CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 2002, p. 374–377 Vol. 9, No. 2
1071-412X/02/$04.00+0 DOI: 10.1128/CDLI.9.2.374–377.2002

Heterologous Expression, Purification, and Immunological Reactivity of a Recombinant HSP60 from *Paracoccidioides brasiliensis*

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Received 26 July 2001/Returned for modification 25 September 2001/Accepted 20 November 2001

The complete coding cDNA of HSP60 from *Paracoccidioides brasiliensis* was overexpressed in an *Escherichia coli* host to produce high levels of recombinant protein. The protein was purified by affinity chromatography. A total of 169 human serum samples were tested for reactivity by Western blot analysis with the purified HSP60 recombinant protein. Immunoblots indicated that the recombinant *P. brasiliensis* HSP60 was recognized by antibodies in 72 of 75 sera from paracoccidioidomycosis patients. No cross-reactivity was detected with individual sera from patients with aspergillosis, sporotrichosis, cryptococcosis, and tuberculosis. Reactivity to HSP60 was observed in sera from 9.52% of control healthy individuals and 11.5% of patients with histoplasmosis. The high sensitivity and specificity (97.3 and 92.5%, respectively) for HSP60 suggested that the recombinant protein can be used singly or in association with other recombinant antigens to detect antibody responses in *P. brasiliensis*-infected patients.

Paracoccidioidomycosis is a fungal disease caused by *Paracoccidioides brasiliensis*, a thermal dimorphic fungus which is geographically confined to Latin America (13). The significance of paracoccidioidomycosis results from its high prevalence in areas of endemicity and from the severity of its clinical forms (7). It is estimated that 10 million people may be infected by *P. brasiliensis* in those areas, and up to 2% of them might develop the infection (15). Acute and subacute forms are found predominantly in children and young adults, and chronic forms predominate in infected adult men (21). The fungus grows as yeast at body temperature and as mycelium at 22 to 26°C. The mycelia produce conidia that differentiate into yeast cells when inhaled by the host, thus establishing the infection (12).

Members of the heat shock protein (HSP) family participate in several cellular processes, including acting as molecular chaperones (6, 11). In addition to their central role in transferring peptides through cells, HSPs are recognized as important molecules in the modulation of the immune system. Of the HSP family members, HSP60 has been shown to be a major immunodominant antigen in parasites and a target of the cell-mediated and humoral immune responses to infections (9). In fact, immune responses to HSPs have been reported in infectious diseases caused by bacteria, protozoa, and fungi and in models of experimental infection (5, 24, 25, 29). Vaccination using a *Histoplasma capsulatum* recombinant HSP60 induces a protective cellular immune response in experimental mice against intranasally administrated sublethal doses of fungal cells (10). HSP60 from the human-pathogenic fungus *Coccidioides immitis* triggers proliferation of T cells isolated from immunized mice (27). Furthermore, studies have suggested that antibodies to HSPs from microbes play an important role in protection against infection (14, 20). For instance, sera from patients with American cutaneous leishmaniosis reacted with the recombinant *Leishmania major* HSP60 (22).

Our laboratory is engaged in a program to identify immunogenic components of *P. brasiliensis.* Because HSPs are dominant and conserved antigens from several infectious agents, with a potential role in the interaction with the host, we focused our analysis on HSP60 of *P. brasiliensis.* We have previously reported the cloning and characterization of the *P. brasiliensis* HSP60 gene and its cDNA. The HSP60 gene from *P. brasiliensis* encodes a 62-kDa protein, a putative mitochondrial molecule as determined by its signal peptide. We also reported the reaction of native and recombinant glutathione *S*-transferase–HSP60 proteins to sera from infected patients (23). In the present study we report the expression and purification of the recombinant protein. The protein is recognized by an anti-HSP60 monoclonal antibody. We report the recognition of the recombinant purified HSP60 by a group of sera from 75 individuals with *P. brasiliensis* infection. In addition, we evaluated the reactivities of the purified HSP60 to sera from individuals with several other diseases.

MATERIALS AND METHODS

Expression of recombinant HSP60. An HSP60 cDNA clone was obtained by reverse transcription-PCR as described previously (23). In order to overproduce the *P. brasiliensis* HSP60, the cDNA obtained from isolate *P. brasiliensis* 01 (ATCC MYA-826) was cloned into the expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, Buckinghamshire, England). *Eco*RI and *Not*I restriction sites were introduced in the oligonucleotides prior to the cDNA synthesis. The expression construct pGEX-4T-3-HSP60 was introduced into *Escherichia coli* XL1-Blue. The cDNA was cloned in frame, as confirmed by sequencing, into the expression vector pGEX-4T-3, which gives a recombinant protein with a fusion to glutathione *S*-transferase. The synthesis of the fused recombinant

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protein (91 kDa) was induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

Purification of recombinant HSP60. Purification of the recombinant HSP60 from glutathione *S*-transferase was performed according to the instructions of the manufacturer (Amersham), with modifications. Bacterial extracts were prepared by growing cells to an absorbance of 0.6 at 600 nm. The final concentration of IPTG was 0.1 mM. The bacteria were pelleted and resuspended in phosphate-buffered saline (PBS) (50 μl of PBS for 1 ml of culture). The cells were incubated with lysozyme (100 μg/ml) at 4°C for 1 h. The IPTG-induced cells were extensively sonicated for 30 min at 4°C, and the cell lysate was filtered through 0.45-μm-pore-size nitrate filters. The recombinant protein was purified by affinity chromatography using glutathione-Sepharose 4B (Amersham). After unbound proteins were washed from the column with PBS, the fusion protein was cleaved by the addition of thrombin (50 U in 950 μl of PBS for each 1 ml of Sepharose). The reaction mixture was incubated for 16 h at room temperature, and the recombinant HSP60 was recovered. The protein concentration was measured by the Bradford protein assay (1). The proteins were analyzed on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel. The gels were stained with Coomassie blue (18) or transferred to nitrocellulose sheets.

Immunoblot assays. The proteins were subjected to electrophoresis and transferred to nitrocellulose membranes, as described previously (28). The membranes were blocked with Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.6]) containing 5% nonfat dry milk. The membranes were reacted with a mouse monoclonal antibody raised to a human recombinant HSP60 (H-3524; Sigma Aldrich, Inc., St. Louis, Mo.) or to human sera. The secondary antibodies were, respectively, anti-mouse immunoglobulin G (IgG) and anti-human IgG, both alkaline phosphatase coupled (Sigma). The reactions were developed with BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium).

Sera. A total of 121 serum specimens from patients with proven mycotic diseases (75 with paracoccidioidomycosis, 26 with histoplasmosis, 8 with sporotrichosis, 8 with aspergillosis, and 4 with cryptococcosis) were included in this study. Among the paracoccidioidomycosis serum specimens, 45 samples were from patients with the chronic form and 30 were from patients with the acute form of paracoccidioidomycosis. Forty-two control serum samples from healthy individuals and six samples from patients with confirmed tuberculosis, previously tested by immunodifusion against *P. brasiliensis* exocellular antigens, were also included in this sampling.

RESULTS

Overproduction and purification of the recombinant HSP60 from *P. brasiliensis*. *Eco*RI and *Not*I restriction sites covering the ATG and AUG codons, respectively, were created to join the cDNA to pGEX-4T-3 (23). The *E. coli* transformants accumulated the recombinant HSP60 mostly as inclusion bodies. For solubilization of the protein, treatment of the cell lysates with lysozyme and cell sonication were performed. The fusion protein was cleaved by addition of thrombin and purified using glutathione-Sepharose 4B. The eluted product migrated as a protein with an apparent molecular mass of 62 kDa (Fig. 1A, lane 2) that reacted in immunoblot assay with the anti-HSP60 monoclonal antibody (Fig. 1B, lane 2).

Immunological reactivity of recombinant HSP60 protein. Seventy-five serum samples from *P. brasiliensis*-infected patients were tested against 2.5 μg of the recombinant HSP60 protein. Of these 75 serum samples, 73 specimens (97.3%) recognized HSP60 protein by immunoblot assay (Table 1). Figure 2A presents the typical reaction of serum samples obtained from patients with the chronic form to the recombinant HSP60 (lanes 2 to12), and Fig. 2B (lanes 2 to 12) shows the reactivity of *P. brasiliensis* HSP60 to acute-phase serum samples obtained from symptomatic paracoccidioidomycosis patients. In both cases, strong reactivity was detected for the analyzed sera.

To evaluate the specificity of recombinant HPS60 in the immunoblot assay, we tested sera from negative controls and from patients with other mycoses and tuberculosis. A total of 4 serum specimens (9.52%) from the 42 controls were reactive to the recombinant HSP60, as shown in Table 1. Figure 2C presents the reactivities of some control sera to the recombinant HSP60. Most of the heterologous sera did not react with recombinant HPS60. Cross-reactivity was observed in only three serum samples (11.5%) from histoplasmosis patients. Figure 3 shows representative immunoblots for recombinant HPS60 probed with histoplasmosis (Fig. 3, lanes 2 to 4), aspergillosis (Fig. 3, lanes 5 to 7), cryptococcosis (Fig. 3, lanes 8 to 10), sporotrichosis (Fig. 3, lanes 11 to 13), and tuberculosis (Fig. 3, lanes 14 to 16) serum samples.

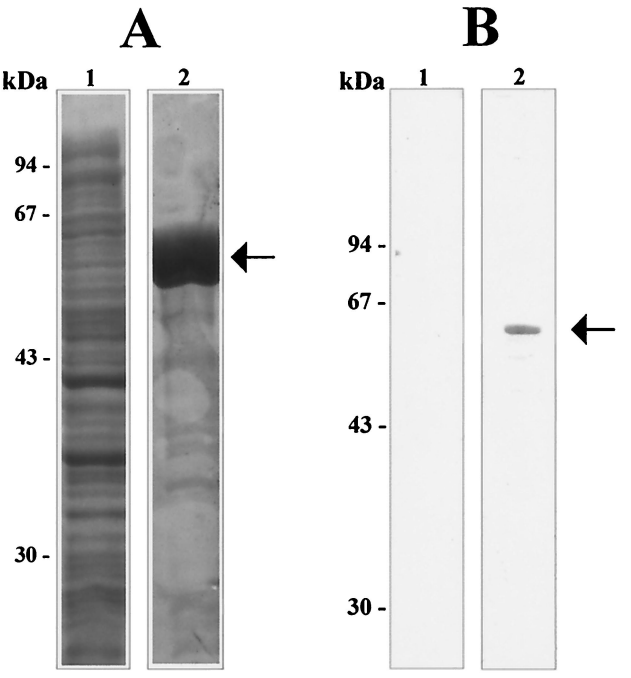

FIG. 1. SDS-PAGE and immunoblot analysis of the recombinant *P. brasiliensis* HSP60. *E. coli* XL1 Blue cells harboring the pGEX-4T-3 plasmid were grown at 30°C to an A_{600} of 0.6 and harvested before (lanes 1) and after (lanes 2) a 2-h incubation with 0.1 mM IPTG. The cells were concentrated by centrifugation and lysed by extensive sonication. After centrifugation, the supernatant was absorbed to a glutathione-Sepharose affinity column in the presence of thrombin for 16 h. The eluate was analyzed (lanes 2). (A) SDS-PAGE analysis. Lane 1, 25 μg of total protein; lane 2, 6 μg of purified recombinant HSP60. (B) Reaction to the monoclonal anti-HSP60 antibody. Lane 1, 25 μg of total protein; lane 2, 500 ng of purified HSP60. The arrows indicate the HSP60 recombinant protein.

TABLE 1. Nature of sera and reactivity with the recombinant HSP60

Patient group	No. of serum samples	No. of reactive samples
Paracoccidioidomycosis	75	73
Normal human serum	42	4
Histoplasmosis	26	3
Aspergillosis	8	0
Cryptococcosis	4	0
Sporotrichosis	8	0
Tuberculosis	6	0

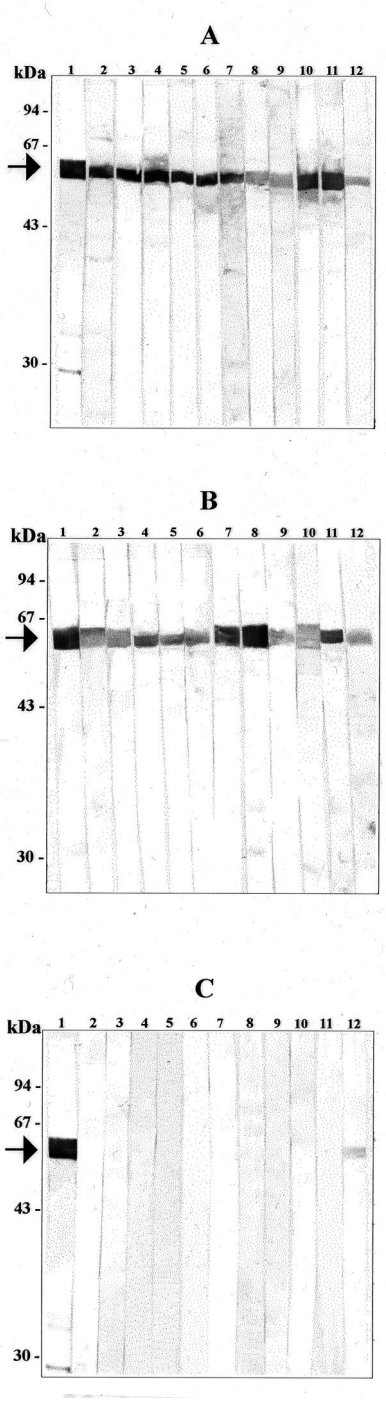

FIG. 2. Reactivities of individual serum samples from *P. brasiliensis*-infected patients to the recombinant HSP60 as determined by Western blotting. Recombinant purified HSP60 (2.5 μg) was fractionated on an SDS–15% polyacrylamide gel and transferred to a nitrocellulose membrane. (A and B) Reactivity to serum samples (at a 1:500 dilution) from *P. brasiliensis*-infected patients with chronic and acute disease, respectively. (C) Reactivity of human control sera. Alkaline phosphatase-conjugated anti-human IgG antibody was used at a dilution of 1:1,000. The reactions were developed with BCIP-NBT. Lanes 1, reaction of the purified *P. brasiliensis* HSP60 to the monoclonal antibody. Lanes 2 to 12, reaction to individual sera at a 1:500 dilution. The numbers on the left are molecular masses in kilodaltons. The arrows indicate the relative gel migration of the purified recombinant HSP60.

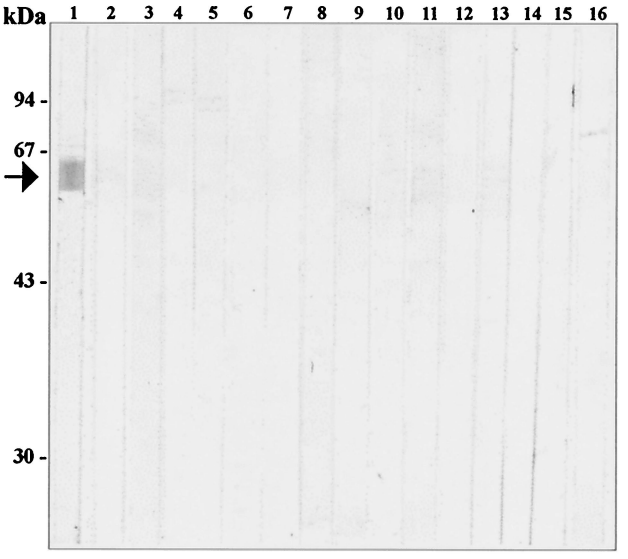

FIG. 3. Reactivities of sera from individuals with different diseases to the recombinant *P. brasiliensis* HSP60. Purified recombinant protein HSP60 (2.5 μg) was fractionated by one-dimensional gel electrophoresis (SDS–15% PAGE) and transferred to nitrocellulose membranes. Reactivities of serum samples from individuals with histoplasmosis (lanes 2 to 4), aspergillosis (lanes 5 to 7), cryptococcosis (lanes 8 to 10), sporotrichosis (lanes 11 to 13), and tuberculosis (lanes 14 to 16) are shown. Lane 1, reaction to the monoclonal anti-HSP60 antibody. The arrow indicates the relative migration of the purified recombinant HSP60.

DISCUSSION

We have described a system that permits overexpression of the *P. brasiliensis* HSP60 and allows efficient purification of the recombinant protein. We originally identified and characterized the HSP60 gene and cDNA from *P. brasiliensis*. In addition, the native HSP60 and the recombinant protein were efficiently recognized in immunoblots by sera from patients with paracoccidioidomycosis (23).

This study was performed to characterize the immunogenicity of the full-length recombinant purified HSP60. The detection of antibody by serological methods is very useful in the diagnosis of paracoccidioidomycosis (2, 3). However, the lack of antigen standardization may be a limitation (8, 17). Therefore, recombinant forms of purified proteins are required as an alternative reagent to replace the crude antigenic preparations.

Evidence is accumulating that HSPs serve as target antigens, and antibodies reactive to them have been found (14, 20, 22). When the recombinant purified HSP60 of *P. brasiliensis* was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblot assay with serum samples from paracoccidioidomycosis patients, reactivity was observed in 73 of the 75 serum samples analyzed, showing 97.3% sensitivity. The recombinant HSP60 did not react with sera from patients with aspergillosis, cryptococcosis, sporotrichosis, or tuberculosis. Therefore, cross-reactivity was demonstrated in a small number of healthy individuals (4 of 42). In addition, there was also a detectable IgG response in some sera from histoplasmosis patients (3 of 26). Since the HSPs are very conserved proteins and almost every human subject has been

confronted with microbial infection, it can be suggested that the positive reactions detected in the heterologous sera are directed against conserved regions of HSP60. Supporting this suggestion, we have found that the deduced amino acid sequence of *P. brasiliensis* HSP60 is 89% identical to that of the *H. capsulatum* protein (10, 23).

For diagnostic purposes, an immune response to *P. brasiliensis* HSP60 should be directed against nonhomologous epitopes. In this context, the finding of no cross-reactivity to sera from individuals with several diseases is relevant. In addition, the 60-kDa antigenic protein demonstrated 92.5% specificity for *P. brasiliensis*. The high frequency of HSP60 recognition (97.3%) by serum from patients with paracoccidioidomycosis and the high specificity suggest usefulness of this antigen in the serological diagnosis of paracoccidioidomycosis.

To our knowledge, this description is the third one related to recombinant antigens of *P. brasiliensis*. The exoantigen gp43 and the p27 protein have been characterized and are suitable molecules for the diagnosis of paracoccidioidomycosis (4, 16, 19, 26). Despite those descriptions, there is a paucity of purified cloned antigens. The characterization of new antigenic proteins and their heterologous production will allow a broader spectrum of molecules to be used in the diagnosis of paracoccidioidomycosis. In conclusion, the recombinant form of the antigen HSP60 of *P. brasiliensis* evaluated in this study shows IgG binding ability and may be of value for specific diagnosis of paracoccidioidomycosis. Further analysis of this recombinant protein in experimental animal models may shed new light on its role in the pathogenesis of the disease.

ACKNOWLEDGMENTS

This research was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and by grants AI-34361 and AI-42747 from the National Institutes of Health (to G.S.D.).

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