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

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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 leishmaniasis 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 (Amer sham Pharmacia Biotech, Buckinghamshire, England). EcoRI and NotI 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...
protein (62 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 incubated 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).

**RESULTS**

Overproduction and purification of the recombinant HSP60 from *P. brasiliensis.* EcoRI and NotI 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, lanes 1) and after 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.

**Immunological reactivity of recombinant HSP60 protein.** Seventy-five serum samples 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 sampling. 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).

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**B** 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).

**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                      |
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 forms of purified proteins are required as an alternative reagent to replace the crude antigenic preparations.

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**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).

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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, 25).

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.

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