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Quantitation and Intracellular Localization of the 85K Heat Shock Protein by Using Monoclonal and Polyclonal Antibodies

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Two monoclonal antibodies have been produced against the human 85,000-molecular-weight heat shock protein (hsp85). One of these, 16F1, cross-reacts with the murine homolog and is shown by peptide map immunoblots to be directed against an epitope different from that recognized by the other monoclonal antibody, 9D2. Both monoclonal antibodies recognize only a single M_r-85,000 species in two-dimensional immunoblots. Immunoprecipitation did not reveal an association of this heat shock protein with any other protein in HeLa cells. Immunoperoxidase staining showed a purely cytosolic distribution at both light and electron microscopic levels and no association with membranes, mitochondria, or other organelles. The 9D2 monoclonal and a polyclonal antimurine hsp85 antibody were used to identify the antigens and to quantitate their levels in a variety of normal tissues by immunoautoradiography. Relative abundance in the various tissues as determined by Coomassie blue staining correlates reasonably well with the immunoreactivity. Testis and brain, for example, have high hsp85 levels, whereas heart and skeletal muscle have little or none. The M_r-85,000 sodium dodecyl sulfate-polyacrylamide gel band in testis and brain lysates was further confirmed to be hsp85 by one-dimensional partial proteolytic peptide mapping. Based on these data and our previous observations showing that synthesis and levels of the protein are altered by depriving cultured cells of glucose, we speculate that intracellular hsp85 levels depend on differences in the intermediary metabolism of glucose in the various tissues. Furthermore, it appears that high basal levels of this heat shock protein may not necessarily protect cells against heat shock, since testis is one of the most heat-sensitive tissues and has the highest hsp85 level.

For several years, work in our laboratory has been concerned with the characterization of an abundant cytoplasmic protein migrating with an apparent M_r of 85,000 (85K protein) in sodium dodecyl sulfate (SDS)-polyacrylamide gels. We described the purification and preliminary characterization of the denatured protein (22) and established that its synthesis and turnover are regulated when L929 cells are maintained in the absence of glucose (14, 21). Based on mobility in SDS-polyacrylamide gels and one-dimensional partial proteolytic peptide maps, we concluded that this protein is identical with one of the pp60^{src}-associated proteins (23). Subsequently, we showed that synthesis of this protein is induced, along with several others, by exposure to elevated temperatures (15, 21, 24), i.e., that it is a heat shock protein (hsp85).

The heat shock proteins are synthesized by a wide variety of cells after exposure to elevated temperatures. Although it has been suggested that these highly conserved proteins may participate in opposing the lethal (12, 22, 26, 35) or metabolic (10, 21) effects of hyperthermia, their function is still essentially unknown. Since certain functions might be excluded or suggested by a unique intracellular location, e.g., intranuclear, intramitochondrial, or intramembranous, the distributions of several heat shock proteins have been studied. Thus, hsp69 is present predominantly in the cytosol and translocates to the nucleus after heat shock (25, 40, 43). In contrast, the hsp100 is located predominantly in the Golgi apparatus (27).

Homogenization in low-ionic-strength buffers or lysis with nonionic detergents easily extracts hsp85 from cultured murine (22), human (44), and *Drosophila* (25) cells, suggesting that it is a component of the cytosol. This localization is supported by sedimentation velocity (22) and equilibrium (42) studies showing that the protein behaves as a monomer. However, it is also known to complex with the Rous sarcoma virus transforming protein, pp60^{src} (23), so a labile association with other structures, e.g., plasma membrane, cannot be entirely excluded. The present study describes the production and characterization of monoclonal antibodies to human hsp85, which are then used to determine the ultrastructural location of this antigen. The data suggest a purely cytosolic distribution consistent with the results of less definitive approaches.

Even if hsp85 does play a role in protecting cells against hyperthermia, this is probably not the only function since it is present at relatively high concentrations in cells grown at normal temperatures (21, 22). The situation in vivo is even more obscure, since despite the fact that heat shock protein induction has been observed in intact animals (6, 10, 41), the normal levels of these proteins in various tissues are not known. Therefore, in this report, one of the monoclonal antibodies directed against human hsp85 and an affinity-purified polyclonal antiserum against the murine homolog are used to determine the hsp85 concentration in normal tissues. The significance of these findings for the role of hsp85 in normal and heat-shocked cells is discussed.

MATERIALS AND METHODS

General. BALB/c P3U-1 myeloma cells were kindly provided by T. Easton (State University of New York—Downstate Medical Center) and maintained in Dulbecco modified Eagle minimal essential medium containing 10% fetal calf serum. L929 cells from MA Bioproducts (Bethesda, Md.) and HeLa cells provided by R. Bablanian (State University

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of New York—Downstate Medical Center) were maintained in Dulbecco modified Eagle minimal essential medium containing 10% newborn calf serum. Carrier-free $Na^{125}I$ was from Schwarz/Mann (Orangeburg, N.Y.) and [^{35}S]methionine (1.2 Ci/mmol) was from Amersham Corp. (Arlington, Ill.). Disposable polyvinyl plates were from Cooke Laboratories (Alexandria, Va.), and nitrocellulose sheets (0.45-μm pore size) were from Millipore Corp. (Bedford, Mass.). All chemicals were reagent grade.

hsp85 purification. Murine hsp85 was purified from L1210 cells as previously described (22). Preparation of human hsp85 was from therapeutic orchiectomy specimens. The tissue was homogenized in 10 V of 50 mM Tris-hydrochloride buffer (pH 7.6) containing 5 mM 2-mercaptoethanol and 100 mM NaCl, lysed with Nonidet P-40 (Shell Chemical Co.) at a final concentration of 1%, and centrifuged at 100,000 × *g* for 1 h. Chromatography of the supernatant soluble proteins on DEAE-Sephadex A-50 was performed as described for the murine protein. Fractions were assayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (3, 22). Peak fractions containing the abundant 85K protein band which coelectrophoresed with murine hsp85 were pooled and subjected to chromatography on a hydroxylapatite column (2.2 × 13 cm; Bio-Rad Laboratories, Richmond, Calif.) as described by Welch and Feramisco (42). Peak fractions were pooled, assayed for total protein (29), and stored at 0 to 4°C.

SDS-PAGE. One-dimensional 5 to 15% gradient slab gel electrophoresis was based on the discontinuous Tris-glycine system of Laemmli (19) and modified as previously described (3). The method of O'Farrell (32) was used for two-dimensional PAGE.

Immunization procedures and hybridoma production. Twelve male Wistar rats each were immunized with various preparations of purified human hsp85. The protein was either emulsified with polyacrylamide after being subjected to SDS-PAGE and visualization by the method of Higgins and Dahaus (11), or it was subjected to performic acid oxidation (38) before emulsification with Freund complete adjuvant. The SDS-denatured protein in polyacrylamide was also emulsified with Freund complete adjuvant. Aliquots of these preparations (100 μg) were injected intraperitoneally and followed by five booster injections, including one of SDS-denatured and four of performic acid-oxidized protein, which were given in Freund incomplete adjuvant at biweekly intervals. Sera were assayed for anti-hsp85 antibodies by radioimmunoassay (RIA) and immunoblot before administering the last booster injection.

Four days after the last booster, the spleen of the rat having the highest titer by RIA was aseptically removed and teased. The fusion system contained 10^8 spleen cells and 3×10^7 P3U-1 mouse myeloma cells in 1 ml of 30% (vol/vol) polyethylene glycol 4000 (18). The fused cells were transferred to Costar 96-well microculture plates (10^5 cells per well) and cultured for 2 weeks in Dulbecco modified Eagle minimal essential medium with 10% fetal calf serum containing hypoxanthine, aminopterin, and thymidine. After RIA screening, positive hybridomas were cultured in medium containing hypoxanthine and thymidine. Hybridomas that were subsequently shown to be positive by immunoblot were subcloned by limiting dilution.

Three preparations of purified murine hsp85, i.e., native, SDS-denatured, and performic acid oxidized, were used to immunize separate groups of male Wistar rats. Each preparation was emulsified with Freund complete adjuvant, and 100 μg of antigen was injected intraperitoneally into each of four rats. Eight booster injections were subsequently given, each in Freund incomplete adjuvant. Blood was collected at intervals, and sera were assayed by solid-phase RIA.

Solid-phase RIA. The solid-phase RIA employed 96-well U-bottomed polyvinyl plates which had 1 μg of purified murine or human hsp85 dried onto the surface of each well (33). To minimize nonspecific adsorption, wells were rinsed twice with 1% bovine serum albumin in phosphate-buffered saline (PBS). Each well was incubated sequentially at room temperature for 30 min with 50 μl of hybridoma culture supernatant or a 1:10 dilution of rat serum and then with 30 μl of ^{125}I-labeled rabbit anti-rat immunoglobulin G (IgG) (10^7 cpm/ml). The wells were rinsed with PBS and 1% bovine serum albumin in PBS after each incubation. Binding was determined by cutting the individual wells from the plates and measuring the radioactivity in a gamma counter.

Radioiodination of rabbit anti-rat IgG. The radioiodination method was based on that of Greenwood et al. (9) as modified by T. Easton (State University of New York—Downstate Medical Center). To a column containing 100 μl of rat IgG-Sepharose (1 mg/ml) was added 100 μl of rabbit anti-rat IgG (1 mg of specific antibody per ml; Cappel Laboratories, Downingtown, Pa.), followed by 5 ml of PBS. Then, 2 mCi of $Na^{125}I$, 20 μl of 0.5 M sodium phosphate (pH 7.5), and 25 μl of chloramine-T (1.6 mg/ml) were mixed, transferred to the column, and stirred for 90 s. The reaction was stopped by addition of 25 μl of sodium metabisulfite (8 mg/ml), and unbound reactants were removed by washing the column with PBS. ^{125}I-labeled rabbit anti-rat IgG was eluted with 0.2 M glycine-hydrochloride (pH 2.3) containing 1% bovine serum albumin, and fractions were collected into an equal volume of 0.2 M Tris-hydrochloride (pH 8.6) containing 1% bovine serum albumin and 0.2% sodium azide. After radioactivity determination in a gamma counter, peak fractions were pooled and stored at −20°C.

Purification of antibodies to murine hsp85 by affinity chromatography. Purified native murine hsp85 (0.5 mg) was coupled to 1 g of CNBr-activated Sepharose 4B. After extensive washing, the coupled gel was suspended in PBS and packed in a 1-ml column. Anti-murine hsp85 serum (R4) diluted 1:1 with PBS was applied to the column, and the beads were stirred gently and then washed with PBS until the absorbance at 280 nm of the eluate returned to base line. The specific anti-hsp85 antibodies were eluted with 200 mM glycine-hydrochloride (pH 2.3), and each fraction was collected into an equal volume of 200 mM Tris-hydrochloride (pH 8.6). The fractions having the maximum absorbance at 280 nm were pooled and dialyzed against PBS. The purified antibody was subsequently designated R4-C.

Immunoblot procedures. The procedure of Towbin et al. (37) was used essentially without modification to determine the specificity of anti-hsp85 antibodies in undiluted hybridoma supernatants. Various antigens were subjected to one-dimensional SDS-PAGE, two-dimensional PAGE (32), or one-dimensional partial proteolytic peptide mapping (4) and then were electrophoretically transferred to nitrocellulose sheets. After brief rinsing in 10 mM Tris-hydrochloride (pH 7.4) in saline, the sheets were incubated in 50% newborn calf serum in 10 mM Tris (pH 7.4)–saline buffer for 1 h at 37°C. Sheets were then incubated at room temperature with the various monoclonal supernatants or control medium for 30 min, followed by ^{125}I-labeled rabbit anti-rat IgG (5×10^6 cpm/ml) for 1 h. The sheets were washed extensively with Tris-saline buffer after each incubation and were exposed for autoradiography after air drying.

Various tissues were removed from male mice, homoge-

nized in ice-cold PBS, and solubilized with SDS and 2-mercaptoethanol (each to a final concentration of 1%). The lysates were subjected to immunoblot analysis by using the affinity-purified antibody (1:40). Human tissues obtained at autopsy were processed similarly, and immunoblots were performed with a monoclonal antibody (9D2) to human hsp85. Binding of labeled rabbit anti-rat IgG to the hsp85 band was quantitated by densitometry of the corresponding autoradiograms and integration of the peak areas or by cutting out the immunoblot bands themselves and counting in a gamma counter. The values thus obtained were compared with standards subjected to immunoblot analysis in the same experiment.

Peptide mapping. Unlabeled protein bands were cut from briefly stained SDS-polyacrylamide gels as previously described (3). One-dimensional peptide mapping by limited proteolysis was performed in 12.5% SDS-polyacrylamide gels as described by Cleveland et al. (4) with *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.). Unlabeled peptide bands were detected by using the silver stain of Oakley et al. (31), including the initial prefixing step. After staining, gels were immersed in Kodak Rapid Fixer and lightly wiped to remove surface precipitates.

Immunoprecipitation. The procedure was based on that of J. Lewis (State University of New York—Downstate Medical Center) and employed rabbit anti-rat IgG coupled to CNBr-activated agarose (Sigma Chemical Co., St. Louis, Mo.) to capture the IgG and bound antigen. HeLa cells labeled with [^{35}S]methionine were lysed in 50 mM Tris-hydrochloride (pH 7.15) containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride and centrifuged in a Microfuge (Brinkman Instruments, Inc., Westbury, N.Y.). A portion of the supernatant containing 5×10^6 cpm was incubated with 100 μl of the monoclonal supernatant (diluted 1:10) for 2 h at 37°C and then at 4°C overnight. Rabbit anti-rat IgG-agarose (50 μl) was then added, and the suspension was mixed continuously for 3 h at room temperature. The beads were washed five times with lysis buffer containing 1 mg of ovalbumin per ml, but no sodium deoxycholate, suspended in 50 μl of 50 mM Tris-hydrochloride (pH 6.7) containing 2% SDS, 1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 500 μg of ovalbumin per ml, and heated for 3 min at 90°C, and the supernatant was analyzed by SDS-PAGE.

Immunoperoxidase staining. HeLa cells grown on glass slides were rinsed with Dulbecco PBS and fixed overnight in 0.1 M sodium phosphate buffer (pH 7.4) containing 2% paraformaldehyde and 50 mM sucrose. The monolayer cells were then further fixed for 30 min at 4°C in the above buffer containing 0.05% glutaraldehyde, washed in phosphate-sucrose buffer, and permeabilized by incubation for 30 min in phosphate buffer containing 0.2% gelatin and 0.05% saponin. The saponin-containing buffer was used in all subsequent washes and incubations with either monoclonal antibodies or rabbit anti-rat IgG-peroxidase conjugate (diluted 1:100; Miles Laboratories).

Stained monolayers to be examined by electron microscopy were incubated for 30 min in 2% OsO_4, passed through serial alcohols to absolute ethanol, and embedded in Epon 812 in inverted Beem capsules. After polymerization for 72 h, the embedded monolayers were separated from the original glass slides by immersion in an ethanol-dry ice bath, followed by rapid immersion in boiling water. After the blocks were trimmed to appropriate sizes, thin sections were cut directly from the block face.

RESULTS

Purification of human and murine hsp85. The hsp85 from both sources was present in the 100,000 × *g* supernatant and eluted as a symmetrical peak from DEAE-Sephadex A-50 at 0.17 M to 0.19 M NaCl. No other bands of this apparent molecular weight were seen elsewhere in the elution profile, and the yield at this step was 75% based on the amount present in the 100,000 × *g* supernatant. The proteins eluted from hydroxylapatite as single peaks at ca. 0.2 M phosphate. The protein in most of the fractions from these peaks migrated as a single band in SDS-polyacrylamide gels, and the overall yield of the homogeneous proteins was 60%.

Hybridoma generation and selection. Of the 12 rats injected with the human hsp85, only 2 produced antibodies detectable by both RIA and immunoblot; since immunoblot analysis showed that 1 rat also produced high titers against minor contaminants in the hsp85 preparation, the spleen of the remaining rat was used for the fusion. Of 1,824 wells plated, 187 contained hybridomas and were screened by RIA. A positive RIA reaction was found for 11 hybridomas, and 2 of these were also positive by immunoblot. These cultures were expanded, subcloned, and frozen in liquid nitrogen. There was no absolute correlation between the RIA and immunoblot reactivity: some supernatants were strongly positive by RIA but negative by immunoblot, whereas others were weakly positive by RIA but positive by immunoblot. All of the monoclonal antibodies were of rat origin and were of the IgG type, since the screening method was specific for that species and immunoglobulin class.

Characterization of anti-human hsp85 monoclonal antibodies. Reactivity of the antibodies 9D2 and 16F1 against the hsp85 originally used as antigen, as well as against total HeLa and L929 cell lysates, is shown in Fig. 1. The purified protein and the corresponding polypeptide in HeLa cell lysates were recognized by both monoclonal antibodies; in addition, 16F1 recognized the homologous murine hsp85 in an L929 cell lysate. Thus, at least two epitopes are present on hsp85 molecules from widely different sources. Of these, neither is present on the rat homolog, whereas only one is present on the murine L929 cell homolog. Several other

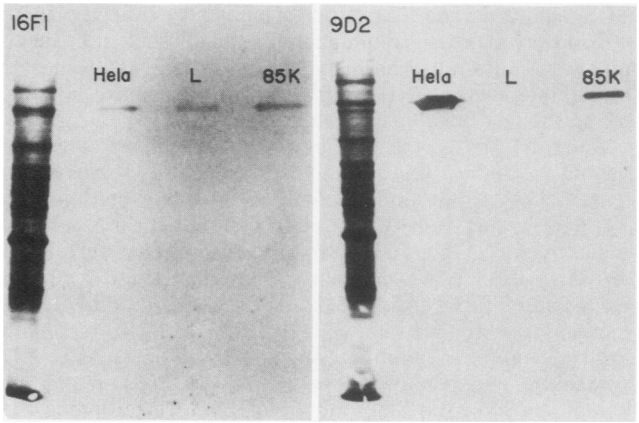

FIG. 1. Immunoblot analysis of anti-human hsp85 monoclonal antibody specificity. In each panel, [^{35}S]methionine-labeled L-cell marker proteins, unlabeled HeLa cell lysate, unlabeled L-cell lysate, and purified hsp85 (left to right) were subjected to SDS-PAGE, transferred to nitrocellulose sheets, exposed to either 16F1 (left panel) or 9D2 (right panel) culture supernatants, developed with ^{125}I-labeled rabbit anti-rat IgG, and autoradiographed.

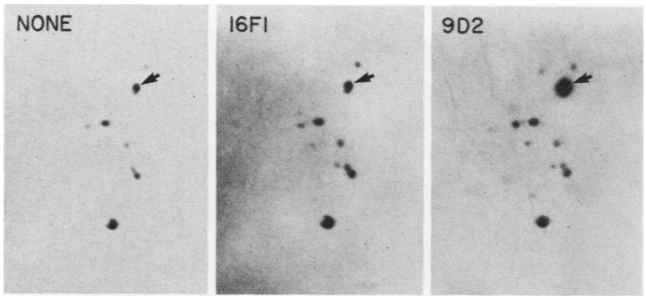

FIG. 2. Two-dimensional immunoblot analysis of anti-human hsp85 monoclonal antibody specificity. Unlabeled HeLa cell lysates were coelectrophoresed with [^{35}S]methionine-labeled L-cell marker proteins, transferred to nitrocellulose sheets, exposed to medium alone (left), 16F1 (center), or 9D2 (right) culture supernatants, and developed as described in the legend to Fig. 1. Arrows indicate the hsp85 spots.

polypeptides (apparent molecular weights of 80,000, 65,000, and 50,000) in the HeLa cell lysate were very weakly defined by the 9D2 monoclonal antibody. Although it is not known with certainty whether these are distinct polypeptides sharing common epitopes with hsp85 or are simply fragments of the heat shock protein itself, the latter possibility is preferred because similar bands appeared in preparations of the purified protein after storage for ca. 1 year.

Two-dimensional immunoblots (Fig. 2) demonstrated unequivocally that the 85K antigen recognized by the monoclonal antibodies was indeed hsp85. In such experiments, unlabeled HeLa cell lysates were coelectrophoresed with [^{35}S]methionine labeled L929 cell lysate proteins as markers to facilitate orientation of the ^{125}I-labeled immunoblot pattern. Identical results were obtained with HeLa lysate proteins alone, but precise orientation of the immunoblot pattern was impossible. Both 16F1 and 9D2 recognized a single 85K spot which corresponded exactly with that of hsp85.

The 9D2 monoclonal antibody also immunoprecipitated the native HeLa cell antigen (Fig. 3). These data further demonstrate the specificity of the antibody and confirm previous observations that the bulk of hsp85 is not stably associated with any other cell protein.

Final characterization was done by a partial proteolytic peptide map immunoblot technique to establish that the two monoclonal antibodies indeed recognize different epitopes. Figure 4 shows that the sets of hsp85 proteolytic fragments recognized by 9D2 as compared with 16F1 are quite different, regardless of the protease used. Whereas both antibodies bind to some proteolytic fragments and not others, this is especially true of 16F1, which binds only to those of high molecular weight.

hsp85 intracellular distribution. Immunoperoxidase staining with the 9D2 monoclonal antibody showed that the distribution of the antigen was exclusively cytoplasmic. Although numerous unstained cytoplasmic areas could be seen, no organization into filaments or other structures was evident, and an exclusive association with membranes, mitochondria, or lysosomes could be eliminated (Fig. 5A). Nuclear staining was also absent in stained sections of paraffin-embedded HeLa cells pellets (data not shown). Electron micrographs of similar preparations (Fig. 5A and B) largely confirmed this conclusion and also demonstrated that this heat shock protein was absent from mitochondria.

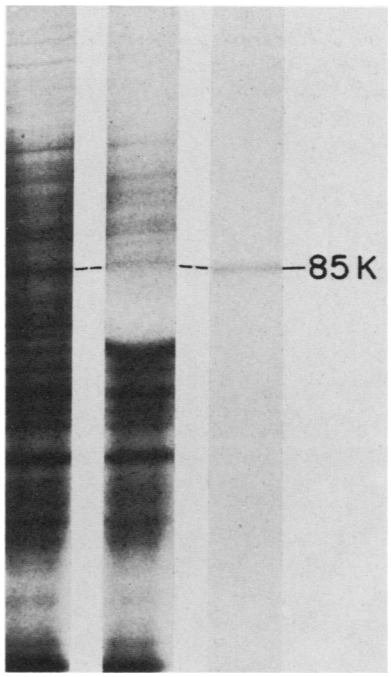

FIG. 3. Immunoprecipitation of [^{35}S]methionine-labeled HeLa cell proteins by 9D2 anti-human hsp85 monoclonal antibody. Left lane, HeLa cell lysate before precipitation; right lane, material captured in immune complexes and eluted with SDS; center lane, unbound material remaining in the supernatant after immunoprecipitation.

Although the distribution of essentially all of the antigen was cytosolic, a small fraction could still be membrane associated, since occasional patchy peroxidase staining superimposed on the osmium-stained plasma and endoplasmic reticulum membranes could not be entirely eliminated.

Production of anti-murine hsp85 antisera. The RIA results

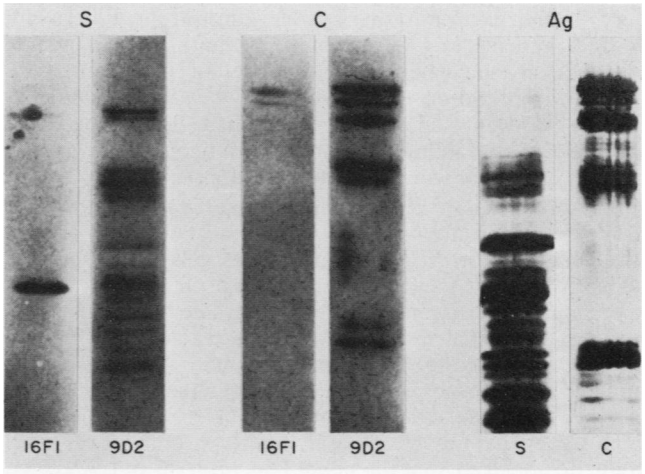

FIG. 4. Immunoblot reactivity of 16F1 and 9D2 monoclonal antibodies against peptides obtained by partial proteolytic digestion of human hsp85 and separation by SDS-PAGE. Each lane contained 4.0 μg of the purified protein and 0.5 μg of either *S. aureus* V8 protease (S), or chymotrypsin (*C*). The protein in the lanes labeled Ag was digested with either V8 protease (S) or chymotrypsin (C) and silver stained (31).

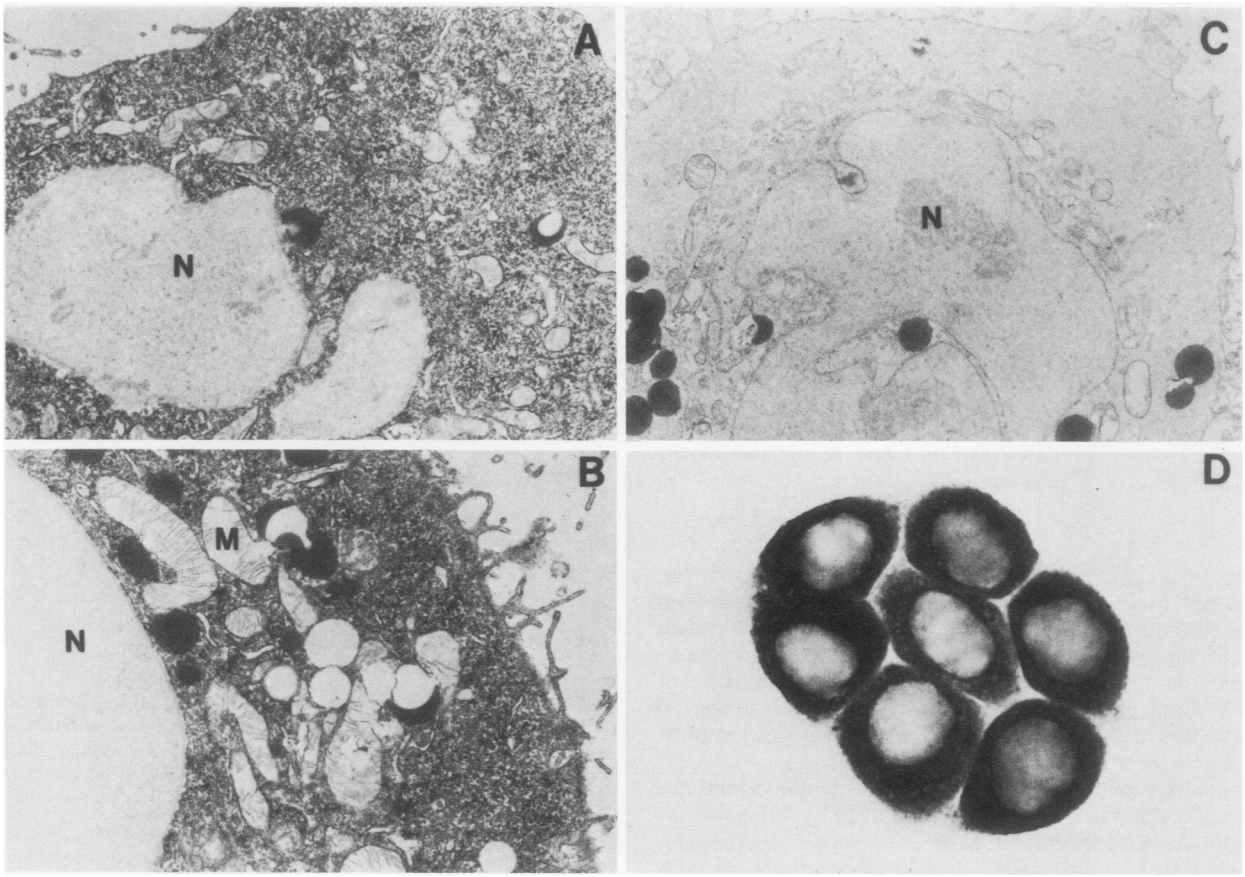

FIG. 5. Immunohistochemical determination of hsp85 distribution in human cells. HeLa cells were immunoperoxidase stained by using the 9D2 monoclonal antibody. Electron micrographs of stained and osmicated monolayer cells are shown: (A) ×5,670; (B) ×7,350. (C) Electron micrograph of control exposed to antibody-free medium followed by peroxidase conjugate and development as in (A) and (B). (D) Light micrograph (×950) of monolayer cells prepared as in (A) and (B).

obtained after the last booster injection showed that the native and the SDS-denatured protein are very poorly antigenic, whereas the performic acid-oxidized hsp85 evoked a very strong reaction in one rat. This antiserum, R4, gave a 14-fold increase in ^{125}I-labeled rabbit anti-rat IgG binding over background when the native protein was used as the fixed antigen and an 8-fold increase when guanidine-denatured hsp85 was used as the fixed antigen. Interestingly, the response of the animals toward this antigen varied greatly even though they were from an inbred strain.

SDS-PAGE Analysis of murine tissues. Figure 6 shows that a number of normal murine tissues have a visible protein band migrating in SDS-polyacrylamide gels with the same apparent molecular weight as purified hsp85 and the corresponding band in a total L929 cell lysate. Although it is possible to say that several tissues, e.g., skeletal and cardiac muscle, contain little or none of the protein, it is not possible on the basis of these data alone to say whether the band seen in other tissues is actually hsp85.

However, peptide mapping by limited proteolysis (Fig. 7) could demonstrate that the major 85K proteins in L929 cells, testis, and brain yielded fragment patterns very similar to that of the purified hsp85. It should be noted that the relative intensities of several small fragments varied with the source of the protein, suggesting the presence of two or more hsp85 species differing slightly with regard to primary structure or secondary modifications.

Quantitation of hsp85 tissue levels by immunoblot analysis. The reactivity of the affinity-purified antiserum was examined by testing it against all of the antigens in an L929 cell lysate by immunoblot analysis. Before affinity purification the R4 serum bound to two lower-molecular-weight polypeptides in addition to hsp85. In contrast, the affinity-purified antiserum stained only the hsp85 band both in the lane containing the purified protein and in that containing the L929 cell lysate (data not shown).

Using the affinity-purified antiserum to stain immunoblots of the various murine tissues, we obtained the results shown in Fig. 8. Because of significant variations in background staining, such as those clearly visible in this figure, it was necessary to scan the autoradiograms densitometrically to estimate the labeling intensity relative to that of purified hsp85 standards electrophoresed in parallel. This value was directly related to the amount of the antigen (Table 1) and is probably accurate to within ±20%. In all the tissues except testis, the affinity-purified antiserum bound to only the hsp85 band, and no cross-reactivity with other polypeptides was observed. In testis, some lower-molecular-weight bands were also labeled, but this was not seen in other experiments, and we attribute their appearance to partial proteolysis of hps85 before or during preparation of the tissue lysate. This suggests, but does not prove, that the 85K band contained similar antigenic determinants in all the tissues examined. The relative amount of the antigen in the various

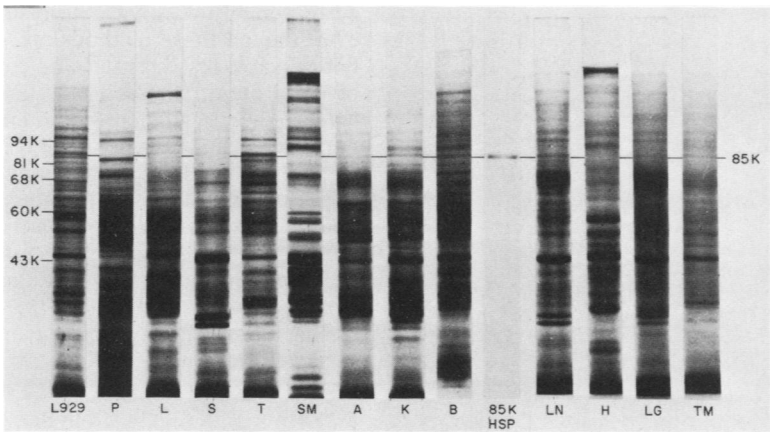

FIG. 6. Coomassie blue-stained SDS-polyacrylamide gels of normal murine tissue lysates, L929 cells, and purified hsp85. The lanes were loaded with 150 μg of cell or tissue lysate protein and show (from left to right) the polypeptide patterns of L929 cell lysate (L929), pancreas (P), liver (L), spleen (S), testis (T), skeletal muscle (SM), adrenal gland (A), kidney (K), brain (B), 5 μg of purified hsp85 (85K HSP), lymph node (LN), heart (H), lung (LG), and thymus (TM).

tissues as quantitated by this method correlates fairly well with the results obtained by staining the SDS-polyacrylamide gels with Coomassie blue. It should be noted that levels of hsp85 in thymus were highly variable by both methods. Thus, the sample stained by Coomassie blue in Fig. 6 also had a barely detectable level of the protein by immunoblot, whereas the sample analyzed by immunoblot in Table 1 showed a prominent 85K band by Coomassie blue staining.

The problem of high and variable background binding was eliminated by using the anti-human hsp85 monoclonal antibody 9D2 (Fig. 9). In these experiments, it was possible to directly quantitate binding to the standard bands by cutting them out and determining the radioactivity in a gamma counter. The data indicate that, for the most part, levels of human hsp85 are similar to those in the corresponding murine tissues. Levels in human lung and liver were significantly lower than those in the murine tissues, and this difference was reproducible. Although the level in human heart was 0.3 μg/100 μg of protein and was undetectable in murine heart, this difference may reflect the relatively high detection limit of ca. 0.5 μg/100 μg of protein imposed by the polyclonal antiserum.

DISCUSSION

One major accomplishment of the present work is the production of monoclonal antibodies against human hsp85, a protein that has proven to be very poorly antigenic because of its evolutionary conservation (30). Antisera have been produced against all of the major heat shock proteins, including the chicken homolog of hsp85 (16), and monoclonal antibodies against the hsp69-70 have been described (39). However, this is the first instance in which monoclonal antibodies have been produced by using the mammalian homolog of hsp85. Performic acid oxidation was probably responsible for increasing the immunogenicity of the protein, since repeated prior attempts to raise antisera against the SDS-denatured protein were unsuccessful (E. J. Kasambalides and K. W. Lanks, unpublished data).

Characterization of the two monoclonal antibodies showed that they were directed against different epitopes. Whereas 9D2 recognized even relatively small proteolytic fragments, binding of 16F1 to only high-molecular-weight proteolytic fragments suggests that its epitope may be complex and easily destroyed by proteolysis. Nevertheless, both monoclonal antibodies revealed only a single reactive 85K species in two-dimensional immunoblots. These data, along with the finding that one-dimensional partial proteolytic peptide maps of hsp85 synthesized both before and after heat shock are identical (21), suggest that synthesis of a single protein is being regulated. This is in contrast to the case of hsp69-70, in which additional isoelectric species are synthesized after heat shock (21, 28).

The failure of immunoprecipitation to reveal an association of hsp85 with other cellular proteins is consistent with previous studies showing that the bulk of the protein in L929 cells sediments as a monomer (22) and that even in Rous sarcoma virus-infected cells very little of the heat shock protein is complexed with the transforming protein, $pp60^{src}$ (23). This situation also contrasts with that of hsp69-70,

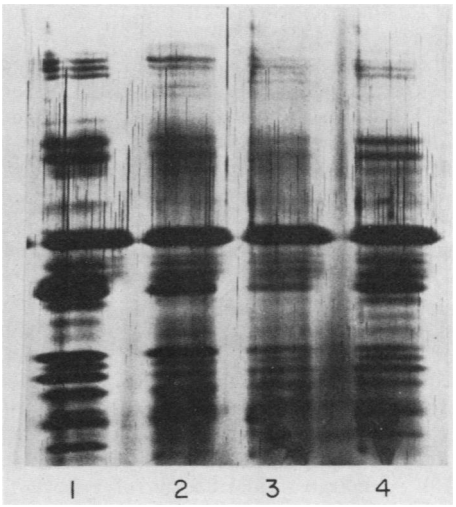

FIG. 7. Partial proteolytic peptide maps of purified hsp85 (lane 1), the L929 cell 85K band (lane 2), the testis 85K band (lane 3), and the brain 85K band (lane 4). The intense silver-stained band present in all of the lanes is 0.5 μg of *S. aureus* V8 protease.

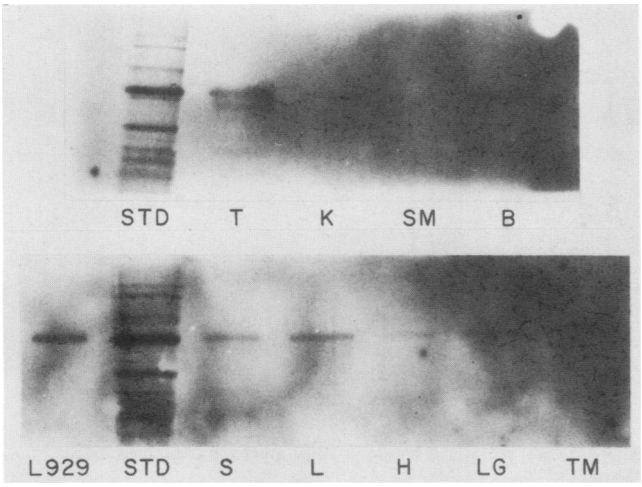

FIG. 8. Detection of hsp85 in normal murine tissues by using the affinity-purified antiserum (R4-C). The tissue lysates are identified as described in the legend to Fig. 6. Each lsyate (150 μg of total protein) was subjected to SDS-PAGE, transferred to a nitrocellulose sheet, and processed for immunoautoradiography as described in the text. STD, Molecular weight standard. [^{35}S]methionine-labeled L-cell lysate was used as the molecular weight standard.

which has been found to associate with a 90K cell surface glycoprotein (13), as well as intranuclear structures (25, 39).

The subcellular distribution revealed by immunoperoxidase staining is consistent with that suggested by detergent extraction, i.e., purely cytoplasmic with no increased localization in membranes, mitochondria, or other organelles. The question of whether there is any membrane-associated hsp85 cannot be answered by conventional means and could probably be best approached by a quantitative electron immunohistochemical method with ferritin-labeled antibodies. Nevertheless, the cytosolic distribution of the bulk of hsp85 suggests that its function is different from that of the other major heat shock proteins. Thus, we suspect that the data on intracellular distribution and the availability of monoclonal reagents will facilitate further work on the function of this heat shock protein and the metabolic consequences of heat shock.

Although the native murine hsp85 is poorly antigenic in rats, an antiserum could be raised after performic acid oxidation. This antiserum was affinity purified, and its specificity was demonstrated by using L929 cell lysates in an immunoblot procedure. Reactivity was demonstrated against the antigen from a variety of murine tissues, but no cross-reactivity could be detected by immunoblot against human hsp85 (unpublished data). It is of interest that this antiserum failed to react with the native antigen in immunoprecipitaton assays. This further confirms the poor antigenicity of the native protein and suggests that even though the native species was used for solid-phase RIAs, reactivity may have actually been directed against denatured material either initially present or produced during adsorption and drying. In contrast, an antiserum raised in rabbits against the corresponding avian protein (hsp89) does precipitate the antigen (16). Although this antiserum is reported to cross-react by immunoblot assay with the homologous protein from species as distantly related as humans and mice, it is not clear whether the cross-reactivity suggested by immunoblot extends to immunoprecipitation systems.

Quantitation of hsp85 by Coomassie blue staining correlated reasonably well with the results of immunoblot binding, even though these methods are semiquantitative. Thus, by both assays, testis, brain, and cultured cells had high levels of the protein, whereas heart and skeletal muscle had low levels. The abundance was intermediate in other tissues of both human and murine origin. Thus, in contrast with hsp69-70, which is undetectable in normal rat tissues (6) and unstressed murine cells, the abundance of hsp85 varies from quite high to relatively low levels in comparable human and murine tissues. Interestingly, rat cardiac muscle cells have very low levels of hsp85 and apparently are not induced to synthesize it by heat shock (10). This is consistent with the finding that quail myoblasts can synthesize a full complement of heat shock proteins, but that more differentiated myotubes are not inducible for hsp85 and require more severe heat treatment to induce the hsp69-70 complex (2).

How do these tissue distribution data relate to the question of whether or not heat shock proteins protect cells against heat shock? This function was suggested in part because of the good correlation between heat shock protein synthesis and thermotolerance, i.e., sublethal heat shock and other treatments that induce heat shock proteins protect cultured cells against killing by subsequent exposure to temperatures that would otherwise be lethal (e.g., see references 20, 26, and 35). However, it is well known that testis is one of the most heat-sensitive tissues, with the germinal epithelium undergoing complete atrophy at normal mammalian body temperature. Although it might be said that basal hsp85 levels are high because the tissue is heat sensitive, the argument as generally understood, i.e., that heat shock proteins protect against heat shock, may rquire modification. This conclusion is reinforced by our finding that even though simian virus 40-transformed mouse embryo cells have higher basal heat shock protein levels than their untransformed counterparts, they are much more sensitive to killing by heat (32a).

On the other hand, the high levels of this protein in unstressed cells suggest that this heat shock protein may have a "housekeeping" function in mammalian cells. A clue to the nature of this function could come from the metabolic differences known to exist among the various tissues. Some of the proposed heat shock protein functions, e.g., those that are required only when mitochondrial function is inhibited (1) or only under conditions of oxidative stress (24), have no obvious correlation with the distribution in unstressed nor-

TABLE 1. Quantitation of the murine and human hsp85 by immunoblot analysis[a]

Source	Human hsp85 (μg/100 μg of protein)	Murine hsp85 (μg/100 μg of protein)
Testis	2.1	2.6
Thymus	2.0	2.5
Brain	1.5	2.8
HeLa cells	1.6	
L929 cells		2.4
Lymph node	1.7	2.2
Liver	1.3	2.4
Kidney	1.5	1.4
Adrenal	1.1	1.5
Pancreas	1.2	1.3
Spleen	1.1	1.3
Lung	0.4	1.4
Heart	0.3	ND[b]
Skeletal muscle	ND	ND

[a] Each value is the mean of two separate determinations.
[b] ND, Not detectable.

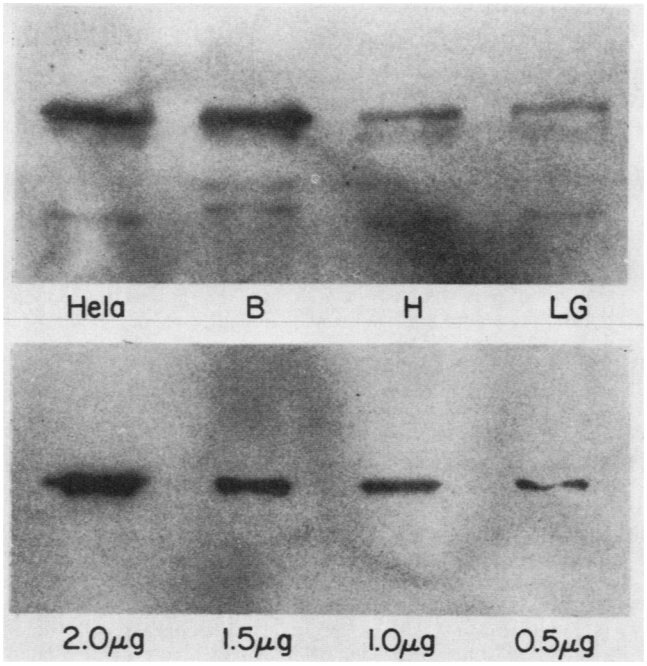

FIG. 9. Immunoautoradiographic detection of hsp85 in normal human tissues with the 9D2 monoclonal antibody. The samples were identified and processed as described in Fig. 8. Binding to various quantities of purified hsp85 is shown in the lower panel.

mal tissues. However, since we have shown that in L929 cells hsp85 intracellular levels are regulated by the availability of glucose (14, 21), we can ask whether the tissue distribution correlates with some aspect of glucose metabolism.

In fact, testis and brain differ enormously from muscle with regard to glucose metabolism. Whereas testis and brain are almost totally dependent on glucose for energy production (7, 8), cardiac and skeletal muscle derive only 30 and 50%, respectively, of their energy requirement from glucose, with the bulk coming from lactate and fatty acids (17). Also, whereas testis and brain utilize large quantities of citric acid cycle intermediates for lipid and protein synthesis, these pathways are not prominent in muscle. Although the situation in testis is not so clear, brain also differs from other tissues in the synthesis of complex carbohydrates and glycolipids derived from glucose. Such pathways may also be relevant to heat shock protein function in view of the association between hsp85 and pp60^{src}, since the latter protein may be capable of phosphorylating phosphatidylinositol (36) and the high levels of pp60$^{c\text{-}src}$, its normal cell homolog, found in brain (5).

Therefore, we may speculate that hsp85 functions in one of several possible metabolic pathways which are related to glucose metabolism and which vary in activity from tissue to tissue. Although the pathways that are relevant to heat shock protein induction are presently unknown, heat shock might be expected to perturb many aspects of glucose metabolism, and this hypothesis is currently being tested in our laboratory.

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