An 80-Kilodalton Antigen from *Histoplasma capsulatum* That Has Homology to Heat Shock Protein 70 Induces Cell-Mediated Immune Responses and Protection in Mice

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An extract of the cell wall and cell membrane from *Histoplasma capsulatum* yeast cells was assayed by Western blot (immunoblot) for reactivity with two monoclonal antibodies to heat shock protein 70. Four bands with molecular masses of 80, 66, 54, and 32 kDa bound both antibodies. The 80-kDa protein was isolated, analyzed for homology to heat shock protein 70, and tested for antigenicity and immunogenicity in C57BL/6 mice. The 80-kDa protein reacted with monoclonal antibody to heat shock protein 70. Sera from mice immunized with the antigen recognized *H. capsulatum* heat shock protein 70. Moreover, the amino-terminal sequence of the 80-kDa protein revealed substantial homology with heat shock protein 70 from several species. The 80-kDa protein induced delayed-type hypersensitivity responses in mice immunized with either viable yeast cells or antigen. Splenocytes from mice immunized with yeast cells or with antigen responded in vitro to the 80-kDa antigen. Immunization of mice with the antigen enhanced host resistance against a sublethal inoculum of *H. capsulatum* yeast cells, but it did not reduce the mortality of mice given a lethal challenge of yeast cells. Thus, this antigen manifests homology with members of the heat shock protein 70 family. Furthermore, the 80-kDa protein elicits cellular immune responses to *H. capsulatum*, and it mediates protective immunity.

Living organisms respond to adverse environmental conditions such as temperature elevation by a rapid increase in the synthesis of heat shock proteins (hsp). Production of these proteins presumably protects cells against the inimical effects of stress. Several families of hsp have been identified, and hsp 70 is one of the most abundant (19, 20).

Studies of hsp from the thermally dimorphic fungus *Histoplasma capsulatum* have focused on their role in the conversion of mycelia to yeast cells. This fungus exists as mycelia in soil but transforms into yeast cells once it has invaded mammalian tissues. The transition from mycelium to yeast cell is a heat-inducible event. When mycelia are exposed to elevated temperatures (34 to 40°C), there is intense synthesis of hsp, including hsp 70 (27). In addition, Caruso et al. (5) have shown that expression of the hsp 70 gene from *H. capsulatum* varies among strains that differ in pathogenicity and thermotolerance. Thus, hsp appear to play an important role in the mycelial phase-to-yeast phase conversion of *H. capsulatum*.

In recent years, it has been observed that members of the hsp family, including hsp 70 from several pathogenic microbes, are antigenic (for a review, see reference 33). Moreover, hsp of an invading microbe often are immunodominant targets of the cellular immune response as well as the humoral response (33). In the present study, we probed an extract of the cell wall and cell membrane of *H. capsulatum* yeast cells (CW/M) with monoclonal antibodies (MAb) to hsp 70 for the presence of immunoreactive bands. This extract was selected since previous work in this laboratory and others have demonstrated that it elicits in vivo and in vitro cellular immune responses and confers protective immunity (9, 11, 12).

Western blot (immunoblot) analysis of CW/M with MAb

to hsp 70 revealed the presence of four immunoreactive bands with molecular masses of 80, 66, 54, and 32 kDa. Since we previously reported that a murine CD4⁺ T-cell line recognized antigens in the extract that ranged in molecular mass from 69 to 82 kDa (12), the 80-kDa protein from CW/M that reacted with MAb to hsp 70 was isolated, analyzed for homology to members of the hsp 70 family, and tested for antigenicity and immunogenicity. The data demonstrate that this protein manifests homology to the hsp 70 family and induces cell-mediated immune responses in C57BL/6 mice. Furthermore, immunization with the 80-kDa antigen enhances resistance in mice given a sublethal challenge of *H. capsulatum* yeast cells.

MATERIALS AND METHODS

Mice. Male C57BL/6 mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Injection of mice with *H. capsulatum* yeast cells. Preparation of *H. capsulatum* yeast cells was performed as described previously (12). Mice were immunized with viable yeast cells by using the following regimen. On day 0, animals were injected subcutaneously (s.c.) with 10⁶ yeast cells. On day 14, mice received an intravenous (i.v.) injection of 6 × 10⁵ yeast cells, and on day 35 an intraperitoneal injection of 5 × 10⁶ yeast cells was administered. In experiments in which protective immunity was examined, mice were challenged i.v. with either a sublethal inoculum of 6 × 10⁵ yeast cells or a lethal inoculum of 4 × 10⁶ yeast cells.

Preparation of CW/M and the 80-kDa antigen. CW/M was isolated as described previously (12). Briefly, yeast cells were disrupted in a bead beater (Biospec Products, Bartlesville, Okla.), and the pellet was separated by differential centrifugation. The particulate material was boiled in an extraction buffer consisting of 125 mM Tris, pH 6.9, 6 M urea, 20 mM 2-mercaptoethanol, and 1% (vol/vol) Tween 20

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for 5 min and then incubated overnight at 4°C. The soluble material was separated by centrifugation and dialyzed against phosphate-buffered saline (PBS) for 36 h.

To isolate the 80-kDa antigen, 4 to 8 mg of CW/M was electrophoresed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels that were 3 mm thick. Following electrophoresis, the gels were stained for 20 min at room temperature with 0.3% Coomassie brilliant blue that was dissolved in 10% glacial acetic acid and 30% isopropyl alcohol (16). The gels were destained with a solution containing 5% glacial acetic acid and 16.5% methanol for 2 to 3 h at 4°C (16). The 80-kDa band was excised and soaked in water for 2 h. The section of gel was minced and placed in an electroelutor (ELUTRAP; Schleicher & Schuell, Keene, N.H.). Elution was performed at room temperature for 8 h at 200 V in a buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. The purity of the eluted fraction was checked on SDS-polyacrylamide minigels, and if there were contaminating proteins, the material was electrophoresed and electroeluted a second time. SDS and Coomassie blue were extracted from the protein by the method of Konigsberg and Henderson (17). The antigen was suspended in PBS, pH 7.4, and filter sterilized prior to use. The protein content of the 80-kDa antigen was determined by the Bradford method (3).

ATP affinity chromatography and isolation of hsp 70 from *H. capsulatum*. Yeast cells were grown for 36 h at 37°C, heated for 2 h at 42°C, and then returned to 37°C for an additional 2 h. They were disrupted with a bead beater, and the cytosol was harvested by differential centrifugation. To isolate hsp 70, 20 to 25 mg of cytosol (3 to 5 ml) was applied directly to an ATP-agarose column (1.0 by 5.0 cm; Sigma Chemical Co., St. Louis, Mo.) (32). The column was washed with 40 ml of low-salt buffer (50 mM Tris, 0.1 NaCl, 0.1 M EDTA, 5 mM MgCl₂, 15 mM 2-mercaptoethanol [pH 7.5]) and then with 40 ml of high-salt buffer (low salt buffer containing 0.5 M NaCl). The column was rinsed with 10 ml of 10 mM ATP (Sigma Chemical Co.) in high-salt buffer. The ATP eluate was collected, dialyzed extensively against 2 mM ammonium bicarbonate, lyophilized, and resuspended in 0.5 ml of PBS, pH 7.2. Protein concentration was determined by the Bradford assay (3).

SDS-PAGE of the 80-kDa antigen. One microgram of antigen was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gradient polyacrylamide gel (19), and the gel was stained with silver (28).

Antibodies. Rat MAb 7.10, which recognizes members of the hsp 70 family of *Drosophila melanogaster* (18, 31), was kindly provided by Susan Lindquist, Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Ill. This immunoglobulin G (IgG) MAb chiefly reacts with denatured hsp 70 and does not bind hsp 70 in immunofluorescence or immunoprecipitation assays. Mouse MAb N27F3-4, specific for the human hsp 72/73, was provided by William Welch, University of California at San Francisco. This mouse IgG1 recognizes both the constitutive (72-kDa) and inducible (73-kDa) forms of human hsp 70 under both native and denaturing conditions. MAb N27F3-4 appears to recognize hsp72/73 isolated from all mammalian sources that have been examined (30). Control antibodies included a rat IgG and a mouse IgG1.

Western blot analysis of CW/M, the 80-kDa antigen, and hsp 70. One hundred twenty-five micrograms of CW/M or 1 µg of purified proteins was electrophoresed (10% polyacrylamide gels, 1 mm thick), transferred to nitrocellulose, and stained with Ponceau S. Lanes of nitrocellulose were cut and incubated in Tris-buffered saline (TBS), pH 7.5, containing

5% powdered milk for 1 h at room temperature to block nonspecific binding sites. Lanes were then incubated in 1 ml of TBS containing a 1:800 dilution of either MAb 7.10 or MAb N27F3-4 at 4°C overnight. In some experiments, the 80-kDa antigen or hsp 70 was incubated with a 1:1,000 dilution of sera from mice injected with the 80-kDa antigen or with bovine serum albumin (BSA). The strips were washed twice with TBS plus 2% Tween 20 for 10 min and incubated for 2 h with 1 ml of a 1:1,000 dilution of the appropriate peroxidase-labelled second antibody in TBS at room temperature. Blots were washed twice in TBS-detergent and developed with 0.6 mg of 4-chloro-1-naphthol (Bio-Rad Laboratories) per ml and hydrogen peroxide (0.015%).

Amino-terminal sequencing. Fifteen micrograms of the 80-kDa protein was electrophoresed in a 10% polyacrylamide minigel and electroblotted onto a polyvinylidene difluoride membrane (Schleicher & Schuell) in 3-[(cyclohexylamino)-1-propane-sulfonic acid] (CAPS) buffer, pH 11, at 250 mA for 1 h. The membrane was stained with Coomassie brilliant blue and destained with methanol (22). The band was excised and was sequenced with a gas phase microsequencer (Applied Biosystems; model 475A).

In vivo injection of mice with the 80-kDa antigen. The 80-kDa antigen was emulsified with an equal volume of complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) (Difco Laboratories, Detroit, Mich.). Mice were injected s.c. with 40 µg of antigen in CFA; 2 weeks later, they were injected s.c. with 40 µg of antigen in IFA.

Induction and measurement of DTH responses to the 80-kDa antigen. Groups of six mice immunized with viable *H. capsulatum* yeast cells or with the 80-kDa antigen were challenged intradermally with 1 µg of antigen in a volume of 0.05 ml. Footpad swelling was measured 24 h later. The delayed-type hypersensitivity (DTH) response was expressed as the mean footpad thickness in millimeters before and after antigen challenge and as a percent increase in footpad thickness (12). As a control, DTH was measured in age-matched littermates injected with an equal quantity of BSA in Freund's adjuvant. The percent increase was calculated as follows: [(thickness 24 h after injection of antigen – thickness before injection)/thickness before injection] × 100.

Organ culture for *H. capsulatum*. Spleens from mice infected with *H. capsulatum* were homogenized in PBS, and the homogenate was serially diluted. One hundred microliters of homogenate was dispensed onto plates containing brain heart infusion agar (2% [wt/vol] agar) supplemented with 5% (vol/vol) defibrinated sheep erythrocytes, 1% (wt/vol) glucose, and 0.01% (wt/vol) cysteine hydrochloride. Plates were incubated at 30°C, and CFU were enumerated after 7 to 10 days.

Splenocyte preparation and splenocyte proliferation assay. Spleen cells from mice were prepared as described previously (12). In the proliferation assay of splenocytes, 4 × 10⁵ cells in 0.2 ml of RPMI 1640, containing 10% fetal bovine serum and 10 µg of gentamicin per ml, and 0.05 ml of antigen (20 µg/ml) or medium were added to each well of a microtiter plate. For the proliferation assay of the murine T-cell line, 2 × 10⁴ resting cells in 0.1 ml of medium were incubated with 5 × 10⁵ irradiated splenocytes suspended in 0.1 ml of medium and 0.05 ml of antigen (20 µg/ml) or medium. Cultures of splenocytes were incubated for 144 h at 37°C in 5% CO₂, whereas cultures of the T-cell line were incubated for 72 h; 16 h before cell harvest, 0.5 µCi of [³H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) was added to each well. The final concentration

FIG. 1. Western blot analysis of CW/M with MAb to hsp 70. One hundred twenty-five micrograms of CW/M was electrophoresed in 10% gels, transferred to nitrocellulose, and reacted with a 1:800 dilution of either MAb 7.10 (lane A), which was raised against *Drosophila* hsp 70, or MAb N-27F3-4 (lane B), which was raised against human hsp 70. Molecular weight markers (10^3) are on the left.

of the 80-kDa protein in a well was 4 $\mu\text{g}/\text{ml}$, which was an optimal concentration. Responses by cells to the 80-kDa antigen were considered positive when they were greater than or equal to three times the response of cells incubated in medium alone.

Statistics. The Mantel-Haenszel test was used to analyze survival of *H. capsulatum*-infected mice immunized with the 80-kDa antigen or BSA (24). The Wilcoxon rank sum test was used to analyze differences between groups. For both tests, $P < 0.05$ was considered significant.

RESULTS

Western blot analysis of CW/M. We sought to determine by immunoblot analysis if CW/M contained determinants recognized by MAb to members of the hsp 70 family. As shown in Fig. 1, MAb to *Drosophila* hsp 70 and to human hsp 70 reacted identically with CW/M. Four bands with various staining intensities were evident at molecular masses of 80, 66, 54 and 32 kDa. The curvilinear appearance of the 80- and 66-kDa bands was caused by intentional overloading of the gel with protein. This was done in order to ensure transfer of the many proteins in CW/M. Moreover, the patterns of

FIG. 2. SDS-PAGE analysis of the 80-kDa antigen. One microgram of protein was electrophoresed in a 10% gel and then stained with silver. Molecular weight markers (10^3) are on the left.

bands produced by incubating MAb to hsp 70 with CW/M were similar whether CW/M was produced from yeast cells subjected to heat shock (42°C for 2 h) or not (data not shown). No immunoreactive bands were apparent if CW/M was exposed to control antibodies. Thus, CW/M contained a determinant or determinants that were recognized by two disparate MAb to hsp 70.

Electrophoretic appearance of the 80-kDa protein and immunoblot analysis. We had reported previously that a murine T-cell line derived from C57BL/6 mice responded to a region of CW/M that encompassed 69 to 82 kDa (12). Therefore, it was postulated that the 80-kDa band that recognized MAb to hsp 70 might be one of the antigens within that region. The 80-kDa protein was isolated from SDS-PAGE gels by electroelution, and 1 μg was electrophoresed. Figure 2 illustrates that the electroeluted protein migrated as a single band with a molecular mass of 80 kDa in a silver-stained gel. All assays of the 80-kDa protein were conducted with preparations that had a single band, as determined on silver-stained gels.

To determine whether the 80-kDa protein was recognized by MAb to hsp 70, 1 μg of the protein was electrophoresed, transferred to nitrocellulose, and incubated with either rat MAb 7.10 or murine MAb N27F3-4 to hsp 70. The results of an immunoblot with MAb 7.10 are depicted in Fig. 3. The 80-kDa protein reacted with MAb to hsp 70. Thus, the 80-kDa protein possesses an epitope recognized by MAb to hsp 70.

Sera from mice immunized with the 80-kDa antigen recognize *H. capsulatum* hsp 70. Since the 80-kDa antigen con-

FIG. 3. Western blot of the 80-kDa antigen with MAb 7.10. One microgram of the 80-kDa antigen was electrophoresed in a 10% gel, transferred to nitrocellulose, and incubated with a 1:800 dilution of MAb 7.10. Molecular weight markers (10^3) are depicted on the left.

tained a determinant that was immunoreactive with MAb to hsp 70, the possibility was explored that sera from mice immunized with the 80-kDa antigen would recognize *H. capsulatum* hsp 70. Therefore, pooled sera from mice immunized with the 80-kDa antigen were tested for reactivity with *H. capsulatum* hsp 70.

Initially, we sought to isolate hsp 70 from *H. capsulatum*. Since hsp 70 binds to ATP (32), cytosol from yeast cells was subjected to ATP affinity chromatography. A 70-kDa protein eluted from the column with ATP (Fig. 4, left lane). This protein was transferred to nitrocellulose and immunoblotted with MAb 7.10. The 70-kDa protein reacted with this MAb (Fig. 4, right lane).

By Western blot analysis, sera from mice immunized with the 80-kDa antigen reacted with the 80-kDa antigen and hsp 70 (Fig. 5, lanes 1 and 3), whereas sera from mice immunized with BSA did not recognize either protein (Fig. 5, lanes 2 and 4). Thus, the 80-kDa antigen was serologically cross-reactive with hsp 70.

NH₂-terminal sequence of the 80-kDa antigen. To determine whether the 80-kDa antigen was homologous to members of the hsp 70 family at the amino acid level, the protein was subjected to microsequencing of the NH₂ terminus. Analysis of this sequence revealed a considerable homology at the amino acid level with hsp 70 from *Saccharomyces cerevisiae* (72%), *D. melanogaster* (72%), *Escherichia coli*

FIG. 4. Electrophoretic appearance of hsp 70 from *H. capsulatum* (left lane) and Western blot analysis of hsp 70 with MAb 7.10 (right lane). One microgram of hsp 70 was isolated by ATP affinity chromatography and electrophoresed in a 10% gel. The gel was stained with silver. One microgram was electrophoresed, transferred to nitrocellulose, and incubated with a 1:800 dilution of MAb 7.10. Molecular weight markers (10^3) are depicted on the left.

(56%), and humans (83%) (Table 1). Thus, the 80-kDa protein manifests homology to members of the hsp 70 family at the amino acid level and by serology.

The 80-kDa antigen induces DTH responses in mice immunized with viable yeast cells or with the 80-kDa antigen. Mice were injected with yeast cells or with 80 μ g of the 80-kDa antigen and tested for DTH to this antigen. The DTH response by either group of immunized mice was significantly greater ($P = 0.01$) than that by control animals (Table 2).

Splenocytes from mice injected with yeast cells or with the 80-kDa antigen proliferate in vitro in response to the 80-kDa antigen. Splenocytes from five individual C57BL/6 mice that had been immunized with viable *H. capsulatum* yeast cells or from five controls were tested for their capacity to recognize the 80-kDa antigen. The results for three mice are shown in Table 3. Spleen cells from all immunized mice proliferated in response to the antigen, whereas splenocytes from unimmunized animals did not.

Similarly, splenocytes from five individual C57BL/6 mice that had been immunized with 80 μ g of the 80-kDa antigen recognized this antigen. In contrast, spleen cells from all five mice given BSA did not respond to the 80-kDa antigen (Table 3).

The 80-kDa antigen but not hsp 70 from *H. capsulatum* causes proliferation of a T-cell line. Since the CD4⁺ T-cell line JC1 had responded to nitrocellulose-bound antigens encompassing 70 to 80 kDa (12), we determined whether the more purified 80-kDa antigen stimulated JC1. In several experiments, JC1 responded to the 80-kDa antigen. As an example, the mean ($\pm$ standard error of the mean [SEM])

FIG. 5. Western blot analysis of the 80-kDa antigen and hsp 70, using sera from mice immunized with the 80-kDa antigen (lanes 1 and 3) or BSA (lanes 2 and 4). One microgram of the 80-kDa antigen or 1 μ g of hsp 70 was transferred to nitrocellulose and reacted with a 1:1,000 dilution of sera from mice. Lane 1, immune sera with 80-kDa antigen; lane 2, sera from BSA-injected mice with 80-kDa antigen; lane 3, immune sera with *H. capsulatum* hsp 70; lane 4, sera from BSA-injected mice with hsp 70. Molecular weight markers (10^3) are shown on the left.

proliferative response by JC1 to the 80-kDa antigen that 4 μ g/ml ($8,827 \pm 720$ cpm) exceeded that of cells cultured in medium alone ($1,070 \pm 73$ cpm).

In a separate set of experiments, we tested hsp 70 from *H. capsulatum* for its capacity to stimulate JC1. In three experiments, hsp 70 did not induce proliferation of JC1. In one experiment, the response by JC1 to hsp 70 at 5 μ g/ml ($1,487 \pm 200$ cpm) was not substantially higher than background ($1,203 \pm 197$ cpm). As a positive control, JC1 mounted a brisk response to 5 μ g of CW/M per ml ($20,621 \pm 3,734$ cpm).

Protective efficacy of the 80-kDa antigen. Groups of mice were immunized with 80 μ g of the 80-kDa antigen or with an equal amount of BSA. After immunization, the animals were challenged with a sublethal inoculum (6×10^5) of yeast cells. One week later, the animals were sacrificed, and the number

TABLE 2. DTH responses to the 80-kDa antigen in immunized mice

Immunization ^a	Mean ($\pm$ SEM) footpad thickness (mm) ^b	
	Before antigen challenge	After antigen challenge
None	2.00 $\pm$ 0.03	2.06 $\pm$ 0.01 (3) ^c
<i>H. capsulatum</i> yeast cells	2.20 $\pm$ 0.06	3.15 $\pm$ 0.11 ^d (43)
BSA, 80 μ g	2.05 $\pm$ 0.04	2.09 $\pm$ 0.04 (2)
80-kDa antigen, 80 μ g	2.05 $\pm$ 0.01	2.40 $\pm$ 0.03 ^d (17)

^a Mice were injected with viable yeast cells as described in Materials and Methods. One-half of the total dose of BSA or 80-kDa antigen was injected s.c. in CFA; 2 weeks later, the other half of the dose was given in IFA. Mice were tested 2 weeks later.

^b Mean $\pm$ SEM for groups of six mice. Mice were tested with 1 μ g of the 80-kDa antigen.

^c Figures in parentheses indicate mean percent increase in footpad thickness from that measured immediately before antigen challenge.

^d Differences in footpad thickness between controls and immunized mice were significant at $P = 0.01$.

of *H. capsulatum* CFU in spleens was quantitated. The mean ($\pm$ SEM) number of CFU in spleens of mice ($n = 10$) given the 80-kDa antigen ($43.0 \times 10^4 \pm 2.2 \times 10^4$ CFU) was significantly fewer ($P < 0.01$) than that in spleens of mice ($n = 10$) injected with BSA ($114.1 \times 10^4 \pm 6.4 \times 10^4$ CFU).

Subsequently, groups of six mice immunized with the 80-kDa antigen or with BSA were injected with a lethal inoculum (4×10^6) of yeast cells and were observed daily. In the 80-kDa-antigen-immunized group, three mice died on day 9, two mice died on day 10, and another mouse expired on day 11. In the controls, one mouse died on day 9, three died on day 10, and another died on day 11. Mantel-Haenszel analysis of survival revealed no statistical differences between groups ($P > 0.05$). Thus, the 80-kDa antigen reduced CFU in spleens of mice challenged with a lethal dose but failed to protect mice against a lethal challenge.

DISCUSSION

In this study, we examined the possibility that CW/M contained a determinant or determinants recognized by MAb to hsp 70. The rationale for this set of experiments was based on the previous finding that CW/M contained antigens whose molecular masses were similar to those of members of the hsp 70 family, that is, between 70 and 80 kDa (12). Western blot analysis revealed that two distinct MAb to hsp 70 produced identical patterns of reactivity with CW/M. Four bands with molecular masses of 80, 66, 54, and 32 kDa recognized both MAb. The profile of immunoblots suggests that these MAb recognized degradation products of hsp 70 in CW/M. This finding is not surprising since production of CW/M requires physical disruption of yeast cells. Disruption was performed in a buffer containing protease inhibitors and on ice. Nevertheless, it is still likely that some intact proteins are exposed to proteolytic enzymes and intense heat, which promote degradation.

Although there were several reactive bands, attention was focused on the 80-kDa protein because its molecular mass was in the region of that of nitrocellulose-bound antigen that caused stimulation of a murine T-cell line (12). Therefore, this protein was isolated from CW/M and tested for antigenicity and immunogenicity.

Serological studies strongly suggested that the 80-kDa protein shared a determinant or determinants with *H. cap-*

TABLE 1. Comparison of amino acid sequence of the 80-kDa antigen with sequences of hsp 70 from other species

Protein ^a	Amino acid sequence ^b
Hc 80-kDa Ag	A P A V - G I D L G T T Y S V V G I F
Sc hsp 70	A V - G I D L G T T Y S C V A H F
Dm hsp 70	A V - G I D L G T T Y S C V G V Y
Ec hsp 70	K I I G I D L G T T M S C V A I M
Hu hsp 70	A A A V - G I D L G T T Y S C V G V F

^a Hc 80-kDa Ag, 80-kDa antigen from *H. capsulatum*; Sc hsp 70, hsp 70 from *S. cerevisiae*; Dm hsp 70, hsp 70 from *D. melanogaster*; Ec hsp 70, hsp 70 from *E. coli*; Hu hsp 70, human hsp 70.

^b Single-letter code used. Amino acids that are identical to those in the 80-kDa protein are underlined. Amino acid sequences were obtained from GenBank and reference 20.

TABLE 3. Reactivity of splenocytes from immunized mice to the 80-kDa antigen in vitro

Immunization ^a	Stimulus ^b	[³ H]thymidine incorporation (mean cpm $\pm$ SEM) ^c		
		Expt 1	Expt 2	Expt 3
None	Medium	4,007 $\pm$ 131	5,502 $\pm$ 106	94 $\pm$ 4
	80-kDa antigen	4,675 $\pm$ 306	4,607 $\pm$ 329	134 $\pm$ 28
<i>H. capsulatum</i> yeast cells	Medium	1,139 $\pm$ 80	803 $\pm$ 267	544 $\pm$ 42
	80-kDa antigen	7,157 $\pm$ 618	19,351 $\pm$ 1,838	22,162 $\pm$ 961
BSA, 80 μ g	Medium	538 $\pm$ 47	782 $\pm$ 126	186 $\pm$ 21
	80-kDa antigen	495 $\pm$ 156	896 $\pm$ 158	346 $\pm$ 70
80-kDa antigen	Medium	317 $\pm$ 30	466 $\pm$ 48	210 $\pm$ 21
	80-kDa antigen	6,703 $\pm$ 177	8,350 $\pm$ 473	7,649 $\pm$ 386

^a See Table 2, footnote a.^b The final concentration of the 80-kDa antigen was 4 μ g/ml.^c Mean $\pm$ SEM of triplicate determinations.

sulatum hsp 70. In multiple experiments, we were unsuccessful in isolating the 80-kDa antigen from CW/M by ATP affinity chromatography, although *H. capsulatum* hsp 70 did bind to ATP (13). One potential problem was that CW/M is highly denatured; thus, the ATP-binding site for the 80-kDa protein may have been irrevocably altered. Therefore, to determine whether this antigen was homologous to hsp 70, amino-terminal sequencing was performed. A comparison of the amino acid sequences of the 80-kDa protein and hsp 70 from several species revealed a high degree of similarity. Thus, the 80-kDa antigen appears to be a homolog of hsp 70. The true identity of this antigen will be determined only once the gene has been cloned and sequenced.

The observation that a homolog of hsp 70, viz., the 80-kDa antigen, was present in the cell wall or cell membrane was unexpected since most proteins that are members of the hsp 70 family are present in the nucleus or cytoplasm of cells (23). However, there is precedent for expression of members of the hsp 70 family in the cell wall and cell membrane. A *Plasmodium falciparum* antigen that has 55% sequence homology to hsp 70 appears to reside on the surface of merozoites (1). A 75-kDa protein from *Chlamydia trachomatis* is present on the surface of elementary bodies (7). In addition, the cell membrane of cells of a murine B-cell lymphoma line, LK 35.2, contains a peptide-binding protein that is a member of the hsp 70 family (29). Preliminary experiments indicate by immunofluorescence that the 80-kDa antigen is not on the surface of *H. capsulatum* yeast cells (13). Studies are planned to identify more precisely the location of the 80-kDa protein in *H. capsulatum* yeast cells.

Additional studies of this antigen demonstrated that C57BL/6 mice immunized with viable yeast cells mounted a DTH response to the 80-kDa antigen and splenocytes from these mice proliferated in response to it. Furthermore, inoculation of the 80-kDa antigen in Freund's adjuvant induced cutaneous reactivity and in vitro blastogenic responses to the 80-kDa antigen. This antigen, therefore, is a target of the cellular immune response to *H. capsulatum* and induces cell-mediated immune responses. Moreover, since the antigen did not stimulate normal splenocytes, the data suggest that it is neither a mitogen nor a superantigen.

Members of the hsp 70 family are highly conserved at the amino acid level among eukaryotes and prokaryotes (20, 21, 23). The function of these proteins is to maintain polypeptides in an unfolded state in order to facilitate transport across membranes (6, 8, 10, 23). More recently, hsp 70 or homologs thereof from several species of parasites and bacteria have been shown to be antigenic. Thus, these

proteins from *P. falciparum* (2), *Schistosoma mansoni* (15), *Wucheria bancrofti* (26), and *Mycobacterium* sp. (4, 25) stimulate either a B-cell- or T-cell-mediated response or both. Since the 80-kDa antigen elicited both a cell-mediated and a humoral response, the data indicate that *H. capsulatum* also possesses an antigenic hsp 70 homolog.

Although the 80-kDa antigen manifests homology to hsp 70, it differs antigenically from the latter. The murine T-cell line responded to the 80-kDa protein but not to *H. capsulatum* hsp 70. Thus, the 80-kDa protein contains a peptide recognized by T cells that is not present in hsp 70. However, this finding does not exclude the possibility that cytosolic hsp 70 is antigenic since we did not assess whether splenocytes from mice immunized with *H. capsulatum* react with it.

In studies of protective immunity, the 80-kDa antigen significantly reduced the number of *H. capsulatum* CFU in spleens of mice given a sublethal injection of yeast cells i.v. However, it failed to prolong survival in mice given a lethal challenge of yeast cells. Why immunization with this antigen did not enhance survival among mice given a lethal dose of yeast cells is unclear. It is possible that larger quantities of the 80-kDa antigen might have significantly enhanced its protective capacity. These issues may not be resolved until there is a better understanding of host defense mechanisms that are operative during histoplasmosis. Nevertheless, it is important to recognize that this protein did enhance resistance to *H. capsulatum*.

This study extends the reported number of protective antigens in CW/M to two. There is the 80-kDa antigen and a previously isolated 62-kDa antigen (14). The latter, however, was able to confer protection in mice against a lethal challenge that was identical to the challenge employed in this study (14).

In summary, we have isolated an 80-kDa antigen from the cell wall and cell membrane of *H. capsulatum* yeast cells. This antigen is cross-reactive with MAbs to hsp 70, and it manifests similarity at the amino acid level with hsp 70 from other species. This antigen induced cellular and humoral immune responses and conferred protective immunity. Thus, a homolog of hsp 70 from *H. capsulatum* is antigenic and immunogenic.

ACKNOWLEDGMENTS

This work was supported by grant AI-23017 from the National Institute of Allergy and Infectious Diseases. G.S.D. is the recipient of Research Career Development Award AI-00856 from the National Institute of Allergy and Infectious Diseases.

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