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Cellular Immune Responses to Recombinant Heat Shock Protein 70 from *Histoplasma capsulatum*

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Heat shock protein (hsp) 70 from several microbes is antigenic in mammals. In this study we sequenced and expressed the gene encoding this protein from *Histoplasma capsulatum* to study its immunological activity. The deduced amino acid sequence of the gene demonstrated 71 and 76% identity to hsp70 from humans and *Saccharomyces cerevisiae*, respectively. A cDNA was synthesized by reverse transcription-PCR and was expressed in *Escherichia coli*. Recombinant protein reacted with a mouse monoclonal antibody raised against human hsp70. Splenocytes from C57BL/6 mice immunized with recombinant hsp70 emulsified in adjuvant, but not yeast cells, reacted in vitro to the antigen. Recombinant hsp70 elicited a cutaneous delayed-type hypersensitivity response in mice immunized with protein or with viable yeast cells. Mice were injected with recombinant hsp70 and challenged intranasally with a sublethal inoculum of yeast cells. Vaccination did not confer protection in this model. Thus, recombinant hsp70 can induce a cell-mediated immune response but does not induce a protective response.

Heat shock proteins (hsp) are produced when a cell is confronted with a sudden change in temperature or with other forms of stress. hsp are separated by molecular mass into families of proteins, and the amino acid sequences of each family are strongly conserved. In mammalian cells, hsp70 is one of the most abundant hsp (22). This family plays a major role in the folding, unfolding, and translocation of polypeptides. hsp70 is involved in the unfolding of cytoplasmic proteins and their transport into other cellular compartments. Once inside such a compartment, hsp70 facilitates refolding of the proteins (15).

There is ample evidence that hsp70 is involved in immunity. This protein has been demonstrated to elicit humoral responses during natural infection with various microbes. Anti-hsp70 antibodies have been detected in a significant number of patients with chronic schistosomiasis, malaria, and leishmaniasis (12, 14, 16). Besides its role in humoral immune response, hsp70 from *Plasmodium falciparum*, *Schistosoma mansoni*, and *Mycobacterium* spp. has been shown to induce a cell-mediated immune response (2, 12, 23). hsp70 from a variety of pathogens leads to activation of both $CD4^+$ and $CD8^+$ T cells (1, 3, 5, 19).

Studies of hsp70 from the thermally dimorphic fungus *Histoplasma capsulatum* have been limited. The focus of these studies has been on the expression of hsp70 during the conversion of mycelia to the yeast phase since this is a temperature-dependent phenomenon (20). When mycelia are exposed to elevated temperatures (34 to 40°C), there is intense synthesis of hsp70. Whether this expression is part of the process of differentiation or a reflection of the ability of the fungus to adapt to new environmental conditions remains unclear. Expression of the hsp70 gene from *H. capsulatum* varies among strains that differ in pathogenicity and thermotolerance; that is, maximal hsp70 mRNA transcription occurs in nonvirulent strains at 34°C and in virulent strains at 37°C (7). Studies at the protein level revealed similar results (20). Therefore, hsp70 is an important contributor to the phase transformation of this fungus. Although hsp70 appears to play a prominent role in morphogenesis of *H. capsulatum*, there is little information about its immunological activity.

Therefore, in this study we sought to determine if recombinant hsp70 (rhsp70) participates in the cell-mediated immune response to infection with *H. capsulatum*. The gene encoding hsp70 from *H. capsulatum* had been isolated and cloned (7), and we have sequenced and expressed the gene to study its immunobiological functions. The deduced amino acid sequence was found to be highly homologous with hsp70 members from other species. The recombinant protein was recognized by splenocytes from mice immunized with rhsp70 but not viable yeast cells. Moreover, it induced a delayed-type hypersensitivity (DTH) response in vivo. However, vaccination with the recombinant protein did not protect mice against a sublethal intranasal challenge from *H. capsulatum*. Thus, rhsp70 is antigenic but does not mediate protection.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice were obtained from Jackson Laboratory, Bar Harbor, Maine. All studies were done in accordance with the Animal Welfare Act and guidelines of the National Institutes of Health.

***H. capsulatum* and injection of yeast cells.** The strain of *H. capsulatum* used in this study was G217B. Yeast cells were maintained by biweekly subculture into brain heart infusion agar slants (Gibco BRL, Grand Island, N.Y.) at 37°C in 5% CO_2. For each experiment, a loopful of yeast cells was removed from the agar slant and grown in Ham's F12 (Gibco BRL) medium, pH 7.5, supplemented with glucose (18.2 g/liter), glutamic acid (1 g/liter), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (6 g/liter), and cysteine (8.4 g/liter) for 36 h at 37°C at a gyratory speed of 200 rpm. The cells were harvested and washed three times in Hanks' balanced salt solution. To immunize mice with viable *H. capsulatum* yeast cells, animals were injected subcutaneously with 10^6 yeast cells and then injected intravenously with 6×10^5 yeast cells 2 weeks later. After an additional 2 weeks, mice received an intraperitoneal injection of 5×10^6 yeast cells. In studies of protective immunity, mice were anesthetized via inhalation of methoxyflurane (Pitman-Moore Inc., Mundelein, Ill.) and then challenged intranasally with 2.5×10^6 cells by the placement of 50 μl of the yeast cell suspension into the anterior nares.

Plasmids and bacteria. The *H. capsulatum* hsp70 gene was cloned into the pBR328 vector, generating the clone pMB12 (7). The gene was excised from pMB12 with *Pvu*II and ligated into the *Eco*RV site of pBluescript SK− (Stratagene Corp., La Jolla, Calif.). The plasmid was transformed into *Escherichia coli*

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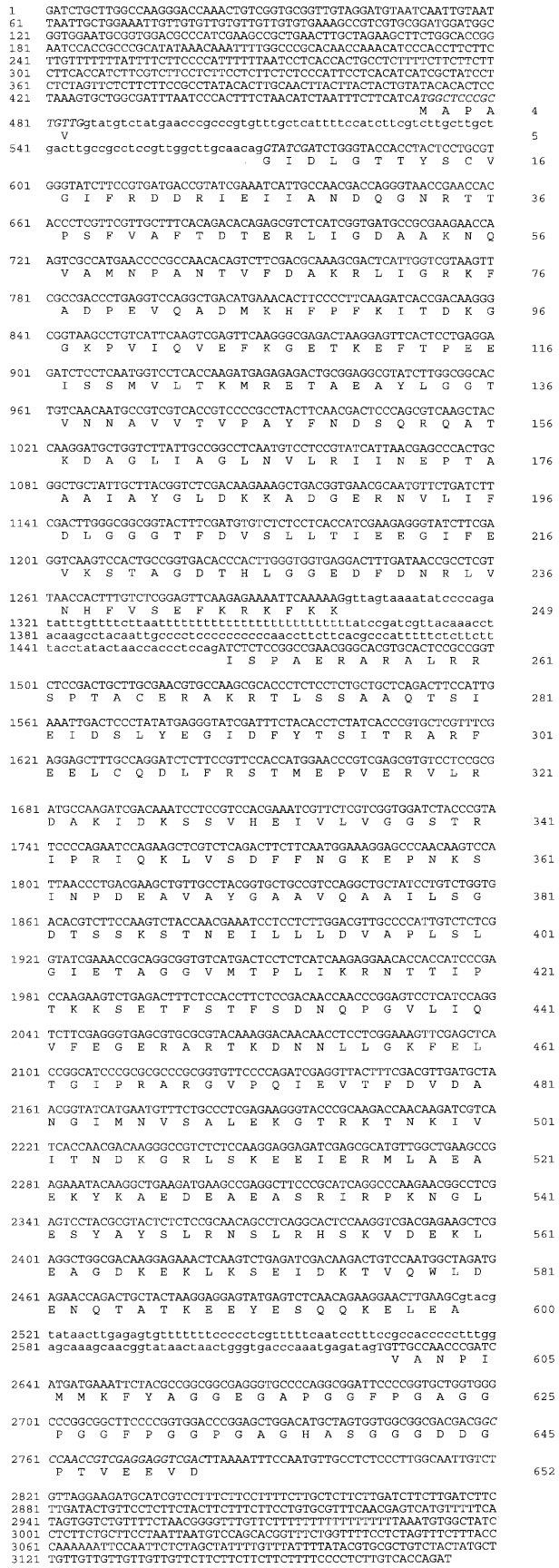

XL1-Blue (Stratagene). The pET19b vector (Novagen Inc., Madison, Wis.) was used for the expression of recombinant protein in *E. coli* BL21(DE3).

Gene sequence analysis. Single-stranded plasmid sequencing was performed by the dideoxy chain termination method with the Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio). Both strands of the cloned gene were sequenced by the strategy of "primer walking." Oligonucleotides (21-mer) were synthesized and used to initiate the sequencing reaction.

Generation of an intron-free gene by using reverse transcription-PCR. A cDNA of the hsp70 gene was generated by reverse transcription-PCR with 5 μg of total *H. capsulatum* G217B RNA prepared as described previously (10). The first-strand cDNA was then amplified by PCR with a sense primer located at the ATG start codon of the gene; the sequence of this primer was 5′-GCTCTAGA CATATGGCTCCCGCTGTTGGTATCGA-3′. The antisense primer used was located at the carboxy terminus, and the sequence of this primer was 5′-CGGG ATCCTCAGTCGACCTCCTCGACGGTTGGGC-3′. A 5′ *Nde*I site was added to the amino terminus primer and a *Bam*HI site was added to the carboxy terminus primer (both underlined) to facilitate cloning into pBluescript SK−. A stop codon was present 3′ of the *Bam*HI site in the carboxy terminus antisense primer. PCR was performed for 35 cycles of 94°C for 45 s, 48°C for 45 s, and 72°C for 2 min with 5 U of Vent polymerase (New England Biolabs, Beverly, Mass.). The PCR product was cloned into pBluescript SK−, restriction mapped, and sequenced to confirm the fidelity of the DNA transcription.

Protein expression. To generate recombinant protein, the intron-free *H. capsulatum* hsp70 gene was excised from pBluescript SK− by digestion with *Nde*I and *Bam*HI. The gene product was gel purified and cloned into the *Nde*I and *Bam*HI sites of pET19b. The plasmids were transformed into BL21(DE3) lysogen host cells.

To express the recombinant protein, transformed *E. coli* cells (500 ml) were grown in Luria-Bertani medium at 37°C in a shaking incubator until an optical density at 600 nm of 0.6 was reached. Subsequently, isopropylthiogalactose was added to cultures to a final concentration of 1 mM, and the cultures were incubated for an additional 2 to 3 h. Cells were harvested by centrifugation at 5,000 × *g*. *E. coli* pellets were resuspended in a buffer consisting of 5 mM imidazole, 500 mM NaCl, and 20 mM Tris-Cl (pH 7.9) and lysed by a freeze-thaw cycle followed by sonication. Soluble and insoluble fractions were separated by centrifugation at 20,000 × *g*. The insoluble pellet fraction was found to contain the recombinant product when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Metal chelate purification of rhsp70. pET19b adds 10 histidines to the NH_2 terminus. This modification allows purification of recombinant protein on nickel columns. The insoluble pellet fraction was resuspended by sonication in a denaturing solubilization buffer consisting of 6 M urea, 500 mM NaCl, 5 mM imidazole, and 20 mM Tris (pH 7.9). Solubilized material was recovered in the supernatant after centrifugation at 20,000 × *g*, and the supernatant was filtered through a 0.22-μm-pore-size cellulose acetate membrane. The material was then loaded into an Ni^{2+}-Sepharose affinity column (His-Bind; Novagen) and washed with 20 mM imidazole, and the recombinant product was eluted with 1 M imidazole in a buffer containing 500 mM NaCl and 20 mM Tris (pH 7.9). Eluted material was dialyzed against 20 mM Tris (pH 7.4)–200 mM NaCl containing decreasing concentrations of urea. The eluate was concentrated by ultrafiltration. For the removal of endotoxin, the protein solution was passed over a polymyxin B affinity chromatography column (Pierce Chemical Co., Rockford, Ill.). The protein concentration was determined by the Bradford method (4).

Western blot (immunoblot) analysis and electrophoresis of rhsp70. Five micrograms of rhsp70 was electrophoresed by SDS–12% PAGE, electroblotted overnight to nitrocellulose membranes, and stained with Ponceau S. Nitrocellulose lanes were cut and incubated in 25 mM Tris-buffered saline, pH 7.5, containing 5% powdered milk for 1 h at room temperature. Lanes were incubated overnight with a 1:1,000 dilution of monoclonal antibody (MAb) N27F3-4 (mouse immunoglobulin G1) at 4°C overnight or, as a control, an equal dilution of anti-human HLA-DR MAb (mouse immunoglobulin G1) (Becton Dickinson, Sunnyvale, Calif.). The strips were washed twice with Tris-buffered saline containing 0.05% Tween 20 for 10 min and incubated for 2 h with 2 ml of 1:1,000-diluted antimouse peroxidase-labeled secondary antibody (Kirkegaard & Perry, Gaithersburg, Md.) at room temperature. Strips of nitrocellulose were washed twice with Tris-buffered saline–Tween and developed with 0.6 mg of 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, Calif.) per ml and hydrogen peroxide (0.015%).

Immunization of mice with rhsp70. Groups of mice were immunized subcutaneously with 200 μg of rhsp70 suspended in an emulsion containing monophosphoryl lipid A, synthetic trehalose dicorynomycolate, and cell wall skeleton (MPL-TDM-CWS) (Ribi ImmunoChem Research, Inc., Hamilton, Mont.). Two doses were given separated by 2 weeks. Concurrently, controls received bovine serum albumin (BSA) emulsified in MPL-TDM-CWS.

FIG. 1. Gene sequence and deduced amino acid sequence of hsp70 antigen. Introns are indicated by lowercase letters. Base numbers are on the left, and amino acid numbers are on the right. The sense and antisense primers used to amplify first-strand cDNA are italicized.

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Hc          MA--PAVGIDLGTTYSCVGIFRDDRIEIIANDQGNRTTPSFVAFTDTERL          48
Sc          -S--K.............AH.AN..VD.......................
Hu          .SKG..............V.QHGKV.............Y.........

Hc          IGDAAKNQVAMNPANTVFDAKRLIGRKFADPEVQADMKHFPFKITDKGGK          98
Sc          ........A....S............N.N..............LI..D..
Hu          .............T............R.D.AV..S....W..MVVNDA.R

Hc          PVIQVEFKGETKEFTPEEISSMVLTKMRETAEAYLGGTVNNAVVTVPAYF         148
Sc          .Q..........N....Q......G..K....S...AK..D.........
Hu          .KV...Y.....S.Y...V........K.I......K..T..........

Hc          NDSQRQATKDAGLIAGLNVLRIINEPTAAAIAYGLDKKADGERNVLIFDL         198
Sc          ............T.........................GKE.H-......
Hu          ............T.........................VGA.........

Hc          GGGTFDVSLLTIEEGIFEVKSTAGDTHLGGEDFDNRLVNHFVSEFKRKFK         248
Sc          ..........S..D......A.....................IQ.....N.
Hu          ........I....D.......................M....IA.....H.

Hc          KISPAERARALRRSPTACERAKRTLSSAAQTSIEIDSLYEGIDFYTSITR         298
Sc          .DLSTNQ-.....LR.................V.....F...........
Hu          .DISENK-..V..LR............ST.A...................

Hc          ARFEELCQDLFRSTMEPVERVLRDAKIDKSSVHEIVLVGGSTRIPRIQKL         348
Sc          .......A......L....K......L...Q.D............KV...
Hu          ......NA....G.LD...KA.....L...QIHD...........K....

Hc          VSDFFNGKEPNKSINPDEAVAYGAAVQAAILSGDTSSKSTNEILLLDVAP         398
Sc          .T.Y.......R.....................T..E...-.QDL.......
Hu          LQ.......L.........................K.EN-VQDL.....T.

Hc          LSLGIETAGGVMTPLIKRNTTIPTKKSETFSTFSDNQPGVLIQVFEGERA         448
Sc          .............K..P..S........I...YA.................
Hu          .............V............QTQT.T.Y...........Y.....

Hc          RTKDNNLLGKFELTGIPRA-RGVPQIEVTFDVDANGIMNVSALEKGTRKT         497
Sc          K............S...P.P.............S...L....V....G.S
Hu          M................P.P...........I.....L....VD.S.G.E

Hc          NKIVITNDKGRLSKEEIERMLAEAEKYKAEDEAEASRIRPKNGLESYAYS         547
Sc          ...T...........D..K.V.....F.E...K.SQ..AS..Q...I...
Hu          ...T...........D....VQ..........KQRDKVSS..S.....FN

Hc          LRNSLRHSKVDEKLEAGDKEKLKSEIDKTVQWLDENQTATKEEYESQQKE         597
Sc          .K.TI--.EAGD...QA..DTVTKKAEE.IS...S.T..S...FDDKL..
Hu          MKATVEDE.LQG.INDE..Q.ILDKCNEIIN...K....E...F.H....

Hc          LEAVANPIMMKFYAGGEGAPGGFPGAGGPGGFPGGPGAGHASGGGDDGPT         647
Sc          .QDI.....S.L.QA.-.....-AAG.A.......A----PPAPEAE...
Hu          ..------------------------------------------------

Hc          VEEVD                                               652
Sc          .....
Hu          -----
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FIG. 2. Alignment of amino acid sequence of *H. capsulatum* (Hc) hsp70 with sequences of human (Hu) and *S. cerevisiae* (Sc) hsp70. The alignment was performed by using PC/Gene (Intelligenetics). Dots indicate identical amino acids. Dashes indicate end of sequence. Amino acid numbers are on the right.

Splenocyte preparation and proliferation assay. Spleen cells from immunized and normal mice were removed aseptically and teased apart between two ground-glass slides, and the cell suspension was washed three times in balanced salt solution. To each well of a 96-well microtiter plate were added 4×10^5 splenocytes in 0.2 ml of RPMI 1640 containing 10% fetal bovine serum and 10 μg of gentamicin per ml. Decreasing concentrations of rhsp70 were added to the cells in a volume of 50 μl, and plates were incubated for 144 h at 37°C in 5% CO_2. Sixteen hours before cell harvest 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.) was added to each well. Cells were collected on glass fiber filters with a semiautomated harvester (Skatron Inc., Sterling, Va.), and uptake of radioactivity was measured by liquid scintillation. Proliferative responses were considered positive when the response of cells incubated with antigen was greater than or equal to three times that of cells incubated with medium alone.

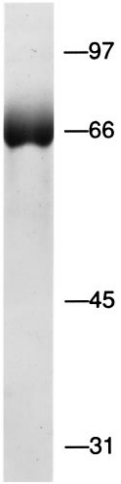

FIG. 3. SDS–12% PAGE gel of purified rhsp70 stained with Coomassie blue. Molecular weight markers (in thousands) are on the right.

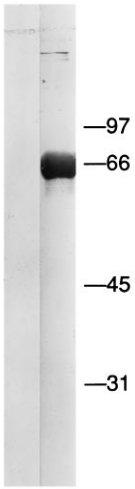

FIG. 4. Western blot analysis of hsp70. Five micrograms of hsp70 was electrophoresed in a 12% polyacrylamide gel and electroblotted to nitrocellulose. Lanes were excised and reacted with anti-HLA-DR (left panel) or anti-hsp70 (right panel). Molecular weight markers (in thousands) are on the right.

Induction and measurement of DTH responses to rhsp70. Groups of six mice that had been immunized with viable yeast cells or with rhsp70 were challenged intradermally with 1 μg of the antigen in a volume of 0.05 ml. Footpad swelling was measured 24 h later with a digital micrometer. The DTH response was expressed as the percent increase in footpad size over that measured immediately before antigen challenge. As a control, DTH was measured both in age-matched control animals that had been injected with an equal volume of buffer emulsified in adjuvant and in animals that had not been injected.

Organ culture of *H. capsulatum*. Lungs, spleens, and livers from groups of six mice each were removed aseptically and homogenized individually by a Teflon tissue grinder in 10 ml of sterile saline supplemented with 10 μg of gentamicin per ml. Homogenates were serially diluted, and 0.1 ml of each dilution was plated in duplicate onto brain heart infusion agar (2% agar) containing 1% dextrose, 0.01% cysteine hydrochloride, 10 μg of gentamicin per ml, and 5% defibrinated sheep erythrocytes. Cultures were incubated at 30°C for 7 to 10 days, and the number of colonies was determined.

Statistics. The Student's *t* test was used to compare groups. A P value of <0.05 was considered significant.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence data reported in this paper is U46464.

RESULTS

Cloning and sequencing of hsp70. The hsp70 gene was excised from its original plasmid, pMB12, by digestion with *Pvu*II and subcloned into the *Eco*RV site of pBluescript SK−. The gene encoding hsp70 was sequenced in its entirety. The gene sequence and the deduced amino acid sequence are illustrated in Fig. 1. On the basis of the homology of the sequence to hsp70 from several sources, three putative introns were noted. The identity of these introns was confirmed by reverse transcription-PCR and sequence analysis of the resultant cDNA. The gene encodes a protein of 652 amino acids.

Homology of *H. capsulatum* hsp70 to other members of the hsp70 family. The deduced amino acid sequence of hsp70 from *H. capsulatum* was aligned with hsp70 from *Saccharomyces cerevisiae* and human samples (Fig. 2). The identity was 76% with *S. cerevisiae* hsp70 and 71% with human hsp70.

Expression of hsp70 antigen. A cDNA was synthesized by reverse transcription-PCR and cloned into the *Nde*I and *Bam*HI sites of pET19b. The plasmid was transformed into *E. coli*. Clones were picked and restriction mapped to verify fidelity. One clone (phsp) was chosen for expression. The re-

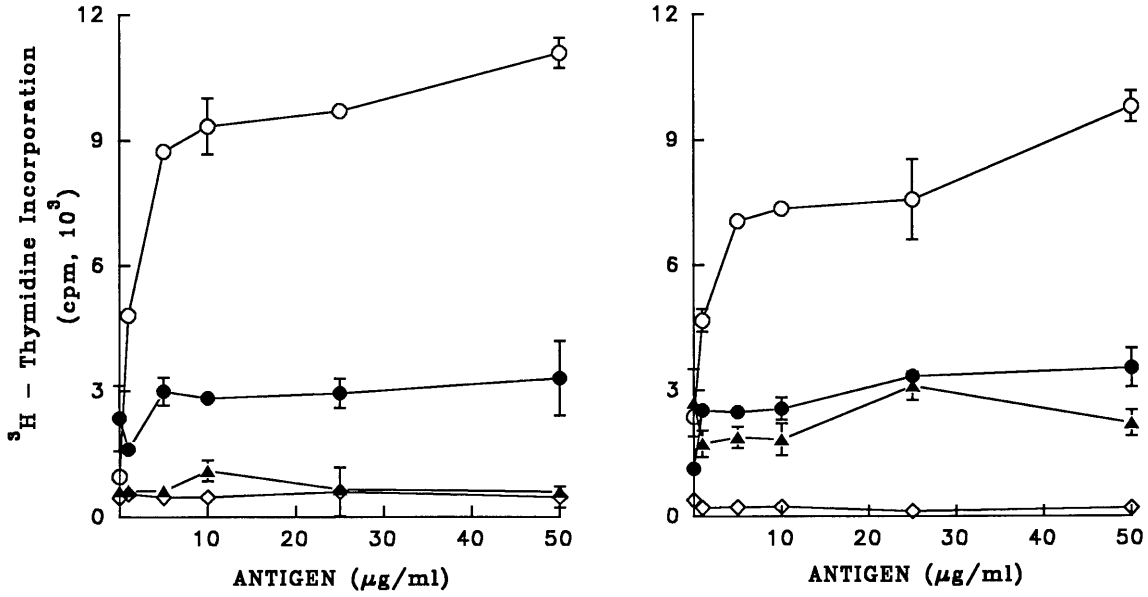

FIG. 5. Splenocytes from hsp70-immunized mice respond to hsp70. C57BL/6 mice were immunized with 400 μg of rhsp70 (○) or BSA (●) suspended in MPL-TDM-CWS, and proliferation was measured 14 days later. Splenocytes from mice immunized with *H. capsulatum* (▲) or unimmunized splenocytes (◇) do not respond to hsp70. Data are the means ± standard deviations of the means of triplicate measurements. Results are shown for two of five experiments.

combinant protein was purified by metal chelate affinity chromatography and analyzed for purity by SDS-PAGE. A single band of approximately 70 kDa was found (Fig. 3). To confirm that the expressed protein was hsp70, 5 μg of the recombinant protein was electroblotted to nitrocellulose and reacted with MAb N27F3-4, which recognizes both the constitutive and inducible forms of human hsp70 (11), or with the isotype-matched MAb which recognizes HLA-DR. The immunoblot, shown in Fig. 4, demonstrates that the protein was immunoreactive with the specific antibody but not with control antibody.

In vitro cellular immune activity of rhsp70. Since others have reported that hsp70 stimulates antigen-reactive T cells, we sought to determine if the recombinant protein was a target of the cellular immune response. C57BL/6 mice were immunized with either viable *H. capsulatum* yeast cells or recombinant antigen, and spleen cells from these animals were tested for their capacity to respond in vitro to rhsp70. Splenocytes from animals immunized with the recombinant protein proliferated in response to the antigen, whereas splenocytes from animals injected with BSA did not. Spleen cells from mice immunized with viable yeast cells and unimmunized control animals failed to mount a proliferative response to rhsp70 (Fig. 5).

Induction of a DTH response in mice immunized with viable yeast cells. Mice were injected with either yeast cells, 200 μg of recombinant antigen, or 200 μg of BSA in adjuvant. Another group of mice remained unimmunized. One day after injection of 1 μg of rhsp70 into the footpad of mice, the DTH response to the antigen was measured. The DTH response (mean ± standard deviation) of animals injected with viable yeast cells (17.5% ± 5.9%) or with recombinant antigen (15.3% ± 5.4%) was significantly ($P \leq 0.01$) greater than that of animals injected with BSA (1.4% ± 1.9%) and that of uninjected mice (1% ± 2.5%).

Course of histoplasmosis in mice immunized with rhsp70. It is generally acknowledged that cell-mediated immunity is the principal host defense mechanism in response to *H. capsulatum*. Therefore, we tested whether immunization with rhsp70 could protect mice from a sublethal inoculum. Mice were injected with either recombinant antigen or an equal amount of BSA emulsified in adjuvant. Two weeks after immunization, the animals were challenged intranasally with a sublethal inoculum containing 2.5×10^6 yeast cells. At 1, 2, and 3 weeks of infection, mice were sacrificed, and the number of CFU in lungs, spleens, and livers was quantified. The number of organisms recovered from the organs at all time periods did not differ significantly ($P > 0.05$) between the group immunized with rhsp70 and the controls (Fig. 6). Thus, rhsp70 did not elicit a protective immune response in this model of pulmonary histoplasmosis.

DISCUSSION

Our laboratory has been engaged in identifying antigens from the pathogenic fungus *H. capsulatum* that elicit a cellular immune response and/or protection. In recent years, it has become clear that members of the hsp families, mainly hsp60 and hsp70, are major antigens of pathogens that trigger an immune response (13). hsp are produced by prokaryotic and eukaryotic cells in response to a variety of stress stimuli, including heat shock and phagocytosis (6). We had previously cloned the hsp70 gene from *H. capsulatum* in an attempt to elucidate the possible role of hsp70 in morphogenesis (7). In this study, we have sequenced and expressed the gene and studied its immunological properties. Sequence analysis of the gene revealed that it has high degrees of identity to hsp70 from *S. cerevisiae* (76%) and human hsp70 (71%). When one compares these three sequences, it appears that the C terminus is hypervariable, while other regions seem more dramatically conserved. These data extend the scope of hsp70 sequences and confirm the extreme conservation in the sequence throughout evolution and across species.

T cells are activated and proliferate in response to hsp70, as has been demonstrated in *Mycobacterium bovis* bacillus Calmette-Guérin vaccines as well as in leprosy and tuberculosis

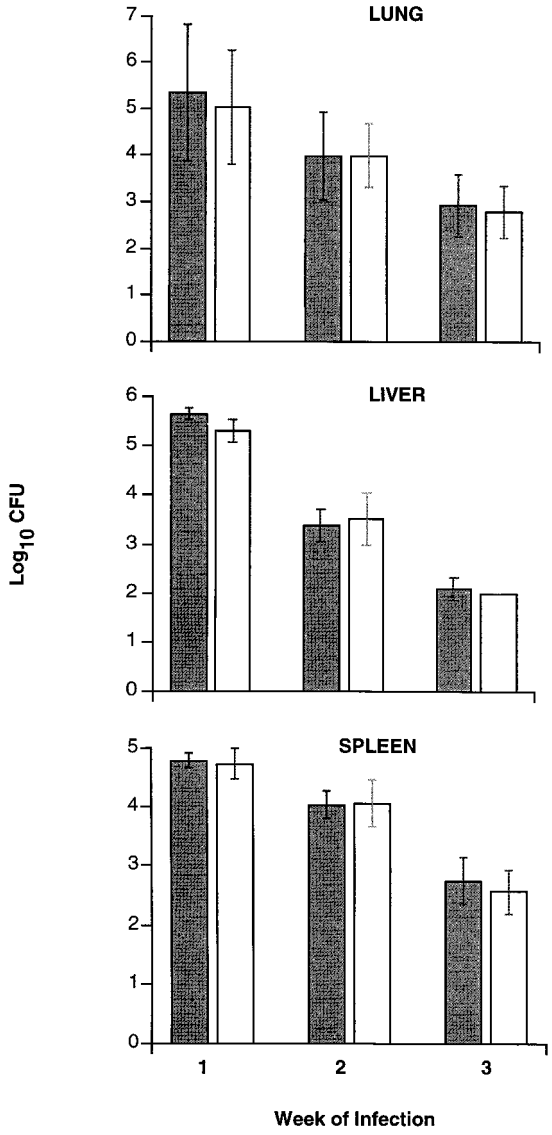

FIG. 6. Recovery of *H. capsulatum* from lungs, livers, and spleens of mice immunized with rhsp70. Groups of C57BL/6 mice ($n = 5$) immunized with 400 μg of rhsp70 (open bars) or with an equal amount of BSA (shaded bars) suspended in MPL-TDM-CWS were infected with 2.5×10^6 yeast cells intranasally. At weeks 1, 2, and 3 of infection CFU detected in organs were enumerated. Data are the means ± standard deviations of the means for five individual mice at each time point.

patients (18), whose T cells react with hsp70. In this study, we sought to determine if hsp70 from *H. capsulatum* could stimulate a cell-mediated immune response since this arm of immunity appears to be critical in host resistance to this fungus. Splenocytes from mice immunized with the recombinant antigen proliferated in vitro in response to rhsp70, whereas splenocytes from animals immunized with viable yeast cells did not. This is not overly surprising, since we had previously reported that native hsp70 from *H. capsulatum* failed to stimulate proliferation of a $CD4^+$ T-cell line (11). However, rhsp70 elicited a cutaneous DTH response in mice immunized with viable yeast cells or with antigen. These findings emphasize the complex nature of measuring cell-mediated immune responses since yeast-immunized mice reacted in vivo to antigen but cells from these animals did not recognize antigen. The reason(s) for this discrepancy is not known. It is possible that the handling of antigen by different populations of antigen-presenting cells alters presentation and recognition.

The number of *H. capsulatum* CFU in lungs, livers, and spleens of mice immunized with rhsp70 was not substantially different from that of control mice. Why immunization with the recombinant antigen did not elicit a protective immune response is unclear. One concern is that the function of an antigen from *H. capsulatum* or any eukaryote expressed in a prokaryote may not resemble that of the native protein. Bacteria may not fold eukaryotic proteins properly or may not modify them normally posttranslation. These subtle changes may alter the immunogenicity of the molecule. Consequently, potential immunogens may be discarded unnecessarily.

rhsp70 does not contain N glycosylation sites, and care was taken to renature it in a conformation similar, if not identical, to that of the native protein. It seems unlikely that the recombinant protein did not exert its immunological function simply because it was expressed in bacteria. The recombinant *H. capsulatum* antigens hsp60 and H, both expressed in pET19b, stimulated lymphocytes from yeast-immunized mice. Moreover, rhsp60 conferred protective immunity when used as a vaccine (10). Thus, in general, prokaryotic expression is a reasonable means by which to produce and test the activity of eukaryotic proteins. The lack of efficacy of a given recombinant protein expressed in bacteria does not necessarily indicate that it does not induce an immune response. Rather, expression in *Pichia pastoris* or insect cells with baculovirus may be alternatives that need to be explored. Studies are under way to express hsp70 in *P. pastoris.*

rhsp70 is the second antigen from *H. capsulatum* which does not elicit a protective response despite being antigenic. We have reported that H antigen from this fungus evokes cellular immune responses in mice, yet it fails to vaccinate animals against histoplasmosis (9). In contrast, rhsp60 from *H. capsulatum* stimulates cellular immune responses and protects mice against a lethal intranasal challenge (10). Thus, a dichotomy has been established: three antigens can prompt a cell-mediated immune response, yet only one of them confers protection. These results provide evidence that the presence of DTH does not precisely correlate with the expression of protective immunity despite the classical association of these two entities.

The apparent functional differences among *Histoplasma* antigens support the concept that linear amino acid sequences are critical in determining the protective efficacy of an antigen. Since T cells and cytokines are pivotal in clearance of the fungus (8, 21), the capacity of an *H. capsulatum* antigen to mediate protection may depend on qualitative and quantitative differences in host response. That is, a protective antigen may cause outgrowth of a T-cell receptor family that is essential for protection. Alternatively, the protective antigen may generate a different profile of cytokines or it may stimulate the release of levels of cytokines that are necessary for elimination of the organism.

We had previously demonstrated that a detergent extract of cell walls and cell membranes from *H. capsulatum* yeast cells (termed CW/M) reacted with two MAb to hsp70, and we subsequently isolated a protein of approximately 80 kDa, His-80 (11). This protein was analyzed for homology to hsp70 and tested for its antigenicity and immunogenicity. His-80 reacted with MAb to hsp70, and the amino acid sequence at the amino terminus revealed that it was highly homologous to the hsp70 family. A murine T-cell line proliferated in vitro in response to His-80, but not in response to a purified hsp70 from *H. capsulatum*. In contrast to the results of the current study, the 80-kDa antigen induced a cellular immune response

and conferred protection against a sublethal inoculum of *H. capsulatum* (11). These findings suggest that members of the hsp70 family, despite their enormous conservation in amino acid sequences, may differ in their immunological properties. Therefore, slight differences in amino acid sequence appear to make substantial differences in the immune response. Thus, His-80 seems to be a member of the hsp70 protein family but it is not identical to hsp70. On the basis of its molecular weight and its homology to the amino terminus of hsp70, His-80 may be a member of the BiP family (17).

In summary, we have sequenced and expressed the gene encoding hsp70 from *H. capsulatum* yeast cells. We have demonstrated that the recombinant protein is antigenic but does not confer protection. Although rhsp70 did not vaccinate mice, it is quite possible that it amplifies the immune response during genesis of protective immunity.

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REFERENCES

1. **Adams, E., R. J. Garsia, L. Hellqvist, A. Basten, and A. S. Inglis.** 1990. T cell reactivity to the purified mycobacterial antigens p65 and p70 in leprosy patients and their household contacts. Clin. Exp. Immunol. **80:**206–212.
2. **Ardeshir, F., J. E. Flint, J. Richman, and R. T. Reese.** 1987. A 75 kd merozoite surface protein of *Plasmodium falciparum* which is related to the 70 kd heat-shock proteins. EMBO J. **6:**493–499.
3. **Behr, C., J. L. Sarthou, C. Rogier, J. F. Trape, M. H. Dat, J. C. Michel, G. Aribot, A. Dieye, J. M. Claverie, P. Druihle, and P. Dubois.** 1992. Antibodies and reactive T cells against the malaria heat shock protein Pf72/Hsp70-1 and derived peptides in individuals continuously exposed to *Plasmodium falciparum*. J. Immunol. **149:**3321–3330.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Britton, W. J., L. Hellqvist, P. Holt, and A. Basten.** 1986. Immunoreactivity of a 70kDa antigen from *Mycobacterium bovis* Bacillus Calmette Guerin purified by monoclonal antibody affinity chromatography. J. Exp. Med. **164:** 695–708.
6. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science **248:**730–732.
7. **Caruso, M., M. Sacco, G. Medoff, and B. Maresca.** 1987. Heat shock 70 gene is differentially expressed in *Histoplasma capsulatum* strains with different levels of thermotolerance and pathogenicity. Mol. Microbiol. **1:**151–158.
8. **Deepe, G. S., Jr.** 1988. Protective immunity in murine histoplasmosis: functional comparison of adoptively transferred T-cell clones and splenic T cells. Infect. Immun. **56:**2350–2355.
9. **Deepe, G. S., Jr., and G. G. Durose.** 1995. Immunobiological activity of recombinant H antigen from *Histoplasma capsulatum*. Infect. Immun. **63:** 3151–3157.
10. **Gomez, F. J., R. Allendoerfer, and G. S. Deepe, Jr.** 1995. Vaccination with recombinant heat shock protein 60 from *Histoplasma capsulatum* protects mice against pulmonary histoplasmosis. Infect. Immun. **63:**2587–2595.
11. **Gomez, F. J., A. M. Gomez, and G. S. Deepe, Jr.** 1992. An 80-kilodalton antigen from *Histoplasma capsulatum* that has homology to heat shock protein 70 induces cell-mediated immune responses and protection in mice. Infect. Immun. **60:**2565–2571.
12. **Hedstrom, R., J. Culpepper, R. A. Harisson, N. Agabian, and G. Newport.** 1987. A major immunogen in *Schistosoma mansoni* infections is homologous to the heat shock protein HSP70. J. Exp. Med. **165:**1430–1435.
13. **Kaufmann, S. H. E.** 1990. Heat shock proteins and the immune response. Immunol. Today **11:**129–136.
14. **Kumar, N., Y. Zhao, P. Graves, J. P. Folgar, L. Maloy, and H. Zheng.** 1990. Human immune response directed against *Plasmodium falciparum* heat shock-related proteins. Infect. Immun. **58:**1408–1414.
15. **Lindquist, S., and E. A. Craig.** 1988. The heat shock proteins. Annu. Rev. Genet. **22:**631–677.
16. **MacFarlane, J., M. L. Blaxter, R. P. Bishop, M. A. Miles, and J. M. Kelly.** 1990. Identification and characterisation of a *Leishmania donovani* antigen belonging to the 70-kDa heat-shock family. Eur. J. Biochem. **190:**377–384.
17. **Munro, S., and H. R. B. Pelham.** 1986. An hsp70-like protein in the ER: identity with the 78kDa glucose regulated protein and immunoglobulin heavy chain binding protein. Cell **46:**291–300.
18. **Mustafa, A. S., K. E. A. Lundin, and F. Oftung.** 1993. Human T cells recognize mycobacterial heat shock proteins in the context of multiple HLA-DR molecules: studies with healthy subjects vaccinated with *Mycobacterium bovis* BCG and *Mycobacterium leprae*. Infect. Immun. **61:**5294–5301.
19. **Rees, A., A. Scoging, A. Mehlert, D. B. Young, and J. Ivanyi.** 1988. Specificity of proliferative response of human CD8 clones to mycobacterial antigens. Eur. J. Immunol. **18:**1881–1887.
20. **Shearer, G., C. Birge, P. D. Yuckenberg, G. S. Kobayashi, and G. Medoff.** 1987. Heat-shock proteins induced during the mycelial-to-yeast transitions of strains of *H. capsulatum*. J. Gen. Microbiol. **133:**3375–3382.
21. **Smith, J. G., D. M. Magee, D. M. Williams, and J. R. Graybill.** 1990. Tumor necrosis factor-alpha plays a role in host defense against *Histoplasma capsulatum*. J. Infect. Dis. **162:**1349–1353.
22. **Welch, W. J.** 1992. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. Physiol. Rev. **7:**1063–1081.
23. **Young, D. B., R. Lathigra, R. Hendrix, D. Sweetser, and R. A. Young.** 1988. Stress proteins are immune targets in leprosy and tuberculosis. Proc. Natl. Acad. Sci. USA **85:**4267–4270.

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