in P. brasiliensis isolate Pb339 (25). Moura-Campos et al. (23) described the existence of four gp43 pI profiles for eight fungal isolates, suggesting that differences can occur both within and among isolates. The antigen from one isolate was peculiarly basic (>8.5), while gp43 proteins from the others had pIs varying from 5.8 to 7.2.

Moraes et al. (22) found a total of 21 informative substitution sites concentrated in exon 2 of the PbGP43 gene, defining genotypes A to E (29). So far, all six samples bearing genotype A have been grouped in a cryptic phylogenetic group, PS2, according to a multilocus study with 65 isolates conducted by Matute et al. (19). Genotype A sequences translate basic gp43 and are highly polymorphic (up to 15
substitution sites) compared with the others. Isolates from PS2 were less virulent than others in the B10.A mouse model of PCM (10).

Serological diagnosis and prognosis of PCM are important tools for clinicians. The most popular test is double immunodiffusion (ID) for its simplicity, high sensitivity, and specificity (9, 31). Whole _P. brasiliensis_ extracellular antigen preparations are commonly used, where gp43 is the antigenic component responsible for ID specificity and positivity over 85% (5, 7, 9, 25). False-negative reactions have been found for patients with intense pulmonary infection and immune depression (5, 9). Purification of gp43 was facilitated after the production of monoclonal antibodies (15, 27), and the antigen, purified or not, has been tested in a variety of immunodiagnostic tests for detection of patient antibodies (4, 8, 26, 35, 37). Antigen detection in sera can also be used for diagnosis of PCM (18). Although gp43 is quite specific for PCM when presented to antibodies in solution (7, 26), in enzyme-linked immunosorbent assays (ELISAs) (8), and in dot blot assays (35), cross-reactions in ELISA can be frequent (1, 26), particularly with _β-2'_Gal/ _β-3'_Gal residues, which are probably more exposed when the antigen is immobilized on plastic.

Regulation of gp43 expression is poorly understood (10) and varies with the isolate (34). However, instability in gp43 expression can occur even in good producers (e.g., strain Pb339). Therefore, standardized whole-antigen preparations are useful for diagnosis/prognosis (4, 7) but are not necessarily reproducible. On the other hand, differences in reactivity related to the gp43 isoforms have already been reported (34). For these reasons, the use of soluble recombinant gp43 (gp43r) would be a step forward not only in the diagnosis of PCM but also in other structural and biological studies.

In the present communication, we report the extracellular expression of soluble gp43r in the yeast _Pichia pastoris_. Purified gp43r was tested for reactivity with sera from patients with PCM and other mycoses. We chose ID and dot blot assays to compare the reactions with those of purified gp43 from Pb339 because they are simple and fairly specific tests according to the literature (7, 35). Dot blotting is a sensitive enzymatic assay (35) and can eventually be quantified (2). We managed to express gp43 isoforms which are characteristic of Pb339 from genotypes A, D, and E (gp43A, -B, and -C, respectively) but not C (22, 29). The expressed isoform from genotype A has a calculated pI of 8.3, while the translated sequences from genotypes D and E have pIs of 6.8 and 7.1, respectively (22). Our results suggest that gp43r produced in _P. pastoris_ may be used in the diagnosis of PCM, especially those isoforms from genotypes D and E.

**MATERIALS AND METHODS**

**Synthesis of PbGP43 cDNA and expression in _P. pastoris_.** The PbGP43 coding sequences corresponding to genotypes A, D, and E (29) were reverse transcribed from DNA-free total RNA isolated from _P. brasiliensis_ Pb3 (PbGP43 genotype A), Pb10 (PbGP43 genotype D), and Pb14 (PbGP43 genotype E) (22). Total RNA was extracted from logarithmically growing yeast cells cultivated at 30°C, with shaking, in 0.5% yeast extract, 0.5% peptone-casein, and 1.5% glucose. The cell pellet was mechanically disrupted by being vortexed with glass beads (425 to 600 μm; Sigma) in the presence of TRIzol reagent (Invitrogen), and total RNA was isolated according to the instructions of the manufacturer. cDNA of the PbGP43 gene was obtained by reverse transcription-PCR (RT-PCR), using the ThermoScript RT-PCR system (Gibco) and the specific 3′ antisense primer 491 (5′-ACGTGCATCTACCCGTTCCACCTACCTT-3′), which has a Sall site immediately after the TGA stop codon. The second strand of the full open reading frame was elongated, using standard PCR conditions, with the 5′ sense primer 490 (5′-CTCAAGATCTATCATGAAATTTGATGCTTTTAAAC-3′), containing a BglII site before the ATG start codon, and with antisense primer 491. The 490/491 PCR fragment (1,250 bp) was extracted from agarose gels by using a Sepha-glass kit (Amerham Pharmacia), cloned into the pGEM-T Easy vector (Promega), and sequenced. cDNA corresponding to processed (secreted) gp43 was synthesized by PCR, using the pGEM-T clone as the template; the upstream primer 690 (5′-CAGTCGACCAAGCAGGATCAGCAATATAT-3′), containing a SalI site before the alanine codon GCA; and the downstream primer 691 (5′-CCGGTACCTCAGCTGATCCACCTACAA-3′), where a KpnI site lies immediately after the TGA stop codon. The 690/691 fragment (1,145 bp) was purified, cloned into the pGEM-T Easy vector, and subcloned into the Sall/KpnI sites of pHis1 (33), which we previously used to express gp43r (His6) in bacteria (not published). Although the purified recombinant _P. pastoris_ culture expressing gp43r in bacteria have not been of further use because of solubilization problems, cloning into pHIS1 was essential for subcloning the 690/691 insert into the EcoRI/NotI sites of a pPIC9 plasmid (Invitrogen) for expression in _P. pastoris_. The correct frame at the 5′ end was verified by sequencing recombinant pPIC9, which was subsequently used to transform _P. pastoris_ G115 according to the manufacturer's instructions.

A total of 10 recombinant clones from each PbGP43 genotype were randomly selected for induction of gp43r expression with methanol. For that purpose, individual colonies were grown for 2 days, with shaking, at 30°C in BMGY (buffered minimal glycerol complex medium), composed of 1% yeast extract, 2% peptone, 0.1 M potassium phosphate, pH 6.0, 1.34% YNB (Difco), 1% ammonium sulfate, and 1% glycerol. Cell pellets were suspended in one-half the initial volume of BMGY (BMGY with 1% methanol instead of glycerol, and incubated with intense shaking at 30°C) and 1% (v/v) 25°C. False-negative reactions have been found for patients with intense pulmonary infection and immune depression (5, 9). Whole antigen ID assay.

**Screening of positive _P. pastoris_ clones and purification of gp43r.** We used dot blotting to screen for _P. pastoris_ gp43r-producing clones upon induction with methanol. Cell-free supernatants (5 μl) were dot blotted onto nitrocellulose membranes and tested for reactivity with rabbit polyclonal anti-gp43 serum (1:5,000), as described below. Purified native gp43 (gp43n) was used as a positive control, and the supernatant from an induced _P. pastoris_ culture expressing irrelevant protein was used as a negative control. Expression of gp43r by positive clones was then verified in SDS-polyacrylamide-stained gels and Western blots. We chose the best-producing clone of each isoform to induce larger cultures of _P. pastoris_ for 2 days for purification purposes. To purify gp43n and gp43r, we used affinity columns of Affi-Gel 10 bound to MAb17c, which is an anti-gp43 monoclonal antibody that recognizes all gp43 isoforms (8; Rosana Puccia and Heloísa A. Carvalho, international patent application PCT/BR2000/000258). Fundação de Amparo à Pesquisa, 27 September 2007; Rosana Puccia and Kátia A. Carvalho, Brazilian patent application priority PI 0604717-3, Brazilian Patent Office, 29 September 2006). ID assay. ID assays were performed on microscope slides covered with a layer of 1% agarose gel diluted in 0.85% NaCl, as described previously (25). Antigens (200 ng) were sero tested in a volume of 10 μl per well. Reactions were incubated overnight at room temperature in a moist chamber; the slides were then washed once in 5% sodium citrate and five times in 0.85% NaCl. The last wash was extended overnight to minimize background staining. Agarose was dehydrated over the slides in an oven, and the reactions were stained with Coomassie brilliant blue.

**Dot blotting.** In order to test the usefulness of gp43r in dot blots, antigens (200 ng) were spotted onto nitrocellulose membranes (1.5 to 2 μl), while for screening purposes, 5 μl of _P. pastoris_ culture supernatant was used. The membranes were left in the air for 5 min and then quenched with sodium phosphate buffered saline (PBS) containing 5% skim milk (Nestlé) (PBS-M) overnight at 4°C. The membranes were washed three times (10 min each) with PBS-T (PBS containing 0.1% Tween 20) and incubated with rabbit polyclonal anti-gp43 serum (1:5,000) to screen for positive _P. pastoris_ clones or with patient sera (1:300 or 1:1,000). Sera were diluted in PBS-M and incubated with shaking for 1 h at room temperature with the membranes, which were then washed three times in PBS-T and incubated with secondary antibodies (peroxidase-labeled goat anti-human immunoglobulin G [IgG] or anti-rabbit immunoglobulin, used at 1:2,000; Amer sham Biosciences) for 1 h at room temperature, with shaking. The membranes were washed three times, and the reactions were developed with diaminobenzidine (Sigma). Preimmune serum or sera from healthy individuals were used as negative controls.
for 2 to 6 days. gp43rD is indicated by an arrow. C, supernatant from *P. pastoris*.

**RESULTS**

We expressed three gp43 isoforms (gp43rA, gp43rD, and gp43rE) as soluble glycoproteins in the culture supernatants of selected recombinant *P. pastoris* clones. They were expressed as major components migrating at about 45 kDa in SDS-PAGE gels from the second day after induction with methanol (Fig. 1A). The peak of expression generally occurred between days 4 and 6 of culture. The major component was identified as gp43r in Western blots incubated with polyclonal anti-gp43 rabbit antibodies (not shown).

The amount of gp43r expressed by *P. pastoris* varied with the isoform. Upon induction of medium-scale yeast cultures with methanol for 2 days, we obtained about 7 mg/liter of purified gp43rD and gp43rE and over 100 mg/liter of purified gp43rA. We chose to work with cultures induced for 2 days to minimize the presence of *P. pastoris* molecules. However, in order to increase the yield of gp43rD and -E, we might process longer-induced cultures in future purifications. Purification was achieved through affinity chromatography with columns containing anti-gp43 monoclonal antibody (MAb17c), and the purified recombinant isoforms can be seen in Fig. 1B in comparison with gp43n. In Fig. 1B, a smear is particularly visible for gp43rD before treatment, suggesting hyper- and heterogeneous glycosylation by *P. pastoris*. The smear is more evident in overloaded silver-stained gels (not shown) and can be seen in the Western blot in Fig. 3C. After enzymatic deglycosylation with endo H, all recombinant isoforms migrated slightly slower than gp43n, probably due to the addition of 10 to 14 vector amino acids at the N terminus. Although we have not sequenced this end of the gp43r isoforms, the constructions predict the addition of a YVEFKGLRQ peptide sequence from pHIS1 and, possibly, the repeat EAEA from pPIC9 before that. This repeat is at the C terminus of the α-mating-factor signal peptide and is left in the recombinant proteins produced in *P. pastoris* after Kex2 cleavage. Peptide EAEA is supposedly cleaved by a dipeptidyl aminopeptidase, Ste13, but cleavage may not occur when the protein is overexpressed (12).

We initially verified the reactivity of each gp43 isoform by double ID assay, which is the test used most often for diagnosis and prognosis of PCM because of its simplicity, sensitivity, and specificity. We started by testing a small sample of PCM patient sera that reacted with gp43n and obtained positive results using 2 µg of all the isoforms. We next titrated the antigen (not shown) in order to use the smallest possible amount to guarantee positive results with pure sera. Based on the results, we chose 200 ng of antigen to screen a larger serum sample. We assayed a total of 90 sera from 78 different patients (Table 1). The samples were tested with gp43rA, gp43rD, and gp43rE in comparison with gp43n. In Fig. 2A, we exemplify these reactions by showing the results for 18 PCM sera. The positive reactions between sera g, h, p, and o and gp43rD were considered weak, while that between gp43rD and serum n was negative (but positive in a second test). Among the negative results, only two were later confirmed, specifically those with isoform gp43rA (Table 1). However, these two sera produced weak reaction bands with the other antigens. The intensities of the precipitation lines were generally similar among isoforms and gp43n, but differences eventually occurred, as represented by those formed between serum a or g and gp43rE. In parallel, we tested sera from patients with histoplasmosis (n = 15), aspergillosis (n = 15), candidiasis (n = 5), and Jorge Lobo’s disease (n = 5), in addition to 30 sera from healthy donors. The antibodies in none of these sera produced ID precipitation bands with gp43r or gp43n.
In order to test the prognostic value of gp43r in ID assays, we titrated (1:1 to 1:32) eight serum samples. As represented by three sera in Fig. 2B, the results among gp43n and gp43r were generally similar, although some differences in band intensity could eventually be observed. Overall, our results showed that for PCM diagnosis using ID, the positivity of gp43rD and gp43rE was 100% comparable to that of gp43n, while for gp43rA it reached 98% (Table 1). The specificity was 100% with all isoforms. We concluded that gp43r produced in \textit{P. pastoris} has ID diagnostic and prognostic value for PCM. A mixture of isoforms is not necessary, and isoform gp43rE apparently produced results most similar to those obtained with gp43n.

We then tested the usefulness of gp43r in dot blotting. Among ID-positive PCM patient serum samples, we assayed 78 with gp43rA, gp43rD, gp43rE, and gp43n (200 ng), treated or not with 10 mM sodium periodate. A total of 33 sera were also tested with the corresponding endo H-treated antigens. At a 1:1,000 serum dilution, the reactions were generally similar among all antigens, treated or not, as shown in the representative dot blots of Fig. 3A. About 13 samples presented weaker dots, as exemplified by sera PCM5 and PCM6. Reaction with PCM5 was the weakest among PCM sera at 1:1,000. Sera from patients with other mycoses were tested at 1:300, and at this dilution about 68% were positive with all the gp43r isoforms, while 20% were positive with gp43n (Table 1). At 1:1,000, cross-reactivity with gp43n was abolished. Dilution to 1:1,000 was also able to eliminate most of the cross-reactions for the samples tested here.

Table 1. Positive reactions between recombinant gp43r isoforms A, D, and E and serum samples from patients with PCM or other mycoses

| Sample group          | % Positive reactions | Total no. of samples |
|-----------------------|----------------------|----------------------|
|                       | gp43rA               | gp43rD or gp43rE     | gp43n     | ID | Dot blot |
|                       | −Per                 | +Per                 | −Per       | +Per | −Per | +Per |
| PCM                   | 98                   | 100                  | 100        | 100  | 100  | 100  | 100  | 100  | 90   | 78   |
| Histoplasmosis        | 0                    | 40                   | 0          | 40   | 0    | 20   | 0    |
| Aspergilosis          | 0                    | 60                   | 0          | 60   | 0    | 20   | 0    | 15   | 5    |
| Candidiasis           | 0                    | 60                   | 0          | 80   | 0    | 0    | 0    | 5    | 5    |
| Jorge Lobo's disease  | 0                    | 100                  | 0          | 100  | 0    | 40   | 0    | 5    | 5    |

Reactivity was tested in ID and dot blot assays. Dot blot reactions were performed before (−Per) and after (+Per) treatment with 10 mM sodium periodate. In ID assays, all sera were tested undiluted, while in dot blots, PCM patient sera were tested at 1:1,000 and heterologous-protein sera were tested at 1:300. The percentage of overall cross-reactivity highly decreased when heterologous sera were assayed in dot blots at 1:1,000 (not shown).
DISCUSSION

We have been able to express soluble gp43r isoforms as glycosylated proteins secreted in the supernatants of *P. pastoris* cultures induced with methanol. The glycoproteins were secreted as major components from day 2 of induction and could be purified in affinity columns containing anti-gp43 monoclonal antibodies. We expressed PbGP43 sequences from genotypes A, D, and E (29) and compared their performances with that of gp43n in immunodiagnostic tests. The correspondent gp43 isoforms were useful in both ID and dot blot assays to detect 100% of PCM sera that were reactive with the native antigen. With gp43r, we obtained total specificity for PCM patient sera in ID assays, while in dot blots, total specificity for PCM patient sera could be achieved when carbohydrate...
epitopes were previously neutralized by treatment with 10 mM metaperiodate or by enzymatic deglycosylation with endo H. In most cases, however, a serum dilution of 1:1,000 was enough to discriminate between PCM and heterologous sera.

We have previously made several efforts to express gp43r in soluble form for use not only as an antigen in immunodiagnostic tests but also as a tool in diverse biological and structural analyses. Our attempts included expression in both bacteria and, unsuccessfully, _Saccharomyces cerevisiae_. For bacteria, we reported the production of insoluble whole and truncated gp43 fused to glutathione S-transferase (13). In immunoblotting, whole gp43-glutathione S-transferase reacted specifically with 27 PCM patient sera, while reactivity with N-terminal, middle, and C-terminal fragments varied. Other vectors used for IPTG (isopropyl-β-D-thiogalactopyranoside)-induced expression of gp43 in bacteria included pET23-a (Novagen) and pHIS1 (33), which resulted in production of insoluble His-tagged proteins of limited antigenic capacity after solubilization with 8 M urea and renaturation using standard protocols (not published).

For production of gp43 in _P. pastoris_, we subcloned PbGP43 genotypes A, D, and E from pHIS1 into both pPIC9K and pPIC9 shuttle vectors, but we managed to express only the gene cloned in pPIC9. We also tried to express gp43 isoform C from Pb339, but so far this has been unsuccessful. Vectors pPIC9 and pPIC9K differ basically in the resistance marker for recombinant yeasts on histidine-deprived selection media, which resulted in production of insoluble His-tagged proteins of limited antigenic capacity after solubilization with 8 M urea and renaturation using standard protocols (not published).

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_Whole gp43r–glutathione was fused to glutathione for recombinant yeasts on histidine-deprived selection media, and renaturation using standard protocols (not published)._
ies from PCM patients are preferentially directed to peptide epitopes (26). The rate of false-negative reactions with gp43n in dot blots was 20% in this work and only 4.3% when Taborda and Camargo (35) standardized the test for diagnosis and prognosis of PCM. They tested 64 sera from non-PCM mycosis patients and 50 sera from healthy individuals; treatment with 10 mM metaperiodate blocked cross-reactions. In the present work, higher percentages of cross-reactivity with gp43r in dot blots were expected due to the ubiquitous nature of mannose epitopes among pathogenic fungi. In gp43r, the main source of cross-reactivity seems to be a unique terminal β-Gal/f residue, at least when ELISA is considered (1, 26).

Overall, our results suggest that gp43r from P. pastoris may replace gp43n in dot blots and ID assays for detection of PCM; although a serum dilution of 1:1,000 eliminated most dot blot false-positive results with this test. We tested three isoforms, among which gp43rD or gp43rE can apparently be used without the need for a mixture of isoforms. The isoform corresponding to Pb339 (genotype C), which is largely recommended for antigen production, has not been expressed successfully in yeast so far. The advantage of gp43r from P. pastoris relies mainly in reproducibility for the production of large amounts of a known sequence of gp43, which is expressed in culture supernatants under inducible conditions in non-pathogenic, fast-growing yeast. Although posttranslational modifications in yeast can be advantageous, we are working on the expression of deglycosylated gp43r, using a point mutation at the glycosylation site. That might render a more specific antigen, but conformational problems could lead to decreased sensitivity.

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