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Antibodies to Two Major Chicken Heat Shock Proteins Cross-React with Similar Proteins in Widely Divergent Species

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Three of the proteins induced by heat shock of chicken embryo fibroblasts have been purified, and rabbit antibodies have been raised against them. These antibodies have been used in radioimmune precipitation reactions and in a solid-phase immune assay to detect antigenic material in non-heat-shocked chicken tissues and in extracts of widely different species ranging from yeast to mammalian tissue culture cells and human erythrocyte ghosts. Antibodies to two of the major chicken heat shock proteins, chsp89 and chsp70, cross-reacted with proteins of similar molecular weights in normal embryonic and adult chicken tissues and in extracts from widely different organisms. These data provide further evidence for the universality of the heat shock response and conservation of proteins induced by this type of stress.

Chicken embryo fibroblasts (CEF) dramatically change their pattern of protein synthesis when incubated for short times at 45°C (12, 13). When examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, extracts of cells treated at high temperatures showed enhanced synthesis of three proteins with molecular weights of 89,000, 70,000, and 24,000. (Molecular weights previously reported were 95,000, 76,000, and 22,000.) This response to heat shock was quite similar to that reported earlier for drosophila (reviewed in reference 2) and more recently described for a variety of organisms including yeast (22), amoebae (32), slime molds (20), and plants (3, 14), as well as mammalian tissue culture cells (13, 27). Procaryotes also show induction of specific protein synthesis after heat shock (17, 34).

Drosophila has been the principal organism used for studying the heat shock phenomenon, and a number of laboratories have established that high temperature activates the transcription of a few specific genes, leading to mRNA's which appear to be selectively translated to yield the heat shock proteins (9, 15, 19, 26, 29). The presence of these proteins or, more likely, an increased level of them protects this organism from the temperature stress (23, 25). A similar kind of protection has been found for heat shock proteins in yeast (22), slime mold (20), and animal cells (J. R. Subjeck et al., personal communication). The precise function of the heat shock proteins and how they provide for thermotolerance are not yet known.

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In extending our studies of the heat shock response in avian cells and seeking a function for these proteins, we have purified the three major chicken heat shock proteins and prepared antibodies to them. A solid-phase immune assay based on the electrophoretic transfer of protein from SDS-polyacrylamide gels to diazotized paper (4, 30) was utilized to determine the distribution of these proteins in various non-heat-shocked tissues of chickens. In addition, we surveyed a wide range of organisms for proteins that might cross-react with our anti-chicken heat shock protein antibodies and found that two of the chicken heat shock proteins are strongly conserved in nature.

MATERIALS AND METHODS

Purification of chicken heat shock proteins. Fifteen roller bottles of CEF primary cells were brought to confluence in minimal essential medium supplemented with 3% fetal bovine serum at 37°C and transferred to an air incubator equilibrated at 45°C. After transfer, 3 to 4 h were required for the media to reach 45°C, and cells were kept at 45°C for an additional 4 h. They were transferred to a 37°C incubator, and the media in each of three bottles were replaced with 10 ml of minimal essential medium-methionine, 20 mM HEPES, pH 7.4 (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), and 50 μCi of [^{35}S]methionine (~1,000 Ci/mmol; New England Nuclear Corp.). After 2 h at 37°C, all cells were washed twice with cold phosphate-buffered saline (PBS; 0.137 M NaCl, 3 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4, pH 7.0), scraped into 300 ml of cold PBS, and washed once more in PBS. The cells were suspended in 50 ml of PBS and disrupted by sonication. The extract was made 0.1 mM with phenylmethanesulfonyl fluoride and centrifuged for 10 min at 10,000 rpm in a Sorvall type SS-34 rotor at 4°C (12,100

× g). The supernatant fractions was centrifuged for 2 h at 45,000 rpm (176,700 × g) in a Beckman type 65 rotor at 4°C. Approximately 34 mg of protein per roller bottle was extracted, and 26% of this protein was recovered in the 176,000 × g supernatant fraction (cytosol). The cytosol was concentrated 10-fold by Diaflo ultrafiltration, using an Amicon PM-30 membrane, and insoluble material was removed by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor. Initial fractionation was carried out on a Sepharose 6B column (86 by 2.5 in. [215 by 6.25 cm]) that had been calibrated with proteins of known molecular weights. Samples from column fractions were precipitated with trichloroacetic acid, suspended in gel loading buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.06 M Tris-hydrochloride, pH 6.8), and analyzed by SDS-polyacrylamide gel electrophoresis (16). The chsp24 protein was eluted from this column with a volume (V_e) equivalent to a protein of 185,000 molecular weight. Fractions enriched in chsp24 were pooled, concentrated by Diaflo ultrafiltration, made 2% in SDS–5% β-mercaptoethanol–10% glycerol, heated for 10 min, and separated on a 10% preparative slab acrylamide gel (0.3 by 14 by 15 cm). The chsp24 protein band was located by staining a small segment of the gel with Coomassie blue, and the entire band was cut from the gel, pulverized by passing the gel through a 5-ml syringe, and eluted in H_2O overnight. The eluted protein was dialyzed against H_2O and lyophilized; this material showed a single band of protein by SDS-gel electrophoresis. After isoelectric focusing, a cluster of two to four proteins was detected at pH 5.4. Four milligrams (0.8% of total protein in the initial extract) of pure chsp24 was obtained from 15 roller bottles.

chsp89 eluted from the Sepharose 6B column with a V_e equivalent to a protein of 510,000 molecular weight. Fractions enriched for this protein were pooled, dialyzed twice against 20 mM HEPES (pH 7.3)–0.02% sodium azide, and pumped onto a column (diameter, 1.2 cm; height, 6 cm) packed with DEAE-cellulose (Whatman DE-52) equilibrated at 4°C in 20 mM HEPES, pH 7.3. After washing with 10 column volumes, protein was eluted with a 500-ml linear gradient of 0 to 0.5 M NaCl in 20 mM HEPES, pH 7.3. chsp70 eluted from the Sepharose 6B column with a V_e equivalent to a protein of 165,000 molecular weight. Fractions enriched in chsp70 were separated by DEAE chromatography as above except that a 0 to 0.4 M NaCl gradient was used. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and those enriched for chsp70 and chsp89 were pooled, dialyzed twice against H_2O, lyophilized, and suspended in gel loading buffer. Samples were boiled for 5 min and separated preparatively on SDS-polyacrylamide slab gels. The chsp89 and chsp70 bands were detected by staining the gel with 4 M sodium acetate (10). Protein bands were cut from the gels and eluted as described for chsp24.

Preparations of antibodies. Six-month-old New Zealand female rabbits were bled to obtain preimmune sera. Purified proteins were mixed in Freund complete adjuvant (Difco Laboratories), and 1 mg of chsp24 or chsp70 and 0.5 mg of chsp89 were injected into footpads. Two weeks after the initial injection, additional protein (0.5 mg of chsp24, 0.25 mg of chsp70 and chsp 89) in complete Freund adjuvant was injected. Ten days after this boost, 30 ml of serum per rabbit was collected. Antibodies were further purified by two precipitations with 40% ammonium sulfate, dialyzed against PBS, clarified by centrifugation, and stored at −20°C.

Radioimmune precipitation. [^{35}S]methionine-labeled cultures (10^7 cells) were washed three times with PBS and lysed with 1 ml of a buffer (RIPA) consisting of 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.05 M Tris-hydrochloride (pH 7.2), and 100 kallikrein inactivator units of Trasylol per ml (7). The lysate was blended in a Vortex mixer and centrifuged for 1 min at 16,000 × g in a microfuge.

Extracts containing about 10^5 cpm were mixed with 5 μl of antibody and incubated at room temperature for 20 min. A 100-μl sample of a 10% solution of Formalin-treated *Staphylococcus* protein A (8) was added, and after 20 min, the mixture was pelleted in the microfuge. Pellets were washed twice with 1 ml of RIPA containing 0.1 M NaCl and once with RIPA. The pellet was suspended in 50 μl of gel loading buffer, boiled for 1 min, and pelleted for 1 min in the microfuge. The supernatant fraction was analyzed by SDS-polyacrylamide gel electrophoresis.

Preparation of cell and tissue extracts. Cell monolayers were harvested by washing twice in PBS, scraping in a small volume of PBS, and pelleting at 2,000 rpm in a Sorvall SS-34 rotor. Animal biopsies, or tissues from other organisms, were stored frozen at −70°C. Tissue culture cells, animal biopsies, or tissues from various organisms were suspended in 2% SDS–50 mM Tris (pH 7.4) and sonicated, and a sample was removed for protein determination (21). Yeast was extracted by methods described by McAlister and Finkelstein (22). The samples were made 5% with β-mercaptoethanol mixed with gel loading buffer, boiled, and analyzed on SDS-polyacrylamide gels.

Solid-phase immune assay. Diazophenylthioether paper was prepared according to Brian Seed (personal communication). SDS-polyacrylamide gels were rinsed in distilled water and transferred to diazophenylthioether paper in a buffer of 25 mM sodium phosphate, pH 6.5, on an apparatus described by Bittner et al. (4). Immunoautoradiography of diazo paper was carried out by the procedure described by Symington et al. (30) modified by incubating antibody and ^{125}I-labeled protein A in Seal-a-meal bags (Dazey Products Co.). Nonspecific binding was reduced by extended overnight washing after the ^{125}I-labeled protein A incubation.

Preparation of ^{125}I-labeled protein A. A 10-μg portion of protein A (Pharmacia Fine Chemicals, Inc.) in 30 μl of 0.5 M phosphate buffer, pH 7.0, was added to 10 μl of ^{125}I (100 mCi/ml) and mixed with 10 μl of chloramine-T (2.25 mg/ml). After 10 s, 10 μl of sodium metabisulfite (6.9 mg/ml) and 10 μl of potassium iodide (10 mg/ml) were added and mixed for 30 to 60 s, and the mixture was loaded on a Sepharose G-25 column (0.5 by 15 cm) previously equilibrated in PBS plus 1% bovine serum albumin. Fractions of 1 ml were collected, and the initial peak tube of ^{125}I radioactivity was reserved for these experiments; it showed a single radioactive band on an SDS-polyacrylamide gel.

Ouchterlony analysis. Ouchterlony plates contained 1% agarose, 50 mM Tris (pH 8.0), and 0.02% sodium azide. Antibody and extracts were used undiluted, and plates were incubated overnight at 30°C. The plates

were washed twice for 12 h in PBS, stained with Coomassie blue (0.025% Coomassie blue, 10% acetic acid, 50% methanol), and destained overnight in 5% acetic acid–10% methanol.

Partial proteolytic peptide analysis. Whole-cell extracts were acetone precipitated, dried, and suspended at 40 μg/40 μl in 0.125 M Tris-hydrochloride (pH 6.8)–0.5% SDS–10% glycerol–0.0001% bromophenol blue. Protease digestion was performed as described by Cleveland et al. (6). The partial proteolytic digests were separated on a 15% SDS gel, transferred to diazophenylthioether paper, and probed with specific antibodies as described above.

$^{32}P_i$ labeling of CEF. Cells were plated and grown to confluence in small glass scintillation vials at 37°C. Media were made 20 mM with HEPES, pH 7.4, and sealed with plastic caps immediately before the experiment. One vial was incubated for 1 h in a 45°C water bath. Cells were washed twice with phosphate-free minimal essential media and incubated for 4 h with 250 μCi of $^{32}P_i$ in 1 ml of phosphate-free minimal essential medium plus 0.1% fetal calf serum at 37°C. Cells were harvested by removing the labeling medium and washing twice with PBS. RIPA buffer (100 μl) was used to solubilize cells. The extracts were centrifuged for 1 min at 16,000 × *g*, and the supernatant fraction was analyzed by radioimmune precipitation.

Phosphoamino acid determination of chsp89. ^{32}P-labeled cell extracts were immunoprecipitated with anti-chsp89, and the proteins were separated on a 10% SDS preparative slab gel. The wet gel was exposed to film, and chsp89 was located, cut from the gel, and electrophoretically eluted into a dialysis bag. A 20-μg amount of bovine serum albumin was added and protein precipitated with acetone at −20°C. The precipitate was collected by centrifugation, dried, and hydrolyzed for 3 h at 100°C in 1 ml of 6 N HCl. This sample was dried under nitrogen, taken up in 100 μl of a buffer containing pyridine-acetic acid-water (1:10:300), and separated by high-voltage paper electrophoresis at pH 3.5. Phosphoserine and phosphothreonine were included in the electrophoresis as standards.

RESULTS

Characterization of rabbit antibodies against heat shock proteins. Preparations of purified, SDS-denatured heat shock proteins were used as immunogens for preparing monospecific antibodies from rabbits. Immunoglobulin fractions of rabbit sera that showed antibody activity were tested initially by radioimmune precipitation of extracts from CEF cells labeled with [^{35}S]methionine. We compared sonified extracts from normal cells, cells heat shocked for 1 h at 45°C, and cells treated with 50 μM arsenite for 3 h (Fig. 1). The latter treatment has been shown to induce proteins identical to the major chicken

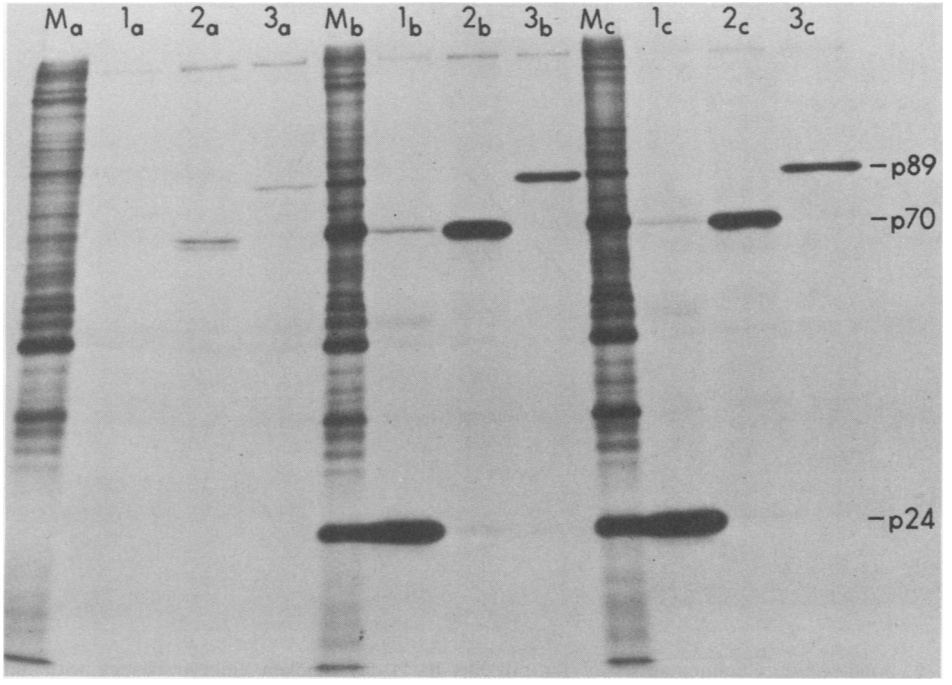

FIG. 1. Specificity of antibodies raised against chicken heat shock proteins. [^{35}S]methionine-labeled cell extracts from untreated CEF (a), CEF heat shocked for 1 h at 45°C (b), and CEF treated for 3 h with 50 μM sodium arsenite (c) were prepared. A total of 10^5 cpm of [^{35}S]protein were mixed with 5 μl of a 1/10 dilution of antibodies, precipitated as described in the text, and separated on a 10% SDS-polyacrylamide gel. M, Total extract. Lane 1 protein precipitated with anti-chsp24; lane 2 protein precipitated with anti-chsp70; and lane 3 protein precipitated with anti-chsp89.

cell heat shock proteins (11). Antibodies against chsp89 precipitated an 89,000-dalton protein in normal cells as well as in the heat-shocked and arsenite-induced cells. Antibodies against chsp70 precipitated two proteins from normal cells with molecular weights of 68,000 and 70,000 as well as the 70,000-dalton protein induced by arsenite and heat shock. Antibodies against chsp24 protein did not detect protein synthesized by normal embryo fibroblasts, but they showed strong reactivity with the chsp24 protein induced by the arsenite and heat treatments. A small amount of the chsp70 protein coprecipitated in these latter reactions (lanes 1_b and 1_c, Fig. 1). The very large effect on biosynthesis (as measured by [^{35}S]methionine incorporation) of these three proteins by heat shock and arsenite poisoning is clearly shown in Fig. 1.

Solid-phase immune assay for heat shock proteins in normal chicken cells. These antibodies were also used to determine whether heat shock proteins could be detected in cells from a nontemperature-stressed 11-day chicken embryo as well as in adult chicken organs. Sonified extracts of various tissues were heated in SDS, electrophoresed in polyacrylamide slab gels, and electrophoretically transferred to diazotized paper. An equivalent set of extracts was electrophoresed in SDS-polyacrylamide slab gels and stained with Coomassie blue. The proteins that had been transferred to diazotized paper were reacted sequentially with the three antibodies, but after each antibody addition, the paper was treated with ^{125}I-labeled protein A. It should be noted that this kind of analysis, in contrast to that described above, measures the levels of nonradioactive protein present in the various tissues. The pattern of proteins from six tissues of the 11-day chicken embryo are shown in Fig. 2 (panel 1) and those proteins reacting with anti-chsp89, anti-chsp70, and anti-chsp24 are shown in the autoradiograms of Fig. 2A, B, and C, respectively. All of the tissues contained protein that cross-reacted with chsp89 and chsp70. There was a relatively small increase in the amount of 89,000-dalton protein after heat shocking fibroblasts for 1 h but a significant increase (about a doubling) in the 70,000-dalton protein (cf. lanes 7 and 8 in Fig. 2A and B). Non-heat-shocked cells showed small amounts of chsp24 (lane 8, Fig. 2C) and a dramatic increase in the level of this protein after heat shock. Cells from chicken muscle, however, contained a protein migrating slightly faster than the chsp24 that reacted with anti-chsp24 antibodies. Analogous results were obtained when the solid-phase immune assay was used with extracts from adult chicken tissues. Only proteins in chicken plasma

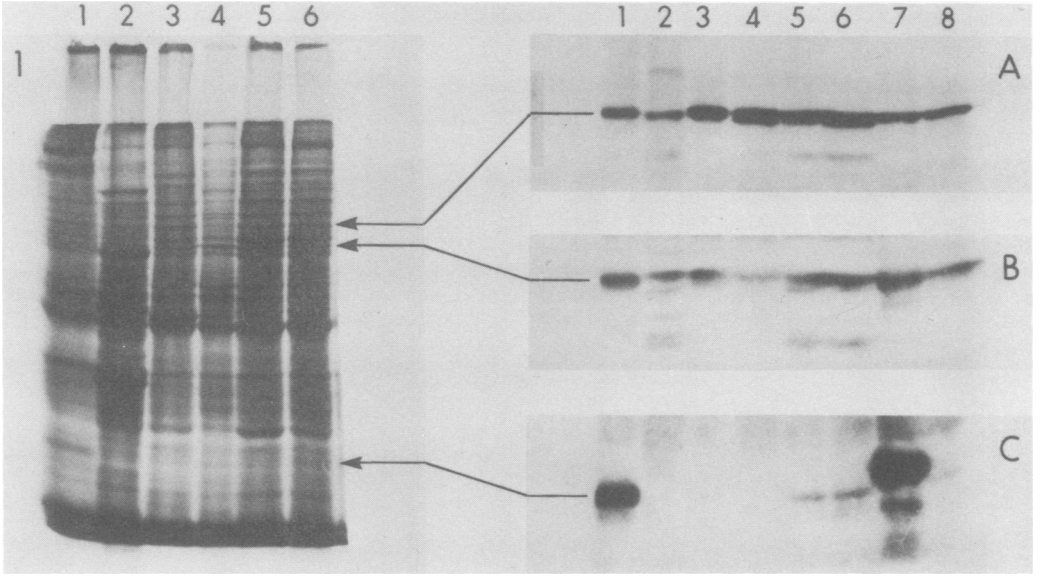

FIG. 2. Solid-phase radioimmune assay for proteins in 11-day chicken embryo tissues with anti-chsp antibodies. An 11-day embryo was dissected to obtain the heart, liver, eye, and brain. The remaining carcass was divided into two parts, head and body. Sonified extracts were made of each biopsy, and equal amounts of protein (100 μg) were loaded onto identical 10% SDS-polyacrylamide gels. One gel was stained with Coomassie blue (panel 1). Proteins in the other gel were transferred to diazophenylthioether paper and probed with anti-chsp89 (A), anti-chsp70 (B), and anti-chsp24 (C). Lanes 1, heart; 2, liver; 3, eye; 4, brain; 5, carcass head; 6, carcass body; 7, heat-shocked CEF cells; 8, normal CEF cells.

failed to cross-react with the anti-chsp antibodies; all other tissues contained 70,000- and 89,000-dalton proteins, and all muscle tissues contained a 22,000- to 24,000-dalton protein which reacted with the antibodies. The weakly reacting bands in Fig. 2A and B are attributed to proteolytic degradation products that retained antigenicity.

We have examined the antigenic relationship between the proteins detected in normal cells and those induced by heat-shocked fibroblasts. Extracts from heat-shocked and normal tissue culture cells and adult chick brain and heart tissue were analyzed by Ouchterlony double diffusion with the three antibodies. Precipitin lines of identity were detected with extracts of the adult tissues and CEF cells that reacted against anti-chsp89 (Fig. 3C). The protein from normal and heat-shocked CEF cells that reacted with anti-chsp70 were also antigenically identical (Fig. 3B). Other data indicated that both the normal and the heat-shocked 70,000-dalton proteins were closely related. We used the anti-chsp70 antibodies and the solid-phase immune assay procedure to examine extracts from normal and heat-shocked cells that had been treated with various amounts of a protease according to the method of Cleveland et al. (6). Four major peptides from the protease-digested extracts of chsp70 and normal 70,000-dalton protein reacted with the antibodies, and these peptides had identical mobilities in SDS gels (data not presented).

With anti-chsp24 a precipitin line was detected with extracts from normal CEF cells and adult heart tissue; no lines were detected for extracts from adult brain tissue (Fig. 3A). Lines of antigenic identity were seen between the normal CEF cells and adult muscle tissue, but the spur detected between heat-shocked and normal CEF indicates that the two cross-reacting proteins share only partial antigenicity.

Proteins in other organisms cross-reacting with anti-chsp70 and -89. Several of the proteins made after heat shock of widely different organisms appear to have similar subunit molecular weights. We were interested, therefore, to determine whether there were proteins in these organisms that could cross-react with our anti-chicken antibodies. We used the solid-phase immune assay and examined extracts from a variety of organisms, some of which had been subjected to heat shock or arsenite treatment. We show in Fig. 4 results with anti-chsp70 antibodies applied to extracts from tissue culture cells of chickens, humans, and hamsters and *Drosophila* embryos. Table 1 lists results of this survey with several other organisms representing fungi, plants, nematode, amphibia, and rodents. Anti-chsp70 reacted with a 70,000-dalton protein in virtually every species examined, and in *Drosophila* and yeast there was about a twofold increase in the mass of this protein after heat shock. Anti-chsp89 had almost as wide a range of cross-reactivity–reacting with an 89,000-dalton protein in fly, frog, mouse, and human cells. Thus, the chsp70 and -89 proteins belong to a class of highly conserved proteins. The antibodies against chsp24 were specific for chicken protein.

The membrane fraction (ghosts) of human erythrocytes was lacking in the 70,000- and 89,000-dalton proteins. This result is consistent with other data indicating that these proteins are not intrinsic membrane proteins (unpublished data). In fact, we purified these proteins from the cell cytoplasm (see Materials and Methods), where they were most abundant.

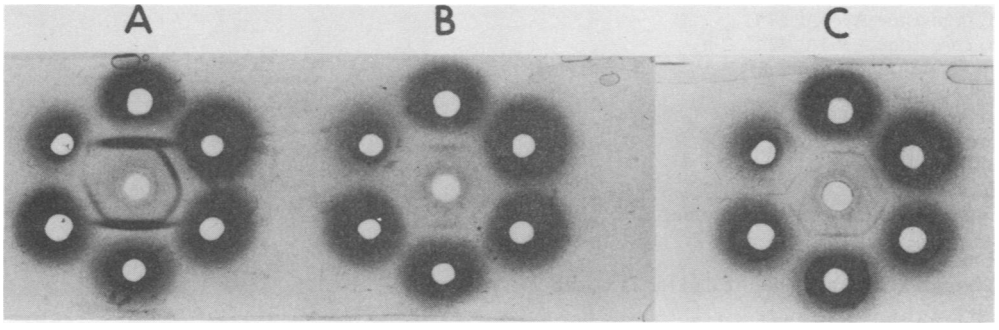

FIG. 3. Relationship of heat shock proteins to normal cell proteins by Ouchterlony double-diffusion analysis. Cell extracts of normal CEF, heat-shocked (4 h) CEF, adult chicken brain, and adult chicken heart were made by sonifying in PBS and centrifuging for 1 min in Eppendorf tubes at 16,000 × *g*. A 5-μl portion of cell extract (3 mg/ml) or 5 μl of chsp antibody (10 mg/ml) was added per well (see text). (A) Anti-chsp24; (B) anti-chsp70; (C) anti-chsp89. Antibody was placed in center wells. Cell extracts were placed in outer wells. Starting with the top well and moving clockwise, the extracts were as follows: 1, heat-shocked CEF; 2, adult chicken heart; 3, normal CEF; 4, same as 1; 5, same as 3; 6, adult chicken brain.

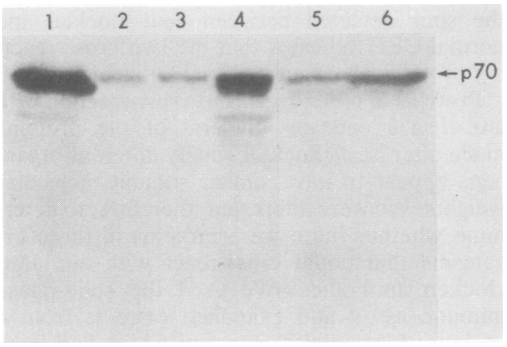

FIG. 4. Solid-phase radioimmune assay of extracts from various organisms that were probed with anti-chsp70 antibodies. All samples tested were sonified in 2% SDS containing 50 mM HEPES, pH 7.4. Lane 1, 50 μg of heat-shocked CEF; 2, 80 μg of human WI38 cells; 3, 100 μg of BHK-21 cells; 4, 100 μg of CEF transformed with Rous sarcoma virus; 5, 50 μg of *Drosophila* embryos grown at 25°C, dechorionated, ground in acetone, and dried before extraction; 6, identical to 5, except embryos were grown at 37°C for 30 min and then at 25°C for 30 min before preparation.

Phosphorylation of heat shock proteins. During a series of experiments designed to test for various enzymatic activities of the heat shock proteins, we labeled cells with $^{32}P_i$ and assayed for label after immunoprecipitation of extracts with the different antibodies. Only anti-chsp89 precipitated a ^{32}P-labeled protein, and this protein comigrated with chsp89 (Fig. 5, lane 3). This protein appeared to be one of the major phosphate-labeled proteins in both normal and heat-shocked fibroblasts. We isolated the ^{32}P-labeled p89, digested it with acid, and determined that the phosphate was bound to serine (data not shown).

DISCUSSION

The most striking result from the studies presented here is that highly diverse eucaryotic organisms have proteins antigenically related to two of the major chicken heat shock proteins. Those cross-reacting proteins had subunit molecular weights almost identical to chsp70 and chsp89. In *Drosophila* and yeast, these same two cross-reactive proteins are ones whose synthesis is induced by heat shock. We predict that other species induce these same proteins when stressed by high temperatures.

What role these two proteins play in a cell's response to this kind of stress is most intriguing. They apparently can protect the cell from thermal stress, but how this occurs is unknown. Our data show that the 70,000- and 89,000-dalton proteins are present in most cells of the chicken under normal conditions. We purified the proteins from high-speed supernatant fractions of tissue culture cell extracts; thus, they appear to be localized primarily in the cell cytoplasm. Others have reported finding some fraction of these particular proteins in the cell nucleus (1, 31). Preliminary results of immunofluorescence of methanol-fixed normal and heat-shocked

TABLE 1. Solid-phase immune assay of cell extracts with antibodies to chicken heat shock proteins[a]

Source of cell extract	Antibody		
	Anti-p24	Anti-p70	Anti-p89
Yeast			
S. cerevisiae A364A, 24°C	−	+	−
S. cerevisiae A364A, 42°C, 2 h		++	
Slime mold (*D. discoideum*)	−	+	−
Dinoflagellate (*P. cinctum*)	−	+	−
Corn seedling roots	−	+	−
Worm (*Caenorhabditis elegans*)	−	+	−
Fruit fly			
D. melanogaster embryos, 25°C	−	+	+
D. melanogaster embryos, 30 min, 37°C	−	++	+
Frog			
Xenopus kidney cell line	−	+	+
Xenopus kidney cell line, treated for 24 h with 50 μM arsenite	−	++	+
Mouse			
L929 cells	−	+	+
L929 cells, treated for 5 h with 50 μM arsenite		++	+
Human			
WI38 cells	−	+	+
Erythrocyte ghosts[b]	−	−	−

[a] 70 to 100 μg of protein was analyzed (see text).
[b] Isolated by the procedure of Steck and Kant (28).

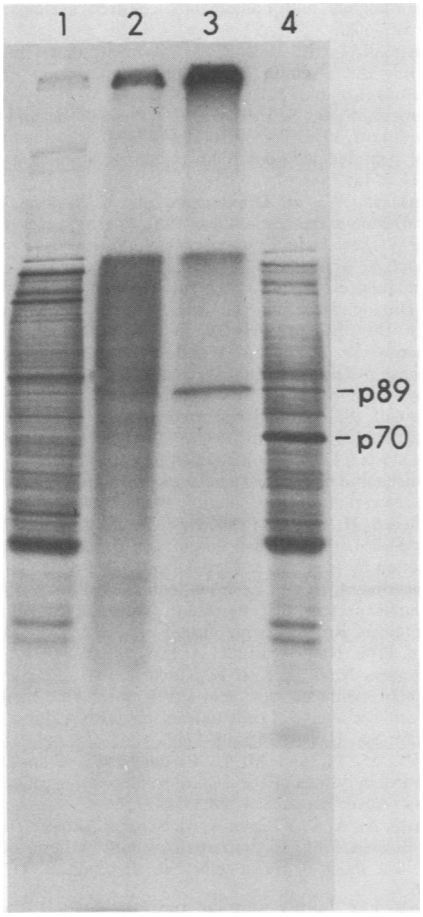

FIG. 5. In vivo phosphorylation of chsp89. Lane 1, Untreated CEF labeled with [^{35}S]methionine; 2, untreated CEF labeled with $^{32}P_i$; 3, ^{32}P-labeled protein from extracts used in lane 2 and immunoprecipitated with anti-chsp89; 4, heat-shocked cells labeled with [^{35}S]methionine. Samples were electrophoresed in a 15% acrylamide–SDS slab gel and autoradiographed.

CEF with anti-chsp70 showed general staining of the cell cytoplasm, with some staining of stress fibers; anti-chsp89 showed a diffuse staining, some of which was over cell nuclei (unpublished data). Wang et al. (33) detected a 70,000-dalton heat shock protein associated with brain microtubule preparations, and some of this protein remained bound to intermediate filaments enriched in Triton-HCl cytoskeleton preparations. This protein was methylated and was also a component of avian myofibril Z disks.

chsp89 is a major phosphorylated protein in the cell, and this protein has been detected in a complex with the *src* protein kinase isolated from virus-transformed CEF cells (5, 24). It is possible that chsp89 is complexed with other cell proteins since the protein fractionated on a Sepharose 6B column as if it had a molecular weight of 510,000.

The chsp24 protein was detected only in avian species. It was made in large amounts in heat-shocked cells and was present in low amounts in normal embryo fibroblasts. Immunofluorescence staining of normal as well as heat-shocked CEF cells with antibodies against this protein showed a pattern similar to that of the anti-chsp70 (unpublished data). A muscle cell protein of similar molecular weight was found to be antigenically related to the chsp24, but we need to purify these two proteins and analyze their tryptic peptide maps to determine their identity. It is noteworthy, however, that Atkinson (2a) has reported a decreased induction of the chsp24 by heat shock as myoblasts differentiated to myotubes.

Proteins identical to the heat shock proteins have been detected in chicken cells given low concentrations of arsenite, heavy metal, and reagents that oxidize sulfhydryl groups (11, 18). Some of these data suggested to us that the heat shock proteins might be key glycolytic enzymes. We measured the activities of several of these enzymes in normal and heat-shocked fibroblasts and also tested for blocking of activity with our antibodies. We failed to show that glycogen phosphorylase, hexokinase, phosphofructokinase, lactic dehydrogenase, pyruvic kinase, and myokinase were significantly changed by heat shock or by the presence of antibodies against the heat shock proteins. There were significant increases in glucose transport and in lactic acid production after heat shock, but these activities do not seem to be directly associated with the heat shock proteins themselves. The ubiquitous presence of these proteins in nature and their induction by temperature stress of many diverse organisms speaks to an important role for them in maintaining vital cellular activities. Precisely how this is done remains an important unsolved problem in cell biology.

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ADDENDUM

When extracts of $^{32}P_i$-labeled cells, incubated at either 37 or 45°C for 3 h, were prepared by boiling with gel loading buffer before diluting 10-fold into RIPA buffer, ^{32}P label was detected in a 70,000-dalton protein immunoprecipitated with anti-chsp70 antibodies and in a 22,000-dalton protein precipitated with anti-chsp24 antibodies. Loomis has found that two forms of a 70,000-dalton protein made in heat-shocked *Dictyostelium* cells were phosphorylated on threonine and serine residues (personal communication).

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