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Expression in Bacteria of the Gene Encoding the gp43 Antigen of *Paracoccidioides brasiliensis*: Immunological Reactivity of the Recombinant Fusion Proteins

Susana N. Diniz,† Kátia C. Carvalho, Patrícia S. Cisalpino,‡ José F. Silveira, Luiz R. Travassos, and Rosana Puccia*

Departamento de Microbiologia, Imunologia e Parasitologia da Universidade Federal de São Paulo, São Paulo, SP 04023-062, Brazil

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gp43 is the major diagnostic antigen of *Paracoccidioides brasiliensis*, the agent of paracoccidioidomycosis (PCM) in humans. In the present study, cDNA of the gp43 gene (*PbGP43*) was obtained by reverse transcriptase PCR, inserted into a pGEX vector in frame with the glutathione *S*-transferase (GST) gene, and expressed in *Escherichia coli* as inclusion bodies. Immunoblotting showed that all sera from patients with chronic pulmonary and acute lymphatic forms of PCM reacted with the recombinant fusion protein of the mature gp43 (381 amino acids). Reactivity with fusion proteins containing subfragments of the N-terminal, internal, or C-terminal regions occurred eventually, and the C-terminal region was the most antigenic. Lack of reactivity with the subfragments may be due to the conformational nature of the gp43 epitopes. Sera from patients with aspergillosis, candidiasis, and histoplasmosis did not react with the gp43-GST fusion protein. Our results suggest that recombinant gp43 corresponding to the processed antigen can be a useful tool in the diagnosis of PCM.

Paracoccidioidomycosis (PCM) in humans is a systemic granulomatous mycosis caused by *Paracoccidioides brasiliensis*, a dimorphic fungus. Infection starts by inhalation of fungal propagules, which reach the pulmonary alveolar epithelium and transform into the parasitic yeast form. Chronic PCM is the most common form and preferentially affects male adults. It evolves gradually in the lungs but can involve other organs, often being associated with mucous membranes and skin lesions. Acute and subacute PCM forms affect both sexes, progress rapidly, and disseminate through the lymphatic system, with lymph node enlargement and, in severe cases, intense hepatosplenomegaly and damage to other organs.

Patients with severe PCM forms are deficient in the protective cellular immunity against *P. brasiliensis*, which can then grow in large scale, increasing the antigenic load and the antibody response against fungal antigens. At this stage the antibodies are no longer protective (8) but are useful in the diagnosis and prognosis of PCM. The main antigenic component described for *P. brasiliensis* is the exocellular glycoprotein gp43 (17), which is recognized by most sera from PCM patients (5, 24). Successful treatment is reflected in a decrease of circulating anti-gp43 antibodies and restoration of cellular immunity (8, 11, 19).

gp43 is also an immunodominant antigen for cellular immunity in humans and experimentally infected animals (20, 21). In murine PCM, native gp43, the 15-amino-acid peptide P10 containing the T-cell epitope, and the gp43 gene were able to protect against intratracheal challenge with virulent *P. brasiliensis* by eliciting a gamma interferon-producing Th-1 response (14, 25). In addition, the glycoprotein may be involved in pathogenesis, since it binds to murine laminin (27), and laminin-coated yeast cells have increased virulence. A murine anti-gp43 monoclonal antibody was able to modulate the infection by these cells in a hamster intratesticular PCM model (9).

The open reading frame of the gp43 gene (*PbGP43*) is within a 1,329-bp DNA fragment comprising two exons separated by a 78-bp intron (6). The gene encodes a protein of 416 amino acids, which includes a signal peptide of 35 residues. The sequence of the mature or exocellular gp43 starts with an alanine residue. The antigen contains a single N-linked oligosaccharide chain (1), but specific antibodies from PCM patients are preferentially directed to conformational peptide epitopes (15). Anti-gp43 monoclonal antibodies could detect at least three sets of epitopes in inhibition assays (9, 16). Polymorphism in the gp43 sequence has been observed at both the protein (13, 17) and gene (12) levels. Isoforms with neutral isoelectric points, like those of the gp43 from isolate B-339 of *P. brasiliensis*, are more commonly found and are mostly reactive with patient's antibodies (22).

Although gp43 is a PCM-specific antigen in immunological tests that preserve its native conformation, it contains terminal galactofuranose units in the oligosaccharide chain (1), which can be a source of cross-reactivity in tests that use the immobilized antigen, such as enzyme-linked immunosorbent assays (15). The expression of recombinant proteins in bacteria can be useful because they are unglycosylated. The present work describes the immunological reactivity of glutathione *S*-transferase (GST) fusion proteins with the entire gp43 sequence

* Corresponding author. Mailing address: Disciplina de Biologia Celular, UNIFESP, Rua Botucatu, 862, oitavo andar, São Paulo, SP 04023-062, Brazil. Phone: 55-11-5084-2991. Fax: 55-11-5571-5877. E-mail: rosana@ecb.epm.br.

† Present address: Instituto Ludwig de Pesquisa do Câncer, São Paulo, Brazil.

‡ Present address: Departamento de Microbiologia do Instituto de Ciências Biológicas Da Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil.

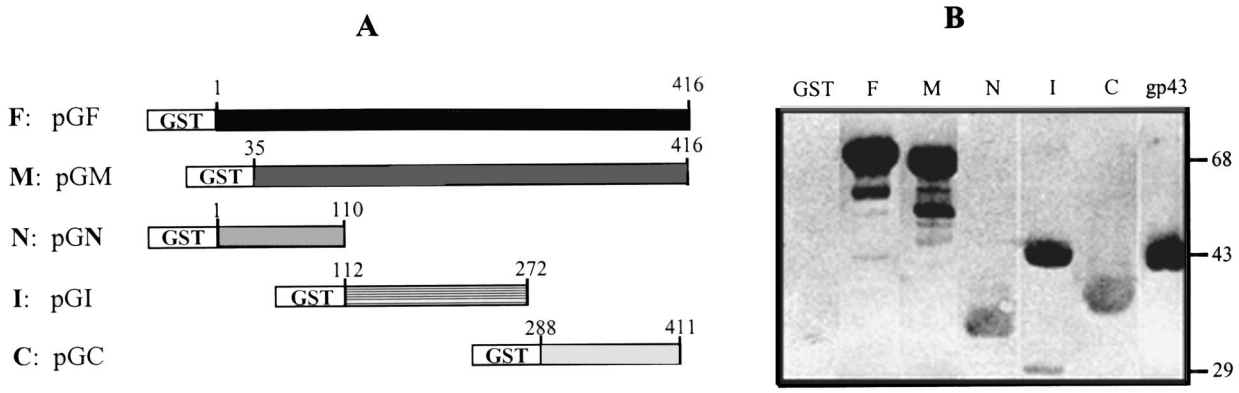

FIG. 1. (A) Schematic representation of the gp43-GST recombinant fusion proteins pGF, pGM, pGN, pGI, and pGC. The numbers correspond to the amino acids in the gp43 sequence, starting from the initial methionine (amino acid 1). (B) The indicated gp43-GST fusion proteins were expressed in *E. coli*; the reactivity of bacterial extracts was tested by immunoblotting with a rabbit polyclonal anti-gp43 serum (1:5,000) and detected with DAB. Reactions with rabbit preimmune serum were negative and are not shown. GST, bacterial extract containing recombinant GST; gp43, native gp43. Sizes of molecular mass markers are indicated in kilodaltons.

and subfragments of the N-terminal, internal, and C-terminal regions, which were expressed in bacteria.

MATERIALS AND METHODS

Synthesis of the gp43 cDNA. The coding sequence of the gp43 antigen was obtained by reverse transcriptase PCR as described previously (14). Briefly, cDNA was reverse transcribed from total RNA isolated from *P. brasiliensis* B-339 (6, 7) by using an oligo(dT) standard primer and was used as a template in PCRs. The full coding region of the gp43 antigen was synthesized by using the internal primer 490 (5′-GTCAGATCTATCATGAATTTTAGTTCCTTAAC-3′), containing a *Bgl*II site before the ATG start codon, and the downstream primer 491 (5′-ACGTCGACTCACCTGCATCCACCATACTT-3′), with a *Sal*I site immediately after the TGA stop codon. The processed or mature gp43 was synthesized with the upstream primer 690 (5′-CAGTCGACAAGCAGGATCAGCAATAT AT-3′), containing a *Sal*I site before the alanine codon GCA, and the downstream primer 691 (5′-GCGGTACCTCACCTGCATCCACCATA-3′), with a *Kpn*I site immediately after the TGA stop codon. The PCR fragments generated with primers 490 and 491 (1,250 bp) and with primers 690 and 691 (1,145 bp) were extracted from the agarose gels by using the Sephaglass kit (Amersham Pharmacia) and cloned using the pMOS T Blue vector kit (Amersham Pharmacia). DNA sequencing of the cloned fragments confirmed their identity with the original *PbGP43* sequence deposited in GenBank (accession number U26160). The gp43 gene subfragment containing the 5′ region (329 bp) was obtained by *Bgl*II and *Bam*HI restriction of the gp43 cDNA encoding the full molecule. The subfragments containing the internal region (492 bp) and 3′ region (375 bp) were obtained as described previously (6).

Expression and serum reactivity of the recombinant gp43. The gp43 cDNA fragments and subfragments were subcloned into pGEX plasmids (Amersham Pharmacia) in frame with the GST gene. The fusion proteins were expressed in *Escherichia coli* DH5α upon induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacteria were cleaved by boiling in reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, the extracts were separated by SDS-PAGE (10) and transferred to nitrocellulose filters (26), and the membranes were used in immunoblot reactions. The reactivity of the recombinant proteins was tested with rabbit anti-gp43 antiserum (1:5,000) (18) and with sera from patients with PCM. Immunocomplexes of rabbit antibodies were evidenced with goat anti-rabbit immunoglobulin G (IgG)–peroxidase conjugates and developed with diaminobenzidine (DAB). Reactions with patients' sera (1:300) were detected with biotinylated anti-human IgG (Amersham Pharmacia) at a 1:1,000 dilution and streptavidin-peroxidase (Amersham Pharmacia) at a 1:1,000 dilution and developed with DAB. Alternatively, the reactivity of recombinant gp43-GST fusion proteins with patients' sera (1:1,000) was detected by chemiluminescence (2) with biotinylated anti-human IgG developed with luminol. Membranes were exposed (3 min) to an X-ray film (Hyperfilm-MP; Amersham Pharmacia).

Sera. Anti-gp43 rabbit immune serum was previously obtained by Puccia et al. (18). Patients' sera were kindly provided by Arnaldo Colombo and Zoilo Pires de Camargo (Universidade Federal de São Paulo), by Carlos da Silva Lacaz (Universidade de São Paulo), and by Gilson Freitas da Silva (Faculdade de Medicina, Ribeirão Preto, Universidade de São Paulo). Patients with chronic PCM were all male, were 44 to 66 years old (except one was 29 years old), and had pulmonary PCM. Patients with acute PCM were 43% female, had the disseminated lymphatic form, and were 10 to 38 years old. Most of the patients had active and severe PCM, with high antibody titers, and many were under treatment.

RESULTS

The *PbGP43* coding sequence was inserted in frame with the GST gene of the pGEX vector and expressed as gp43-GST fusion proteins in *E. coli.* Figure 1A shows a schematic representation of the constructs corresponding to the fusion proteins of the full (pGF) and processed or mature (pGM) gp43 (416 and 381 amino acids, respectively), as well as the subfragments including the first 110 amino acids of the N-terminal region (pGN), the internal sequence from amino acid 112 to 272 (pGI), and the C-terminal region from amino acid 288 to 411 (pGC). The mature protein corresponds to the sequence of the gp43 isolated from culture supernatants, as determined by N-terminal analysis (6). The five recombinant products were expressed as major insoluble cytoplasmic proteins, as revealed by Coomassie blue-stained SDS-polyacrylamide gels of soluble and insoluble components of the bacterial extracts (not shown). Induction of protein expression with lower concentrations of IPTG or at 30°C, for various time intervals, did not reduce the degree of insolubility.

In immunoblots, the bands corresponding to pGF (73 kDa), pGM (70 kDa), pGN (37 kDa), pGI (43 kDa), and pGC (40 kDa), but not GST alone, were all recognized by a rabbit anti-gp43 immune serum, as shown in Fig. 1B. These proteins were also recognized by a rabbit anti-GST antiserum (not shown) and migrated in SDS-PAGE with apparent molecular masses near those expected for GST fusion products. The reactive bands that can be seen in Fig. 1 migrating faster than pGF and pGM are either degradation products or truncated recombinant proteins.

The reactivities in immunoblots of the gp43-GST proteins pGM, pGN, pGI, and pGC were tested with sera from PCM patients, all of which reacted well with native gp43 (not

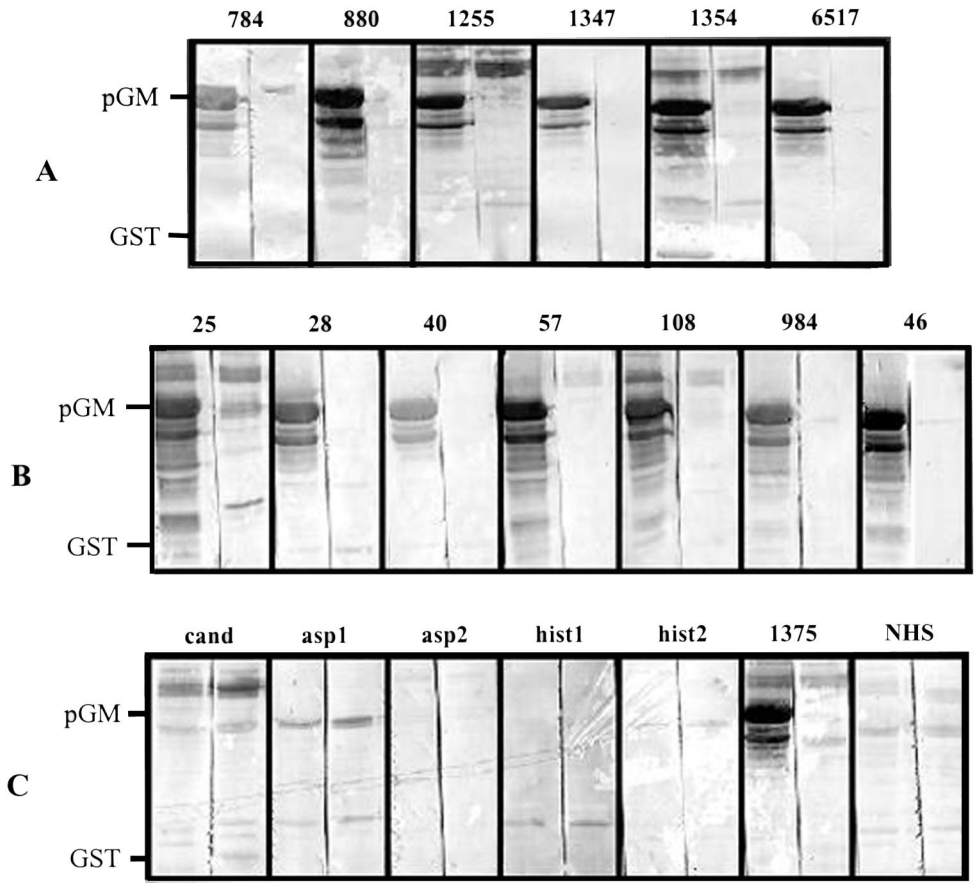

FIG. 2. The pGM fusion protein (lanes on the left) and GST alone (control lanes on the right) were expressed in *E. coli*, and the reactivity of bacterial extracts was tested by immunoblotting. The positions of pGM and GST are indicated. (A and B) Sera (1:300) from patients with chronic (A) and acute (B) PCM. Serum numbers are indicated above the lanes. (C) Sera from patients with other mycoses (cand, candidiasis; asp, aspergillosis; hist, histoplasmosis) and from a healthy individual (NHS). PCM serum 1375, from a patient with chronic PCM, was included in panel C for comparison. The reactions were detected with DAB.

shown). The protein pGM was recognized by all of the sera tested, which included 13 from patients with chronic PCM and 14 from patients with acute PCM. Figure 2A shows the immunoblotting of six sera from patients with chronic PCM (Fig. 2A) and seven sera from patients with acute PCM (Fig. 2B) with total bacterial lysates containing pGM as a major protein component. Some sera (serum 1255, for example) also reacted with bacterial proteins of high molecular weight, which were seen in the bacterial extract expressing only GST (control lanes). Reactive protein bands migrating faster than pGM can be either proteolytic products from pGM or expressed truncated forms. When bacterial lysates containing pGM were tested for reactivity against sera from patients with candidiasis, aspergillosis, and histoplasmosis and from a healthy individual, only nonspecific bacterial components were observed (Fig. 2C).

Since the reactions with pGN, pGI, and pGC were either absent or poorly seen in immunoblots developed with DAB, the assays with individual sera were carried out later using a more sensitive detection system. When the immunocomplexes were detected by chemiluminescence, we observed reactivity with all of the subfragments, although in only a few instances did an individual serum react with all of them. One example can be seen in Fig. 3A: serum 1131 (acute PCM) recognized all of the fusion proteins (pGM, pGN, pGI, and pGC), but the reactivity with pGN was weaker than that with the other recombinants. Serum 4 (acute PCM) reacted well with pGM, pGN, and pGC but little with pGI (Fig. 3A). It is of note that only three sera from patients with chronic PCM reacted with any gp43 subfragment, all of them with pGC, while several sera from patients with acute PCM recognized at least one subfragment. Sera 6442 and 6682 from patients with chronic PCM reacted well with pGC as well as pGM (Fig. 3A). It was interesting that all sera that were able to recognize gp43 subfragments always reacted at least with pGC.

Serum 1293 (acute PCM) reacted with pGM and pGC and weakly with pGI. When the serum of the same patient was tested after the first month of treatment (serum 1293^{t}), the reactivity with pGC was negative after 1 min of exposure and was detectable after overexposure for 6 min (Fig. 3B).

DISCUSSION

The first recombinant clone expressing the gp43 protein recognizable by human antibodies was reported by Taba et al. (23). Subsequently, Cisalpino et al. (6) showed the reactivity of

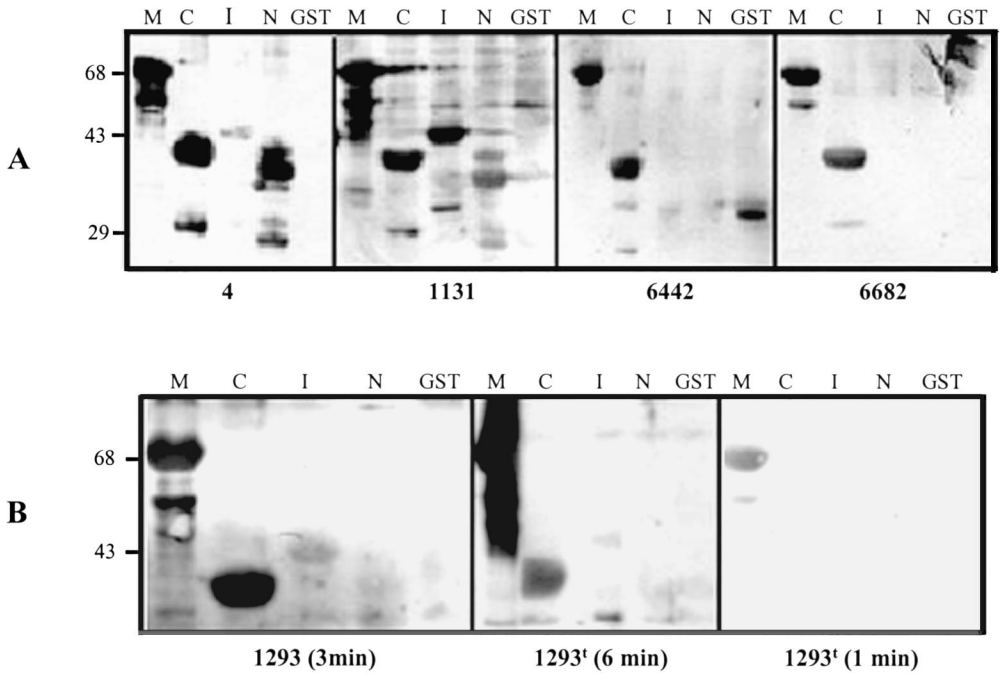

FIG. 3. gp43-GST recombinant proteins (M, pGM; N, pGN; I, pGI; C, pGC) and GST were expressed in *E. coli*, and the reactivity of bacterial lysates was tested by immunoblotting with sera (1:1,000) from PCM patients. Detection was carried out by chemiluminescence assay. (A) Reactions with sera 4 and 1131 (acute PCM) and 6642 and 6682 (chronic PCM). (B) Reactions with serum from patient 1293 (acute PCM) before and after (1293^t) 1 month of treatment, with membrane exposures of 1, 3, and 6 min as indicated. Sizes of molecular mass markers are indicated in kilodaltons.

gp43-GST recombinants of the internal and C-terminal regions of the antigen with a pool of sera from PCM patients and a rabbit anti-gp43 antiserum. This reactivity confirmed that the gene cloned and sequenced by those authors corresponded to that of the gp43 antigen. In the present work, four gp43-GST recombinant proteins were studied, i.e., the mature processed antigen (pGM) and subfragments of the N-terminal (pGN), internal (pGI), and C-terminal (pGC) regions. These proteins reacted in immunoblotting with an anti-gp43 rabbit antiserum, and all the sera from PCM patients recognized the recombinant fusion protein of the mature antigen.

Most of the sera tested reacted only with the mature recombinant protein, and epitope preferences could not be mapped. This general pattern of reactivity was also observed with an anti-gp43 murine monoclonal antibody that reacted with pGM and was negative with the fusion proteins of the distinct regions (not shown). This result is not surprising, since the monoclonal antibody tested recognizes conformational peptide epitopes (9, 16). On the other hand, the gp43-GST proteins were expressed in bacteria as inclusion bodies, and their native conformation could have been changed due to the fusion with GST, lack of glycosylation, insolubility, denaturation in SDS-PAGE, and blotting. Therefore, the low percentage of human sera reacting with the gp43-GST subfragments may be due to loss of conformational epitopes, which are predominantly recognized by human sera as well (15, 16).

Out of the 27 sera tested, however, some (generally from untreated patients with the lymphatic form of PCM) reacted with gp43-GST subfragments, indicating the presence of B-cell epitopes throughout the molecule. The Jameson-Wolf antigenic index predicts several potential antibody binding hydrophilic regions along gp43, according to the Protean graphic analysis of DNASTAR. According to the Emini plot, sequences with highest probability of surface expression in the protein are in the internal (amino acids 222 to 228 and 259 to 264) and, more remarkably, C-terminal (amino acids 356 to 364) regions. Not surprisingly then, many of the PCM patients' sera reacted with the C-terminal region, as well as with the entire molecule. A patient's serum tested after 1 month of chemotherapy lost its reactivity with the C-terminal region, thus correlating this specific antibody response with active infection. In fact, gp43 has been considered a prognostic marker for PCM, so that patients undergoing treatment have decreasing anti-gp43 titers (3, 4, 11). It is possible that the earlier antibodies to disappear in the successfully treated patients are those against the C-terminal epitope(s) of gp43. On the other hand, in our analysis most sera from patients with chronic PCM did not react with subfragments, and that might be due to the fact that these sera were mostly from treated individuals, while the patients with acute PCM were generally untreated when the sera were collected.

In the present study, we found that all sera from PCM patients recognized the mature gp43-GST fusion protein as determined by immunoblotting, while nonrelated sera were negative. Such reactivity confirms that peptide rather than carbohydrate epitopes are immunodominant in gp43 for antibody response, as previously shown with the deglycosylated antigen (15, 16). This analysis also suggests that recombinant gp43 can be a useful tool in the diagnosis of PCM, especially if

expressed as soluble protein that can be purified and used in tests such as enzyme-linked immunosorbent assays.

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Susana N. Diniz and Kátia C. Carvalho contributed equally to this work.

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