A Major Collagen-binding Protein of Chick Embryo Fibroblasts Is a Novel Heat Shock Protein

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Abstract. Heat shock proteins of chick embryo fibroblasts were analyzed on SDS polyacrylamide gradient gels and were found to include not only three previously well-characterized proteins of 25,000, 73,000, and 89,000 D, but also a 47,000-D protein. Two-dimensional gel electrophoresis revealed that this protein was unusually basic (pI = 9.0) and corresponded to a recently characterized, major gelatin- and collagen-binding protein. The induction of synthesis of this 47,000-D membrane glycoprotein after heat stress of fibroblasts was particularly apparent in preparations isolated by gelatin-affinity chromatography. Regulation of this 47,000-D phosphoprotein was more sensitive than that of three major heat shock proteins in that a substantial stimulation of synthesis occurred at even 42°C, as well as at higher temperature. Phosphorylation of the 47,000-D protein was not altered after heat shock. These studies establish this phosphorylated membrane glycoprotein as a member of the heat shock/stress protein family, and they add collagen binding to the unexpectedly diverse spectrum of biochemical activities induced by exposure of cells to stress.

ALMOST all living organisms studied respond to elevated temperature by induction of a small set of proteins termed heat-shock proteins (1, 20). Besides heat shock, a wide variety of physiological stresses, which include the treatment of cells by amino acid analogues (9, 25), sulfhydryl reagents such as arsenite (7), some metals (14), and mitochondrial poisons (11), induce a similar response in a variety of vertebrate and invertebrate cells (1, 8, 20). Thus, "heat-shock proteins" are actually part of a slightly larger set of "stress proteins."

The major mammalian stress proteins with higher molecular masses of 72,000 (69,000–73,000), 85,000 (80,000–90,000), and 110,000 (100,000–110,000) have been shown to be acidic polypeptides with pI values between 5 and 6 (22). In addition to these larger stress proteins, mammalian cells responding to stress also synthesize a set of smaller proteins with molecular masses between 25,000 and 30,000, some of which are reported to lack methionine and to be phosphorylated (11, 24).

Four major stress proteins have already been described in chick embryo cells: three are induced by heat shock (10) and one (35,000 D) is induced mainly by sodium arsenite (7). The three major heat shock proteins, hsp 89 (85,000–89,000 D), hsp73 (68,000–73,000 D), and hsp25 (25,000–27,000 D), are phosphoproteins, and exist predominantly as soluble cytoplasmic proteins whose native forms are dimers or oligomers (19). hsp73 and hsp25 have also been localized in the nucleus. Very recently, ubiquitin was also shown to be a heat shock protein in chick embryo fibroblasts (4).

Although the exact function of these stress proteins is still obscure, it has been shown that heat shock–induced proteins appear concurrently with the acquisition, maintenance, and decay of thermotolerance in stressed cells (1, 20), suggesting that stress proteins in general may function to maintain cellular homeostasis during cellular trauma.

In a previous paper (15), we reported that the synthesis of a major 47,000-D collagen-binding protein is decreased after transformation of chick embryo fibroblasts by Rous sarcoma virus. In addition, we found that the extent of phosphorylation of the 47,000-D protein (47-kD protein) is increased markedly in transformed cells.

In this paper, we report that this 47-kD gelatin-binding protein is a heat shock protein in chick embryo fibroblasts. The induction of the 47-kD protein is more sensitive to temperature than several other major heat shock proteins: this protein is the only prominent protein induced at 42°C, when the other heat shock proteins are only partially induced. This study provides, to our knowledge, the first characterization of a membrane-associated heat shock or stress glycoprotein with a specific binding function. The existence of collagen-binding activity in this 47-kD heat-shock protein suggests a surprisingly diverse range of specific biochemical activities attributable to this class of proteins, which are widely studied, evolutionarily conserved, but as yet rather poorly understood functionally.

Materials and Methods

Chemicals and Reagents

Gelatin-Sepharose 4B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Ampholines of ranges pH 3.5–10, pH 5–7, and pH 9–11 were

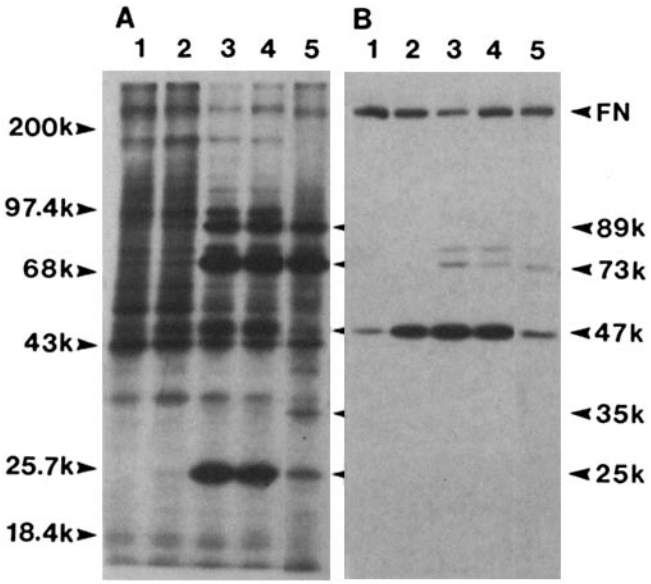

Figure 1. Induction of heat shock proteins in secondary chick embryo fibroblasts treated by heat shock or arsenite. Chick embryo fibroblasts were pre-incubated at various temperatures (37°, 42°, or 45°C) for 4 h, or treated with 0.1 mM sodium arsenite for 4 h. The cells were then labeled at the indicated temperatures for 1 h with 0.1 mCi/ml of [^{35}S]methionine in methionine-free medium supplemented with dialyzed calf serum. The arsenite-treated cells were rinsed, then labeled for 1 h in the absence of inhibitor at 37°C. Cell extracts were prepared by treating the cell monolayer with lysis buffer (1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 2 mM *N*-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride). Aliquots of cell extracts that contained equal amounts of TCA-precipitable radioactivity (*A*) or gelatin-Sepharose bound fraction (*B*) were applied to 8–13% polyacrylamide gradient slab gels containing SDS. Lane *1*, 37°C; lane *2*, 42°C; lane *3*, 45°C; lane *4*, 45°C but labeling at 37°C; and lane *5*, 0.1 mM sodium arsenite. Molecular size markers are indicated at the left and the major stress protein bands at the right (*k*, kilodaltons). *FN*, indicates the position of fibronectin.

obtained from LKB Instruments, Inc. (Gaithersburg, MD). [^{35}S]Methionine (specific activity 1,100 Ci/mol), [^{32}P]orthophosphate, and L-[3,4,5-^{3}H(N)]leucine (specific activity 140 Ci/mmol) were from New England Nuclear (Boston, MA). Nonidet P-40 was obtained from Gallard/Schlesinger Chemical Mfg. Corp. (Carle Place, NY), and dithiothreitol from Calbiochem-Behring Corp. (La Jolla, CA). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO), Bethesda Research Laboratories (Gaithersburg, MD), Gibco (Grand Island, NY), or Bio-Rad Laboratories (Richmond, CA), and were of the highest purity available from each company.

Cell Culture and Metabolic Labeling

Chick embryo fibroblasts were maintained in Vogt's GM medium (23), passaged using 0.05% trypsin-0.02% EDTA (Gibco), and used for experiments between passages two and six.

For short-term labeling, cultures of ~6–10 × 10^5 cells per 35-mm dish were rinsed with Dulbecco's modified Eagle's medium that lacked methionine (prepared by the National Institutes of Health [NIH] Media Unit) and incubated with 0.1 mCi/ml of [^{35}S]methionine in this medium that contained 10% dialyzed calf serum. For long-term labeling, nonradioactive methionine was added to a final concentration of 0.5 μg/ml to the methionine-free medium that contained 10% nondialyzed calf serum and 5 μg/ml of beef embryo extract (Gibco).

To measure phosphorylation in intact cells, cells were incubated in phosphate-free Dulbecco's medium (prepared by the NIH Media Unit) supplemented with 5 μg/ml beef embryo extract, 10% nondialyzed calf serum, and 1 mCi/ml [^{32}P]orthophosphate for 14 h. For double labeling with [^{32}P]orthophosphate and [^{3}H]leucine, cells were incubated in phosphate- and leucine-free Dulbecco's medium (NIH Media Unit) supplemented with beef embryo extract and calf serum as described above, 1 mCi/ml [^{32}P]orthophosphate, and 0.2 mCi/ml [^{3}H]leucine for 18 h.

Isolation of Gelatin-binding Protein

Extraction and isolation of gelatin-binding proteins followed the procedure of Kurkinen et al. (12) with minor changes as described previously (15). Briefly, cells were extracted with lysis buffer (1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 2 mM *N*-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride) on ice for 20 min. After trichloroacetic acid (TCA)[1]-insoluble radioactivity was determined in an LKB Rack-Beta 1215 scintillation spectrometer, aliquots containing equal amounts of radioactivity were either analyzed directly by SDS polyacrylamide gel electrophoresis or mixed with gelatin-Sepharose. After washing the gelatin-Sepharose with 0.4 M NaCl in lysis buffer, gelatin-binding proteins were eluted by boiling the beads for 3 min in Laemmli's SDS gel electrophoresis sample buffer (13). This protocol recovers all 47-kD protein from extracts as determined by fluorograms of two-dimensional gels of original and flow-through fractions from [^{35}S]methionine-labeled extracts. For quantitation, samples were subjected to electrophoresis on 10% polyacrylamide slab gels that contained 0.1% SDS, and radioactivity in the 47,000-D band in dried slab gels was determined by excising the band and scintillation counting as described previously (15).

Gel Electrophoresis

One-dimensional SDS-10% polyacrylamide gel electrophoresis was done according to the methods of Laemmli (13). Pre-stained molecular weight markers (Bethesda Research Laboratories) consisted of myosin (200,000 mol wt), phosphorylase b (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), and lysozyme (14,300). Gels used for radioisotopic quantitation were dried without treatment with autoradiographic enhancers. Otherwise, the slab gels were treated with Autofluor (National Diagnostics, Inc., Somerville, NJ) before drying. Fluorography was performed on Kodak X-Omat AR film.

For the electrophoresis of total cell extracts on one-dimensional slab gels, 8–13% polyacrylamide gradient gels that contained SDS were used to improve the resolution.

Two-dimensional gel electrophoresis using non-equilibrium pH gradient electrophoresis as the first dimension was done exactly as described previously (15, 16).

Results

Induction of 47,000-D Protein by Heat Shock Treatment

When chick embryo fibroblasts were incubated at high temperatures (42° or 45°C) for 4 h, then labeled with [^{35}S]methionine for 1 h at each temperature, the pattern of protein synthesis was altered substantially (Fig. 1 *A*). After incubation at 45°C, three major heat shock proteins were induced, the molecular sizes of which were reported previously to be 85,000–89,000, 68,000–73,000, and 25,000–27,000 D (19). In our gradient gel electrophoretic system, the estimated molecular sizes of these three heat shock proteins were 89,000 (hsp89), 73,000 (hsp73), and 25,000 (hsp25). In addition to these well-known heat shock proteins, we observed a clear increase of incorporation into a 47,000-D band after heat shock. When the cells were incubated at 42°C rather than 45°C, the increases in the three major heat shock proteins were much less prominent, and the increase in the 47,000-D protein (47-kD protein) was, instead, the only major alteration (Fig. 1 *A*, lane *2*).

The 47-kD protein and hsp73 appeared to be synthesized in significant amounts even in extracts of cells cultured at 37°C, and the incorporation into each of these bands was in-

1. *Abbreviation used in this paper*: TCA, trichloroacetic acid.

Table I. Increase in Synthesis of the 47-kD Gelatin-binding Protein by Treatment at High Temperature

Temperature	Preincubation period	Labeling period	Radioactivity in the 47-kD band
°C	h	h	
37	2	1	700 ± 40 (1)
42	2	1	1,130 ± 20 (1.6)
45	2	1	4,100 ± 80 (5.9)
37	4	1	702 ± 37 (1)
42	4	1	3,190 ± 154 (4.5)
45	4	1	4,089 ± 36 (5.8)
37	4	1	700 ± 40 (1)
37 (arsenite)	4	1	1,080 ± 85 (1.5)
37 (arsenate)	4	1	1,250 ± 77 (1.8)

The gelatin-binding proteins from extracts of cells incubated at the indicated temperatures were separated on SDS-10% polyacrylamide slab gels. The 47-kD bands were excised, dissolved in 30% H_2O_2, and incorporated radioactivity was measured by a liquid scintillation counter. Each radioactivity value represents the mean and standard error of three parallel samples. The numbers in parentheses indicate the fold increase in incorporation. The treatments with 0.1 mM sodium arsenite or 0.1 mM sodium arsenate were performed at 37°C for 4 h, and the cells were labeled for 1 h without reagent.

creased at high temperature. In contrast, relatively little hsp89 and hsp25 was detectable in control cultures at 37°C (Fig. 1; reference 19).

We also compared the ability of two compounds known to induce stress or heat shock proteins to induce the 47-kD protein: arsenite, an inhibitor of oxidative phosphorylation, and arsenate, a phosphate analogue inhibitor of cellular phosphate-transfer reactions (11). When cells were treated for 4 h and labeled in the absence of these agents, both arsenite and arsenate induced not only hsp89, hsp73, and hsp25, but also a 35,000-D protein not induced by heat shock, as reported previously (7, 11). However, synthesis of the 47-kD protein was stimulated only slightly (Fig. 1 *A*; Table I).

For increased resolution of protein species, Fig. 2 shows a two-dimensional polyacrylamide gel analysis of [^{35}S]methionine-labeled cell extracts before and after heat shock using non-equilibrium pH gradient electrophoresis as the first dimension. As we showed previously (15), the 47-kD protein has an unusually high isoelectric point of 9.0, with a closely spaced, minor satellite spot. When cells were incubated for 4 h at high temperature, then labeled for 1 h, the synthesis of the 47-kD protein was increased substantially (Fig. 2). The satellite spot was also increased at high temperatures.

Fig. 2 *D* shows the migration of the 47-kD protein purified by gelatin-Sepharose 4B affinity chromatography from heat-treated chick embryo fibroblasts as described previously. By mixing the purified 47-kD protein with total cell extracts, we verified that the spot that increased after heat shock was identical in migration to that of the gelatin-binding protein (data not shown, see reference 15 for similar data).

The alterations in 47-kD protein synthesis were visualized

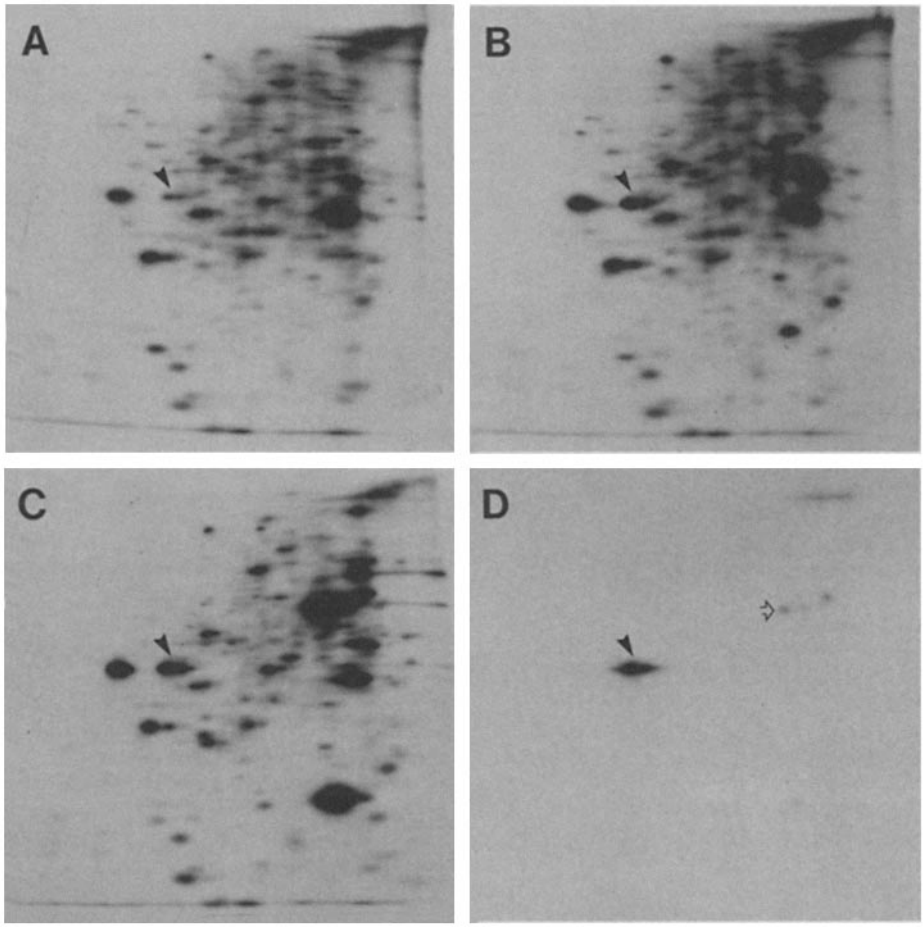

Figure 2. Two-dimensional non-equilibrium pH gradient gel electrophoresis of extracts from cells treated by heat shock. Cells were pre-incubated at various temperatures (37°, 42°, or 45°C) for 4 h, and then labeled at the same temperatures for 1 h with 0.1 mCi/ml of [^{35}S]methionine. Aliquots of cell extracts that contained equal amounts of TCA-precipitable radioactivity were analyzed by two-dimensional gel electrophoresis consisting of non-equilibrium pH gradient electrophoresis as the first dimension (right to left) and SDS-10% polyacrylamide slab gels as the second dimension (top to bottom). (*A*) 37°C; (*B*) 42°C; (*C*) 45°C; and (*D*) 47-kD gelatin-binding protein purified by gelatin-Sepharose 4B from chick embryo fibroblasts cultured at 45°C for 4 h. Arrowheads indicate the 47-kD protein. Open arrow in *D* indicates a small amount of hsp73, which co-migrates with the major hsp73 spot in *C*.

most clearly after gelatin-Sepharose affinity chromatography to examine collagen-binding proteins. Fig. 1 *B* compares the electrophoretic patterns of gelatin-binding proteins of cell extracts that were incubated 4 h and labeled 1 h at 37°, 42°, or 45°C. The 47-kD collagen-binding protein was clearly induced by treatment of the cells at 42° and 45°C (Fig. 1 *B*, lanes *2* and *3*). Labeling the cells at 37°C after heat treatment also showed similar increases in the synthesis of the 47-kD gelatin-binding protein (Fig. 1 *A* and 1 *B*, lane *4*).

Fig. 1 *B* also shows that another, more minor band of 73,000 D, which bound to gelatin-Sepharose, was also induced at 45°C. This minor species co-migrated with hsp73 in two-dimensional gels, but represented only a minor fraction of total incorporation into hsp73 (Fig. 2 *D*, indicated by open arrow). The synthesis of fibronectin, another major gelatin-binding protein, was decreased after incubation of cells at high temperature, which might be due to the general inhibition of cellular protein synthesis accompanying such treatment.

The results of a typical experiment quantitating radioactivities in the 47-kD bands from heat-treated cells are shown in Table I. Although a short pre-incubation (2 h) at 42°C resulted in only a modest increase in the 47-kD protein (Table I, line 2), longer incubation (4 h) at this temperature caused a 4.5-fold increase (Table I, line 5). Incubation at 45°C induced substantial 47-kD protein synthesis even after a short exposure period (Table I, line 3). In some experiments, the increase in synthesis of the 47-kD protein at 45°C was over 10-fold greater than the control value at 37°C (data not shown). Arsenite and arsenate treatments resulted in only slight increases in the synthesis of the 47-kD protein (Table I, lines 8 and 9), which was consistent with the results suggested by analyses of whole-cell extracts (Fig. 1 *A*). Labeling of arsenite- or arsenate-treated cells in the presence of these agents also showed only minor increases in incorporation into the 47-kD protein (data not shown).

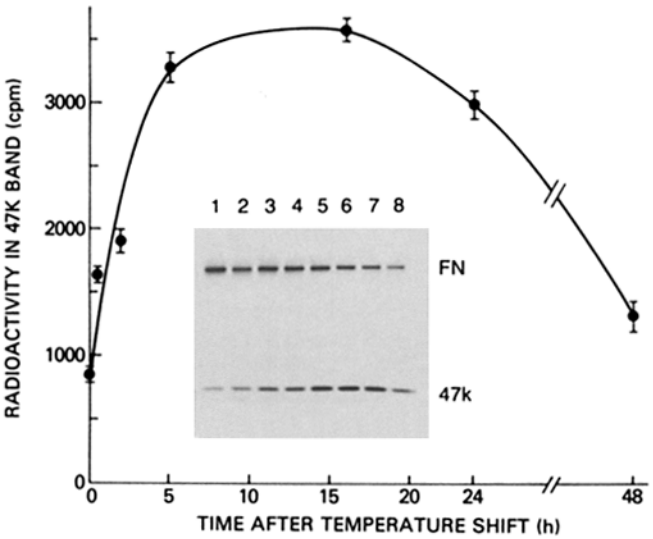

Figure 3. Time course of the induction of the 47-kD gelatin-binding protein by heat shock at 42°C. Cells were pre-incubated at 42°C for various time periods, and then labeled for 1 h with 0.1 mCi/ml [^{35}S]methionine. Aliquots containing equal TCA-precipitable radioactivity were mixed with gelatin-Sepharose. (*Inset*) Gelatin-binding proteins eluted with SDS were analyzed on SDS-10% polyacrylamide gels. Cell extracts from subconfluent (lane *1*) and confluent (lane *2*) cultures incubated at 37°C were also compared. Cells were incubated at 42°C for 0.5 h (lane *3*), 2 h (lane *4*), 5 h (lane *5*), 16 h (lane *6*), 24 h (lane *7*), and 48 h (lane *8*). The bands corresponding to fibronectin (*FN*) and the 47-kD protein (*47k*) are indicated. The 47-kD bands were excised, dissolved in 30% H_2O_2, and radioactivity was determined with a liquid scintillation counter. Lane *1* in inset was counted as zero time. Each value represents the mean ± standard error of three parallel samples.

Time Course of 47,000-D Protein Induction

We examined the kinetics of induction of the 47-kD protein after heat shock. To quantitate its increased synthesis, we analyzed purified gelatin-binding protein preparations on 10% polyacrylamide slab gels containing SDS (Fig. 3, inset) and determined radioactivity in the 47-kD band. As shown in Fig. 3, the 47-kD protein was clearly induced after 30 min of temperature shift to 42°C. The synthesis of the 47-kD protein reached a peak between 5 and 16 h after temperature shift, and gradually decreased after 24–48 h. Even after 48 h of temperature shift to 42°C, cells continued to synthesize the 47-kD protein at a rate higher than did control cells cultured at 37°C. There were no obvious differences between the synthesis of the 47-kD protein in subconfluent and fully confluent cells cultured at 37°C (quantitative data not shown, also see Fig. 3, inset, lanes *1* and *2*).

To compare the kinetics of 47-kD induction with other major heat shock proteins after temperature shift to 45°C, we analyzed total cell extracts labeled with [^{35}S]methionine by SDS gradient (8–13%) slab gel electrophoresis (Fig. 4). Total synthesis of proteins is shown in Fig. 4 *A* where equal volumes of cell extracts prepared from equal numbers of cells were applied to gels, and relative protein synthesis is shown in Fig. 4 *B* in which aliquots of cell extracts that contained equal amounts of TCA-precipitable radioactivity were applied. The synthesis of the 47-kD protein, as well as that of hsp89, hsp73, and hsp25, was induced after incubation of

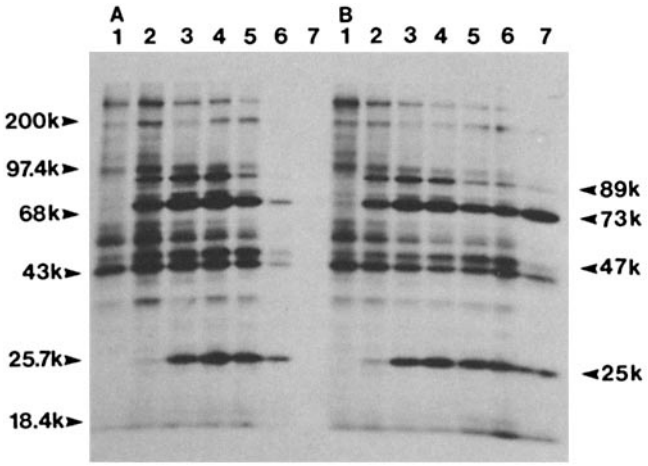

Figure 4. Time course of the induction of heat shock proteins in cell extracts by heat shock. Cells were incubated at 45°C for various times, and then labeled for 1 h with 0.1 mCi/ml [^{35}S]methionine. Equal volumes of cell extracts prepared from equal numbers of cells (*A*), or aliquots that contained equal TCA-precipitable radioactivity (*B*), were applied to 8–13% polyacrylamide gradient slab gels containing SDS. Cells incubated at 37°C were analyzed as the zero-time control (lane *1*). Cells were incubated for 0.5 h (lane *2*), 2 h (lane *3*), 5 h (lane *4*), 10 h (lane *5*), 16 h (lane *6*), and 24 h (lane *7*).

cells at 45°C (Fig. 4). The kinetics of induction differed considerably among these four proteins. hsp89 was induced rapidly (30 min after temperature shift to 45°C) and decreased most rapidly (after ~10 h). hsp73 was also induced rapidly and began to decrease after ~10 h. However, because of the severe inhibition of total protein synthesis after 10 h, the relative synthetic rate remained elevated even after 24 h (Fig. 4 *B*, lane *7*). hsp25 was induced more slowly (2 h after temperature shift) and retained a high level of relative synthesis after 16 h or 24 h. The 47-kD protein was induced clearly after 30 min of temperature shift, reached a peak after 5–10 h, and net synthesis decreased after 16 h (Fig. 4 *A*). The relative ratio of 47-kD protein synthesis to total protein synthesis reached a maximum after 16 h of heat shock (Fig. 4 *B*).

Phosphorylation of the 47,000-D Protein Was Not Altered by Heat Treatment

In a previous paper (15), we reported that the 47-kD gelatin-binding protein was phosphorylated in intact cells, and that the phosphorylation per molecule was increased up to sevenfold in cells transformed by Rous sarcoma virus. Therefore, we investigated whether phosphorylation of the 47-kD protein was affected by heat shock treatment of the cells.

Parallel cultures were labeled for 14 h with either [^{35}S]methionine or [^{32}P]orthophosphate. Aliquots of extracts were adjusted to contain equal quantities of TCA-insoluble [^{35}S]methionine and were applied to gelatin-Sepharose 4B. Identical volumes of cell extract from parallel cultures of cells labeled with [^{32}P]orthophosphate were also applied to gelatin-Sepharose. After electrophoresis of the gelatin-bound proteins on SDS slab gels, the degree of phosphorylation per unit [^{35}S]methionine-labeled 47-kD protein was compared between cells before and after heat shock. Fig. 5 (lanes *1–4*) shows that both the total content and the phosphorylation of the 47-kD protein were increased after a 14 h incubation at 42°C. We calculated the [^{32}P]/[^{35}S] ratio of radioactivity in each 47-kD band to estimate the degree of phosphorylation of the protein, and found that there was no change in the [^{32}P]/[^{35}S] ratio before and after heat shock (Table II).

To confirm that result, we double-labeled the cells with [^{3}H]leucine and [^{32}P]orthophosphate. Aliquots of extracts were adjusted to contain equal amounts of TCA-insoluble [^{3}H]leucine and were applied to gelatin-Sepharose 4B. The spillover radioactivity from incorporated [^{32}P] into the [^{3}H] channel was <5% of total [^{3}H]leucine radioactivity.

After incubation for 18 h at 45°C, both [^{3}H]leucine and [^{32}P]orthophosphate incorporation into the 47-kD band increased ~3–4-fold, resulting in minimal changes in the [^{32}P]/[^{3}H] ratio (Fig. 5, lanes *5* and *6*, and Table II). Lane *7* in Fig. 5 shows the gelatin-bound proteins from cells that were labeled for 17 h at 37°C with both [^{3}H]leucine and [^{32}P]orthophosphate, and then incubated at 45°C for 1 h. Again, we could not detect any significant difference in relative phosphorylation after heat shock (Table II).

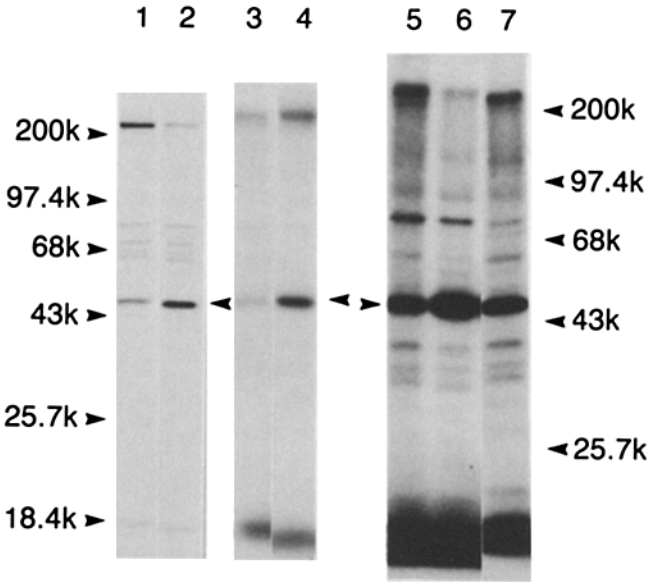

Figure 5. Phosphorylation of the 47-kD gelatin-binding protein. Lanes *1–4*, two parallel cultures were labeled at 37°C or 42°C for 14 h with either 0.1 mCi/ml [^{35}S]methionine or 1 mCi/ml of [^{32}P]orthophosphate. Aliquots of extracts adjusted to contain equal quantities of TCA-precipitable [^{35}S]methionine were applied to gelatin-Sepharose. Identical volumes of cell extracts from parallel cultures of cells labeled with [^{32}P]orthophosphate were also applied to gelatin-Sepharose. The gelatin-binding proteins were analyzed by SDS-10% polyacrylamide gel electrophoresis. Lanes *1* and *2* show the gelatin-binding proteins from extracts of cells labeled with [^{35}S]methionine, and lanes *3* and *4* with [^{32}P]orthophosphate. Lanes *1* and *3*, 37°C; lanes *2* and *4*, 42°C. Lanes *5–7*, cells were double labeled with both [^{3}H]leucine and [^{32}P]orthophosphate at either 37° or 45°C. Aliquots that contained equal TCA-precipitable [^{3}H]leucine were mixed with gelatin-Sepharose. The gelatin-binding proteins were analyzed as described above. Lane *5*, at 37°C for 18 h; lane *6*, at 45°C for 18 h; and lane *7*, at 37°C for 17 h, and then at 45°C for 1 h.

Discussion

The major collagen-binding membrane protein of fibroblasts, a 47,000-D glycoprotein, was found to be heat shock protein in chick embryo fibroblasts. When cells were incubated at 45°C, the synthesis of this 47-kD protein as well as the well-known heat shock proteins hsp89, hsp73, and hsp25 was induced. However, the synthesis of the 47-kD protein was also increased substantially by incubation of cells at 42°C. In contrast, at this temperature, hsp89, hsp73, and hsp25 were not detected as major bands on SDS polyacrylamide gels. This result suggests that the induction of the synthesis of the 47-kD protein was significantly more sensitive to temperature than that of the other major heat shock proteins. The possibility that the major heat shock proteins or the mRNAs for these proteins were more labile at 42°C could be excluded by the results of time course experiments (data not shown) in which the synthesis of hsp89, hsp73, and hsp25 were at lower levels throughout the entire period (0.5–48 h).

Besides this difference in kinetics of induction, the fact that arsenite and arsenate induced three major heat shock proteins as well as a 35,000-D stress protein, yet only slightly stimulated synthesis of the 47-kD protein, suggests somewhat distinct regulatory mechanisms for the induction of these proteins.

The 47-kD protein is quite characteristic as a heat shock protein in at least two respects. First, this protein is an unusually basic protein, while all other heat shock proteins are acidic (pI = 5–6) (19, 22). This fact might account for the

Table II. Incorporation of [^{35}S]Methionine or [^{3}H]Leucine and [^{32}P]Orthophosphate into the 47-kD Gelatin-binding Protein

Exp. 1

Temperature	Radioactivity in the 47-kD band		
	[^{35}S]Methionine	[^{32}P]Orthophosphate	[^{32}P]/[^{35}S] ratio
°C			× 10^{-2}
37	1,732 ± 38 (1)	132 ± 5 (1)	7.6 ± 0.3 (1)
42	5,956 ± 89 (3.4)	478 ± 11 (3.6)	8.0 ± 0.2 (1.1)

Exp. 2

Temperature	Radioactivity in the 47-kD band		
	[^{3}H]Leucine	[^{32}P]Orthophosphate	[^{32}P]/[^{3}H] ratio
°C			× 10^{-2}
37	2,246 ± 8 (1)	283 ± 7 (1)	12.6 ± 0.3 (1)
45	9,658 ± 308 (4.3)	968 ± 18 (3.4)	10.0 ± 0.4 (0.8)
37 → 45	2,201 ± 51 (1.0)	327 ± 5 (1.2)	14.9 ± 0.4 (1.2)

The 47-kD protein was isolated, resolved in SDS gels as described in Fig. 5, excised, and incorporated radioactivity was measured in a liquid scintillation counter. Values in Exp. 1 were obtained from the experiment shown in Fig. 5, lanes *1–4*, and those in Exp. 2 from Fig. 5, lanes *5–7*. Each value represents the mean and standard error of three parallel samples. The relative increases in incorporation are indicated in parentheses.

previous absence of characterization of this molecule as a heat shock protein on two-dimensional gels, because heat shock proteins are usually examined in a pH range between 4 and 7. Second, this protein is a membrane glycoprotein with the ability to bind both to native collagen (15) and to gelatin (denatured type I and III collagen) (12, 15).

Very recently, a glucocorticoid receptor of 90,000 D was reported to be a heat shock protein (18). This protein reacts on Western blots with rabbit antiserum prepared against the 89,000-D chick heat shock protein. As this glucocorticoid receptor is a cytosolic protein, the 47-kD collagen-binding protein is to our knowledge the only heat shock protein with a defined binding activity that is a membrane glycoprotein.

Iida and Yahara recently reported that a yeast heat shock protein of 47,000 D is the yeast enolase (6). Since the isoelectric point of this protein is ~5.8, it is distinct from our gelatin-binding protein. The identification of these diverse activities for some heat shock proteins (collagen-binding, glucocorticoid receptor, and enolase activities) suggests a surprisingly broad range of functions for these proteins.

Protein bands in SDS gels that probably correspond to the 47-kD collagen-binding molecule can be found in the previous literature using chicken cell systems. Robbins et al. briefly commented in their experiments using chick embryo fibroblasts transformed by a temperature-sensitive mutant of Rous sarcoma virus that a 47,000-D protein, termed "delta," was increased slightly on one-dimensional SDS polyacrylamide gels when the cells were incubated at 41°C (17). Kelley et al. also noted that mRNA from cells treated at 45°C produced proteins of ~15, 18, 33, 45, and 55 kD in addition to the three major heat shock proteins in an in vitro translation system (10). A densitometric scan of a 2–20% polyacrylamide gradient slab gel in Fig. 5 *C* of reference 9 shows an induced band by heat treatment that may correspond to the 47-kD protein.

We speculate that the 47-kD protein may not have been characterized previously in chick cells because (*a*) some previous studies did not use gradient gels, which we find necessary to obtain sufficient resolution for separation of the band from the large actin band, (*b*) a second major protein with similar mobility (Fig. 2) may partially obscure any increase in this protein, necessitating two-dimensional gels or isolation of gelatin-binding proteins to observe the increase clearly, or (*c*) because the effect may be stronger in chick embryo fibroblasts from which other, nonfibroblastic cell types have been eliminated by passage.

Biessmann et al. described a 46-kD protein of *Drosophila* that was immunologically related to vimentin and changed in distribution after heat shock treatment; there was a shift of that protein from the microsomal compartment to the nuclear fraction after heat shock (3). It is not clear whether the latter protein has any relationship to the 47-kD collagen-binding protein.

In a previous paper (15), we reported that (*a*) the major collagen-binding proteins in detergent extracts of chick embryo fibroblasts at physiological ionic strength are the 47-kD glycoprotein and fibronectin, (*b*) the synthesis of the 47-kD gelatin-binding protein is decreased in Rous sarcoma virus–transformed cells, (*c*) this protein is phosphorylated in intact cells, and (*d*) the incorporation of [^{32}P]orthophosphate per unit protein is up to sevenfold higher in transformed cells than in normal chick embryo fibroblasts. These findings indicated that the 47-kD protein is also regulated in both total quantity and degree of phosphorylation before and after malignant transformation.

Kurkinen et al. characterized this 47,000-D protein, which they termed "colligin," as a cell surface–associated protein with N-linked oligosaccharide side chains containing mannose, which binds to native type IV collagen and gelatin (12). We found that this 47-kD protein from fibroblasts also binds to native type I collagen (15) and demonstrated its identity to a previously described 47,000- (or 48,000-) D glycoprotein decreased after transformation (5, 17). Although Kurkinen et al. postulated that this protein is a cell surface protein because it could be labeled by lactoperoxidase-iodination, the exact localization and function of this protein are not yet fully known due to the lack of specific antibodies to colligin (12, 21).

Kurkinen et al. also showed that undifferentiated F9 embryonal carcinoma cells synthesize the 47-kD gelatin-binding protein at low levels, and that its synthesis is increased ~15-fold after treatment with retinoic acid and dibutyryl cAMP (12). Since some heat shock proteins are suggested to be involved in morphogenesis or differentiation (1, 2, 20), it should be of interest to examine for changes of the 47-kD protein during development and differentiation. Its induction as part of the stress response appears to underscore the importance of intracellular or extracellular membrane glycoprotein interactions with collagen.

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