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Localization and Characterization of the 86- and 84-kDa Heat Shock Proteins in Hepa 1c1c7 Cells

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The 90-kDa heat shock protein (hsp90) is present in cells at high levels in the cytoplasm and is composed of two separate gene products, hsp86 and hsp84. Rabbit polyclonal antibodies to the murine N-terminal sequences of the 86- and 84-kDa heat shock proteins were isolated from serum by peptide affinity chromatography. Antibodies against each form of hsp90 are capable of immunoprecipitating hsp90. Each antibody preparation is specific against either hsp86 or hsp84 when tested on a protein blot of Hepa 1c1c7 cytosol. The overall ratio of hsp84/hsp86 in Hepa 1 cytosol was estimated to be 2 to 1. Each antibody preparation was used to immunoprecipitate hsp84 or hsp86 from Hepa 1 cytosol to test whether hsp86/84 exists as a homo- and/or heterodimer. After electrophoresis, silver staining revealed that anti-hsp86 antibodies immunoprecipitated both hsp86 and hsp84. This result would suggest that hsp86 forms heterodimers with hsp84. In contrast, the anti-hsp84 antibodies immunoprecipitated almost entirely hsp84, suggesting that hsp84 exists largely as homodimers. Both anti-hsp86 and hsp84 antibodies were able to immunoprecipitate the 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin-labeled *Ah* receptor from Hepa 1 cytosol, indicating that these antibodies are able to bind to hsp90 when it is complexed with other proteins. Both antibody preparations recognize hsp90 in mouse, rat, and human cell lines. Immunofluorescence and confocal microscopy were performed using both antibody preparations, and the results indicated that both hsp86 and hsp84 were located in the cytoplasm and nucleus of Hepa 1 cells. Hsp86 was found to localize unevenly in the cytoplasm, while hsp84 was found evenly dispersed throughout the cytoplasm. Hsp86 also appeared to be localized to a greater degree than hsp84 in the vicinity of the nuclear envelope. © 1993 Academic Press, Inc.

INTRODUCTION

Cells in culture respond to heat and chemical stress by inducing the synthesis of a group of proteins referred to as heat shock proteins [1]. The most highly expressed heat shock protein in unstressed cells is the 90-kDa heat shock protein (hsp90). Hsp90 also appears to have a critical cellular function in *Saccharomyces cerevisiae* under normal growth conditions [2]. The actual function(s) of hsp90 is poorly understood, although a wide number of proteins have been shown to interact with hsp90, including viral tyrosine kinases [3, 4], steroid receptors [5], *Ah* receptor [6], casein kinase II [7], actin [8], and heme-regulated eIF2α kinase [9, 10]. Studies using anti-hsp90 monoclonal antibodies to immunoprecipitate hsp90/protein complexes in Hepa 1c1c7 cells revealed several relatively abundant cytosolic proteins with M_r of 68, 63, 56, and 50 kDa bound to hsp90 [11]. The 68-kDa protein was identified immunochemically as the 70-kDa heat shock protein (hsp70). Hsp70 is a family of proteins that are involved in facilitating protein folding and assembly and are also required to transport at least some proteins across the nuclear membrane [1, 12]. The 56-kDa protein was initially reported as a subunit of nonactivated soluble steroid receptors (e.g., glucocorticoid and progesterone receptors) bound to hsp90 [13]. Recently, the 56-kDa protein was identified as another heat shock protein (hsp56) and has been cloned, with sequence analysis revealing homology with proline isomerases [14, 15]. The viral tyrosine kinase pp60$^{v\text{-}src}$ interacts with a hsp90/p50 complex after translation in the cytoplasm [16]. Pulse–chase experiments have revealed that pp60$^{v\text{-}src}$ transiently binds to hsp90/p50, followed by insertion of the kinase into the plasma membrane. These studies would indicate that hsp90 is found associated with a variety of proteins in large oligomeric complexes and probably serves to stabilize specific proteins in the cell.

Most studies examine hsp90[2] as a single protein, while in fact there are two separate structural genes, referred to as hsp86 and hsp84 in mouse [17, 18] or hsp89α and hsp89β in human cells [19]. The protein sequences of hsp84 and hsp86 are highly conserved among vertebrates. A molecular evolutionary tree of hsp90-related proteins suggests that hsp84 and hsp86

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[2] In this report hsp90 refers to both hsp86 and hsp84.

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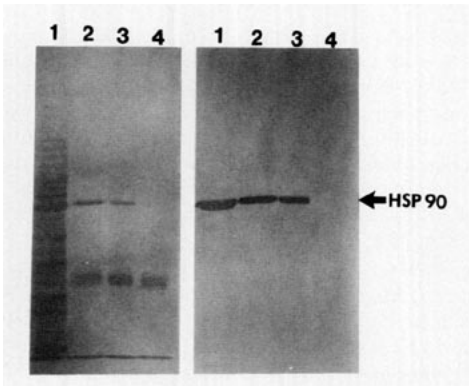

FIG. 1. Immunoprecipitation of hsp90 with anti-N-terminal peptide polyclonal antibodies. Hepa 1 cytosol (200 μg) was incubated with 5 μg of affinity-purified anti-hsp86 or anti-hsp84 antibody immobilized on 50 μl of goat anti-rabbit IgG–Sepharose. After incubation and subsequent washing, the Sepharose pellets were solubilized in 2× SDS sample buffer. Samples were subjected to SDS–PAGE followed by transfer onto PVDF membrane. Each lane in the panel to the left represents the following samples stained with Poneau red: lane 1, 200 μg of Hepa 1 cytosol; lane 2, anti-hsp84 immunoprecipitate; lane 3, anti-hsp86 immunoprecipitate; lane 4, control rabbit IgG immunoprecipitate. After removal of the Ponceau red stain, hsp90 was visualized by the blot with AC88 monoclonal antibody and the bound antibody was visualized as described under Materials and Methods. The lanes in the panel to the right are the same as those given above.

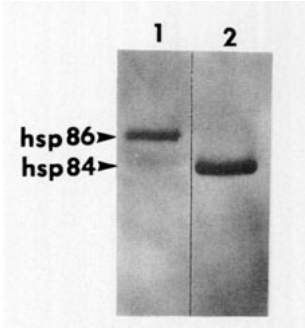

FIG. 2. Immunochemical detection of hsp86 and hsp84 on protein blots. Hepa 1 cytosol (2.5 μg/lane) was applied to 14 × 30-cm polyacrylamide gels with a limiting amount of SDS in the upper running gel buffer as described under Materials and Methods. After electrophoresis the protein was transferred to PVDF membrane and incubated with either 0.5 μg/ml anti-hsp86 antibody (lane 1) or 0.5 μg/ml anti-hsp84 antibody (lane 2) and the bound antibody was visualized as described under Materials and Methods.

diverged 500 million years ago, a time that approximates the emergence of vertebrates [17]. There are several published examples of differential expression of hsp84/hsp84. For example, hsp86/hsp84 are differentially expressed during testicular development [20]. Upon differentiation, the constitutive level of hsp86 was markedly decreased in F9 EC cells compared with hsp84 levels [21]. Examination of the level of expression of hsp86/hsp84 in murine tissues revealed that hsp86 is expressed in brain, testis, and placenta. In contrast, hsp84 is highly expressed in most tissues (e.g., liver, thymus, kidney, etc.) [20]. Despite these regulatory differences, no functional difference between hsp86 and hsp84 has yet been determined.

There have not been any published studies examining the distribution of each form of hsp90 in cells due to the lack of antibodies specific to each form of hsp90. In this report we describe the production and use of polyclonal peptide antibodies to hsp86 and hsp84 and their use in immunocytochemical localization of hsp86/hsp84 in Hepa 1c1c7 cells.

MATERIALS AND METHODS

Peptide synthesis. Synthetic peptides corresponding to the following N-terminal sequences of hsp86 and hsp84 were prepared by Multiple Peptide Systems (San Diego, CA): hsp84-PEEVHHGEEEVEC and hsp86-PEETQTQDQPMC [17, 18]. Each peptide was purified by high-performance liquid chromatography on a Vydac C_{18} reverse-phase column (4.6 × 250 mm). A C-terminal cysteine was added to each peptide to allow coupling to KLH or BSA using the heterobifunctional cross-linking agent *N*-succinimidyl bromoacetate [22].

Antibody production and purification. Each KLH–peptide conjugate in PBS was mixed with an equal volume of complete Freund's adjuvant and injected into rabbits. The immunization and bleeding of the rabbits was performed using standard methods, by the Berkeley Antibody Company (Richmond, CA). Each peptide was coupled to Reacti-Gel, GF-2000 (Pierce, Rockford, IL) at 1 mg/ml, according to manufacturer's instruction. Serum (20 ml) was incubated at 4°C with 5 ml of immunoaffinity gel overnight. The gel was pelleted by centrifugation, washed twice with 10 m*M* Tris, pH 7.5, and packed in a Spectra/Chrom column (1.5 × 20 cm). The column was connected to an HPLC and washed with 10 m*M* Tris, pH 7.5. After a baseline absorbance was obtained, the column was washed with 10 m*M* Tris, pH 7.5, containing 500 m*M* NaCl to elute low-affinity antibodies and nonspecifically absorbed proteins. Antibodies were eluted with 100 m*M* glycine, pH 2.5, and collected in a tube containing 1/10 volume of 1 *M* Tris, pH 8.0. The antibody solution was supplemented with 1 mg/ml bovine serum albumin and 0.02% sodium azide.

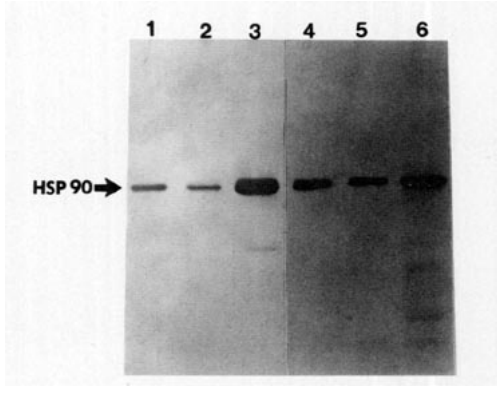

FIG. 3. Cross-reactivity of rabbit anti-hsp86 and anti-hsp84 antibodies to mouse, rat, and human hsp90. Cytosol (5 μg) from murine Hepa 1 cells (lanes 1 and 4), rat McA-RH7777 cells (lanes 2 and 5), and human HeLa cells (lanes 3 and 6) was subjected to SDS–PAGE and Western blotting. The blot was incubated with either rabbit anti-hsp86 antibody (lanes 1–3) or rabbit anti-hsp84 antibody (lanes 4–6) and the bound antibody was visualized as described under Materials and Methods.

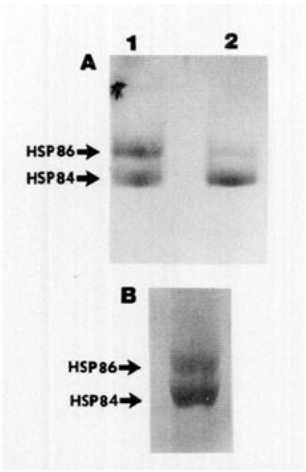

FIG. 4. Immunoprecipitation of hsp86 or hsp84 from Hepa 1 cytosol. (A) Hepa 1 cytosol (200 μg) was incubated with either rabbit anti-hsp86 or rabbit anti-hsp84 antibody as described in the legend to Fig. 1. One-fifth of each immunoprecipitate was applied to a 14 × 30-cm polyacrylamide gel with limiting SDS in the upper running buffer as described under Materials and Methods. After electrophoresis, the gel was silver stained. Lane 1, anti-hsp 86 immunoprecipitate; lane 2, anti-hsp84 immunoprecipitate. (B) Hsp 90 was immunoprecipitated from Hepa 1 cytosol with mAb 8D3. The immunoprecipitate was applied to a 14 × 30-cm polyacrylamide gel with limiting SDS in the upper running buffer. After electrophoresis, the gel was stained with Coomassie blue.

Gel electrophoresis and protein blotting. Polyacrylamide gel electrophoresis (7.5%) was performed essentially as described [23]. In order to separate hsp86 from hsp84 during polyacrylamide gel electrophoresis, the amount of SDS in the upper running electrophoresis buffer was lowered from 0.1 to 0.03% and 14 × 30-cm polyacrylamide gels were used. Protein gels were silver stained according to the procedure of Wray *et al.* [24].

Polyacrylamide gels were blotted onto PVDF membrane (Millipore Corp., Bedford, MA) as previously described [25]. Total protein was detected on each blot by staining with 0.5% Ponceau Red, 1% acetic acid. After destaining, the nonspecific binding sites on each blot were blocked by incubation at room temperature for 60 min with PBS containing 3% bovine serum albumin.

Detection of hsp86 and hsp84 on protein blots. After blocking, each blot was incubated in blotting buffer (0.1% bovine serum albumin, 0.05% Tween 20, 10 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.4) with the following primary antibodies: monoclonal antibody AC88 ascites (1/1000), rabbit anti-hsp86 or anti-hsp84 affinity-purified polyclonal antibody (0.5 μg/ml). After 1 h, blots were washed 3× 5 min with blotting buffer and incubated for 1 h with goat anti-mouse IgG + IgM peroxidase conjugate (1/1000 dilution in blotting buffer). After 3× 5 min washes with blotting buffer, the bound antibodies were visualized by staining with 3,3'-diaminobenzidine (0.3 mg/ml), 0.005% H_2O_2 in 50 m*M* Tris, pH 7.4.

Cell culture and photoaffinity labeling of cytosol. Hepa 1c1c7 mouse hepatoma cells, McA-RH7777 rat hepatoma cells, and HeLa cells were grown as previously described [26]. Each cell line was harvested by exposure to EDTA/trypsin and the cells were washed with PBS twice. Cells were resuspended in MENGM buffer (25 m*M* Mops, 2 m*M* EDTA, 0.02% sodium azide, 20 m*M* sodium molybdate, pH 7.4, plus 10% glycerol) and homogenized with 15 strokes in a tissue grinder (Wheaton Instruments, Millville, NJ). Cytosolic fractions were isolated as previously described [26].

Hepa 1c1c7 cytosolic fractions were photoaffinity-labeled with 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin as previously described [26]. This compound binds with high affinity and specificity to the *Ah* receptor.

Immunoprecipitations. Goat anti-rabbit IgG–agarose (50 μl) was incubated with 5 μg of anti-hsp86 or anti-hsp84 affinity-purified antibodies or control rabbit IgG. After washing with PBS, the immunoaffinity gel was incubated with 200 μg of Hepa 1 cytosol for 90 min. The cytosol was removed and the gel was washed twice with MENGM + 150 m*M* NaCl buffer. For electrophoretic analysis the gel pellets were boiled for 5 min in 100 μl of 2× SDS sample buffer and subjected to SDS–PAGE. Total hsp90 was immunoprecipitated with monoclonal antibody (mAb) 8D3. Goat anti-mouse IgM–Sepharose was incubated with 100 μl of mAb 8D3 ascites for 1 h. After three washes with MENGM buffer, 400 μg (1 mg/ml) of Hepa 1 cytosol was added to the Sepharose pellet and incubated at 4°C for 90 min. The Sepharose was washed three times with MENGM. The Sepharose pellet was subjected to SDS–PAGE after the addition of 2× sample buffer.

Densitometry. Coomassie blue-stained gels were analyzed with a pdi densitometer (protein and dna imageWare systems, Huntington Station, NY).

Indirect immunofluorescence. Sterile poly-L-lysine-coated, HTC-printed microscope slides (Cel-Line Associates, Inc., Newfield, NJ) were placed in 100-mm culture dishes. Hepa 1 cells were plated onto 5-mm wells on the slides at a density of 1×10^4 cells/well. After 4 h, the slides were covered with 25 ml α-minimum essential medium (Sigma Chemical Co., St. Louis, MO) containing 5% fetal bovine serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin and grown

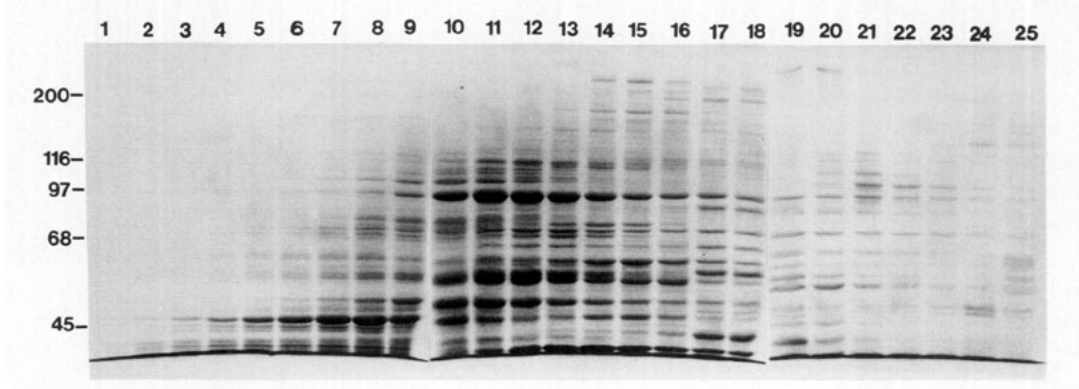

FIG. 5. Sucrose density gradient analysis of Hepa 1 cytosol. Hepa 1 cytosol (3 mg/ml, 300 μl) was applied to a 10–30% sucrose gradient, as described under Materials and Methods. Each fraction was subjected to SDS–PAGE. After electrophoresis the gel was stained with Coomassie blue.

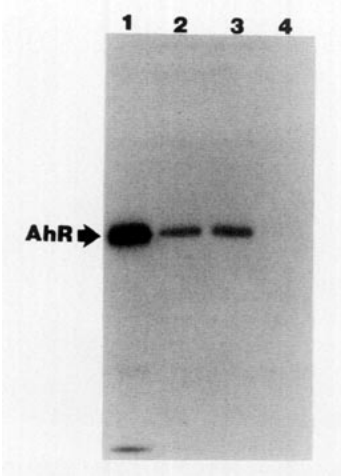

FIG. 6. Immunoprecipitation of the *Ah* receptor from photoaffinity-labeled Hepa 1 cytosol with anti-hsp86 or anti-hsp84 antibodies. Hepa 1 cytosol was photoaffinity-labeled with 2-azido-3-[^{125}I]-iodo-7,8-dibromodibenzo-*p*-dioxin, followed by immunoprecipitation with anti-hsp86 antibody, anti-hsp84 antibody, or control rabbit IgG, as described in the legend to Fig. 1. The immunoprecipitates were applied to a polyacrylamide gel. After electrophoresis the gel was dried and subjected to autoradiography. Hepa 1 cytosol (200 μg) is shown in lane 1, rabbit anti-hsp86 immunoprecipitate, rabbit anti-hsp84 immunoprecipitate, and rabbit control antibody are shown in lanes 2, 3, and 4, respectively.

at 37°C in 94% air/6% CO_2. Twenty-four hours later, the slides were rinsed for 1 min in 90 ml PBS, pH 8.0, and fixed by incubating in 4% paraformaldehyde in PBS for 15 min at room temperature. The fixing solution was washed from the cells with one 2-min rinse in 90 ml PBS, pH 8.0. Permeabilization was carried out for 2 min in methanol at room temperature. The permeabilization solution was washed off with a 2-min rinse in 90 ml PBS, pH 8.0. Incubations with primary and secondary antibody preparations were carried out by placing the slides on water saturated filters in 100-mm culture dishes. Nonspecific binding was blocked by incubating cells with 3% donkey serum in PBS. After 1 h the serum was washed off with five washes of PBS, pH 8.0. The cells were then incubated with either preimmune rabbit sera or affinity-purified anti-hsp84 or anti-hsp86 antibody diluted to 15 μg/ml in 1% BSA, 0.05% Tween 20, and 0.02% sodium azide in PBS for 1 h at room temperature. After removing the antibody solution, the cells were washed five times with PBS, pH 8.0, for a total of 15 min. Lissamine–rhodamine–sulfonyl chloride-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Inc., West Grove, PA) was diluted 1:100 in PBS, pH 8.0, and incubated with the cells for 15 min at room temperature. The cells were then washed with the same protocol used after the primary antibody incubation. The cells were cleared of water using a three-step graded series of glycerol solutions (70 and 90% glycerol in PBS, 100% glycerol, all with 1% propyl gallate), and cells were mounted in the 100% glycerol, 1% propyl gallate solution. Cells were visualized with a Zeiss Ortholux II microscope equipped with epifluorescence optics and a Zeiss Wetzlar 40X (NA 0.65) objective lens. Black and white pictures were taken using a Nikon M-35FA camera attached to a 23.2× magnification photographic tube (total magnification, 464×) and Nikon automatic photometer system using Kodak TMAX 400 film. Exposure times for negative controls were matched with the exposure times used for the experimental samples.

Laser scanning confocal microscopy. The Bio-Rad MRC 600 laser scanning confocal microscope system equipped with a krypton–argon laser was connected to a Nikon inverted microscope using a Nikon 60× objective. Digital images were magnified 2.5× using Bio-Rad software run on an IBM PC XT for a total magnification of 625×. The *xv* image analysis software program (© 1993 by John Bradley, version 3.0) run on a Sun Sparcstation 2 (Sun Microsystems) was used to generate digital images. Black and white pictures were taken with a 35-mm Kodak camera linked to a Sun Sparcstation 2 computer on Kodak TMAX 400 film. The confocal microscope, computer, and software utilized are all property of the Digital Microscopy and Scientific Visualization Laboratory, Department of Anatomy and Cell Biology, University of Michigan (Ann Arbor, MI).

RESULTS

Characterization of peptide polyclonal antibodies to 86- and 84-kDa heat shock proteins. Alignment of hsp86 and hsp84 protein sequences reveals an amino acid homology of 86%. The largest difference in amino acid sequence occurs in the N-terminal region. Peptides containing the N-terminal 12 or 11 amino acids of hsp84 and hsp86 were synthesized and coupled to KLH and injected into rabbits. The animals were bled and the serum fraction isolated. The peptide antibodies were purified by affinity chromatography on peptide columns. Each affinity-purified polyclonal antibody was tested on a Western blot for binding to either hsp86 or hsp84. Polyacrylamide gels were used with 0.03% SDS in the upper running buffer: the limiting amount of SDS causes hsp86 and hsp84 to migrate differentially [23]. Initially, the anti-hsp86 affinity-purified antibody was found to cross-react with hsp84; these cross-reacting antibodies were removed by passage of the antibody fraction through a hsp84 peptide column. Hsp90 was immunoprecipitated from Hepa 1 cytosol with either anti-hsp86 or anti-hsp84 antibodies, as indicated by a single protein band at ~86 kDa after Ponceau red staining of a Western blot. After destaining the blot, hsp90 was visualized using monoclonal antibody AC88 (Fig. 1). This confirms that these anti-peptide antibodies specifically bind to hsp90. Hepa 1 cytosol was separated by SDS–PAGE, followed by transfer to PVDF membrane, followed by incubation with either anti-hsp86 or anti-hsp84 antibody. Bound antibody was visualized with peroxidase–anti-rabbit conjugate; the results revealed that each antibody specifically binds to its respective antigen (Fig. 2). We wanted to determine if these antibodies would cross-react with hsp90 in other species. The results in Fig. 3 demonstrate that both anti-hsp86 and anti-hsp84 antibodies cross-react with mouse, rat, and human hsp90 (Fig. 3).

One important question that we wanted to address is whether hsp86 and hsp84 exist has homo- or heterodimers. Immunoprecipitations from Hepa 1 cytosol were performed with each peptide antibody and subjected to SDS–PAGE. After electrophoresis and silver staining, approximately equal amounts of hsp86 and hsp84 were present in the anti-hsp86 antibody immunoprecipitate (Fig. 4). In contrast, hsp84 is predominantly

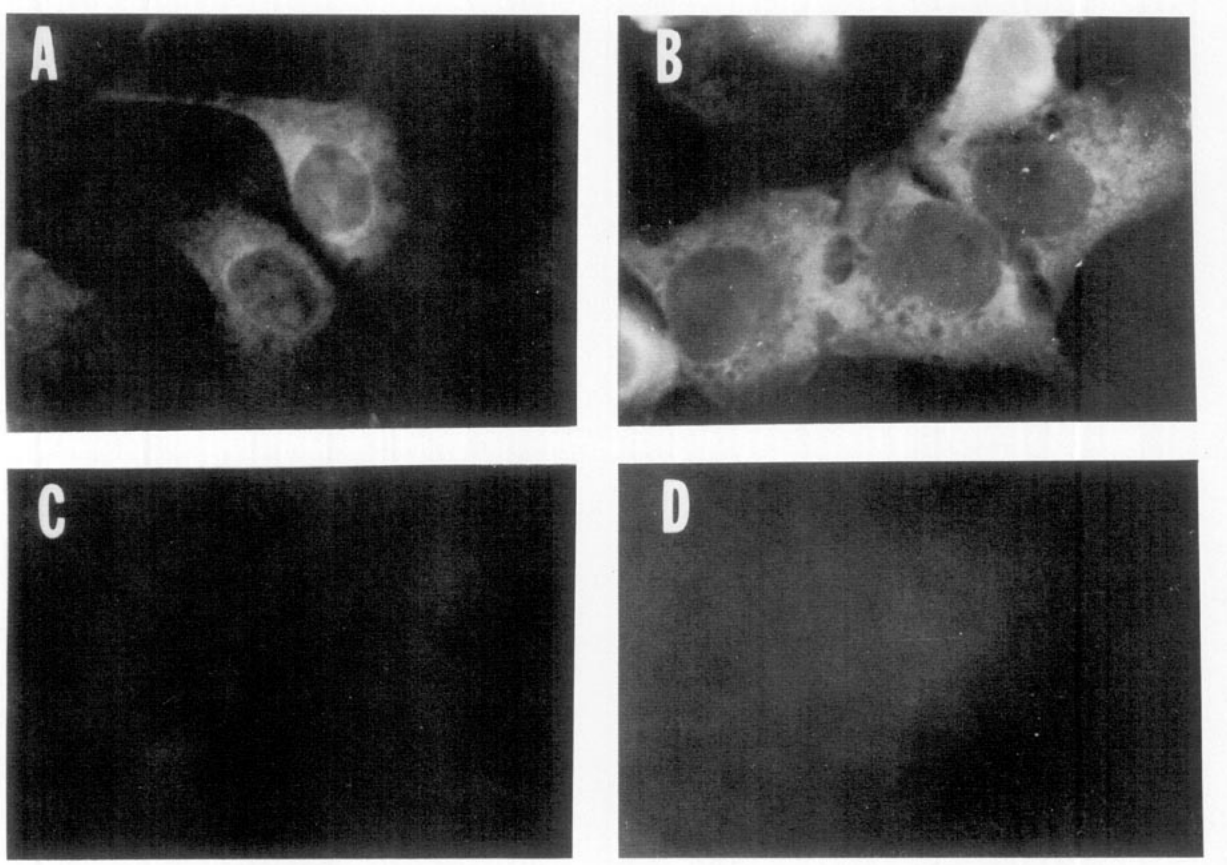

FIG. 7. Immunofluorescence micrographs of Hepa 1 cells stained with anti-hsp86 and anti-hsp84 antibodies. A, cells stained with anti-hsp86 antibodies; B, cells stained with anti-hsp84 antibodies; C, cells stained without primary antibody; D, cells stained with control rabbit IgG.

present in the hsp84 immunoprecipitate. These results would suggest that hsp86 forms heterodimers with hsp84, while hsp84 is present primarily as homodimers. In an effort to insure that we were examining dimeric hsp90 species, Hepa 1 cytosol was subjected to sucrose density gradient centrifugation. The presence of hsp90 in each fraction was assessed by SDS–PAGE and staining the resulting protein gel with Coomassie blue, hsp90 sediments in fractions 10–14 (Fig. 5). Immunoprecipitations of fractions 9–12 and 13–15 were performed to examine whether a different precipitation pattern was obtained with anti-hsp86 or anti-hsp84 antibodies. Results identical to immunoprecipitation of total cytosol were obtained (data not shown), thus suggesting that the heterodimer detected in anti-hsp86 immunoprecipitations is not due to the formation of large hsp90 aggregates. In order to better understand the immunoprecipitation results above we wanted to determine the ratio of hsp86/hsp84 in Hepa 1 cell cytosol. Previously we have determined that mAb 8D3 is able to almost totally deplete Hepa 1 cytosol of hsp90 [11]. An 8D3 immunoprecipitation of hsp90 from Hepa 1 cytosol was performed and subjected to SDS–PAGE with limiting SDS in the upper running buffer. After electrophoresis, the gel was stained with Coomassie blue. Densitometric analysis indicated that the ratio of hsp86 to hsp84 was 1 to 2. These results taken together would suggest that hsp84, being in higher concentration, forms more homodimers than hsp86.

At present, there have been no unique functions found that would explain the necessity of expressing both hsp84 and hsp86, two proteins of high sequence homology. One possible hypothesis would be that each form of hsp90 preferentially interacts with specific proteins. Previously in our laboratory, we have shown that hsp90 is complexed with *Ah* receptor [6]. Immunoprecipitation of the photoaffinity-labeled *Ah* receptor with either anti-hsp86 or anti-hsp84 antibodies revealed that both forms of hsp90 interact, either directly or indirectly, with the *Ah* receptor (Fig. 6). Whether the *Ah* receptor directly interacts with both hsp86 and hsp84 will require additional studies.

Indirect immunofluorescence studies using anti-hsp86 and anti-hsp84 antibodies were performed. As shown in Fig. 7, hsp86 in Hepa 1 cells is found predominantly in the cytoplasm, with a greater intensity of nu-

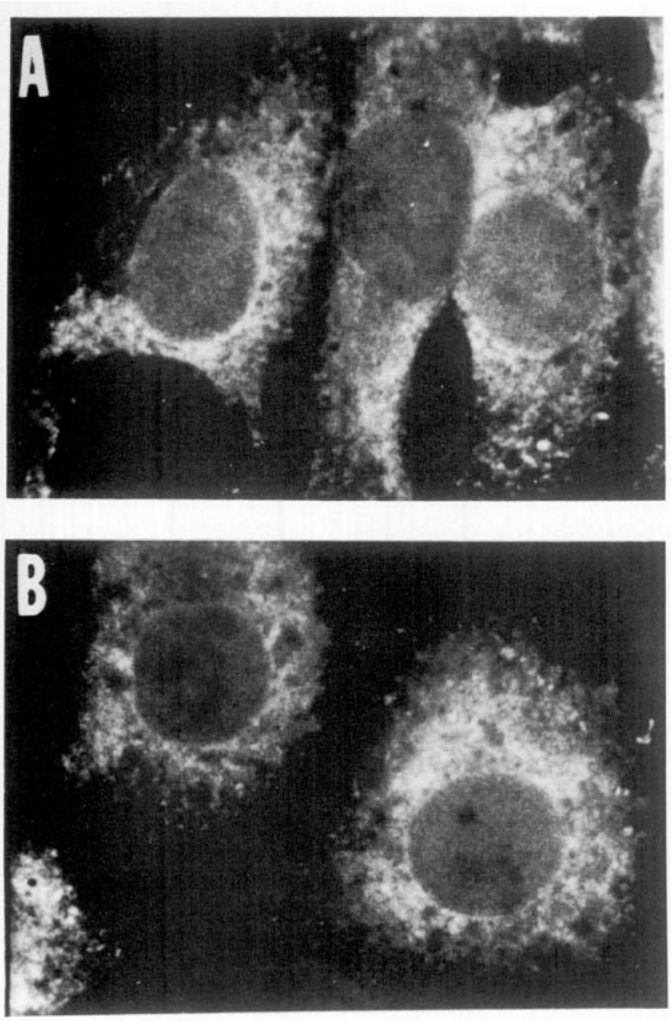

FIG. 8. Confocal micrographs of Hepa 1 cells stained with anti-hsp86 and anti-hsp84 antibodies. Midsectional photographs are shown. A, cells stained with anti-hsp86 antibodies; B, cells stained with anti-hsp84 antibodies.

clear staining relative to the predominantly cytoplasmic fluorescence seen in cells stained with hsp84 antibodies. Exclusion of immunofluorescence from nucleoli was observed with both hsp86 and hsp84 antibodies. Confocal microscopy imaging through the depth of Hepa 1 cells revealed that both hsp86 and hsp84 are found in the nucleus (Fig. 8). Utilizing confocal microscopy, hsp84 localization appeared throughout the cytoplasm. In contrast, hsp86 staining was more perinuclear and was asymmetrically distributed in the cytoplasm. In most cells, hsp86 appears to stain more strongly the cytoplasm near the periphery of the cell. Heat shock treatment of Hepa 1 cells did not appear to affect the distribution of either hsp86 or hsp84.[3]

[3] Data not shown.

DISCUSSION

Most studies on hsp90 have treated hsp90 as a single protein, despite the fact that hsp90 is really two separate gene products. There have been no functional differences described for the two forms of hsp90. Perhaps the main reason for this is the lack of antibodies specific to each form of hsp90. In this report, two rabbit polyclonal, affinity-purified anti-peptide antibodies have been generated. Western blot analysis indicated that each antibody specifically binds to either hsp86 or hsp84.[4] Hsp90 exists predominantly in cytosolic preparations as a dimer [27, 28]. Both hsp86 and hsp84 antibodies were used in immunoprecipitations (Fig. 6), the results suggest that hsp86 forms heterodimers with hsp84 and hsp84 exists in Hepa 1 cytosol predominantly as homodimers. To understand these results, hsp90 was immunoprecipitated from Hepa 1 cytosol, using a monoclonal antibody that binds to both forms of hsp90, and subjected to SDS–PAGE.

The results indicated that the ratio of hsp86/hsp84 is 1 to 2. Presumably, the higher concentration of hsp84 in the cell would lead to more homodimers of hsp84 being formed. In contrast, hsp86 forms largely heterodimers with hsp84, perhaps because of the higher concentration of hsp84 present in the cell. In contrast to these results, studies performed in mouse L cells using a two-dimensional gel electrophoresis system have revealed the presence of mostly homodimers of hsp86 and hsp84 [28]. The differences seen in this study may be due to the use of different cell lines or the methods of analysis. In addition, the amount of hsp86 relative to hsp84 is different in mouse L versus Hepa 1 cells. Whether hsp86 and hsp84 exist as homo- or heterodimers needs to be established in each cell culture system to be studied.

The ability of the antibodies to coprecipitate the *Ah* receptor would indicate that association with at least some proteins does not block the N-terminal end of hsp86/hsp84. This is an important observation when considering the ability of these antibodies to recognize the total pool of hsp90 in the cell.

A number of studies have examined the distribution of hsp90 in various cell types using different antibody preparations, and several have suggested that hsp90 is found largely localized along microtubules [29–31]. In contrast, other investigators also using immunocytochemical techniques have found that hsp90 is distributed throughout the cytoplasm, with some detectable nuclear localization [32, 33]. Most of these studies have used different antibody preparations that probably react with different epitopes. Also, whether these anti-

[4] hsp86 and hsp84 peptide antibodies are currently available from Affinity BioReagents (1-800-527-4535).

bodies are capable of recognizing hsp90 complexed with other proteins is not clear. In this report we wanted to examine whether hsp86 and hsp84 are differentially localized. Since no functional difference has yet been described for each form of hsp90, one potentially important difference could be where each form is localized in the cell. Because hsp86 heterodimerizes with hsp84, a marked difference in localization would not be expected to occur in Hepa 1 cells. The confocal microscopy results, however, suggest that hsp86 and hsp84 are differentially localized.

In summary, these studies have indicated that antibodies specific to hsp86 and hsp84 can be produced that are useful for specific immunoprecipitation of hsp86/hsp84 complexed with other proteins and can be used in immunocytochemical localization of each form of hsp90. These results should stimulate further work on hsp86/hsp84 localization in various tissues and cells. In addition, these antibodies should provide a critical tool to answer the question of whether hsp86 and hsp84 have distinct cellular functions.

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