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HEAT-INDUCED PREFERENTIAL SYNTHESIS AND REDISTRIBUTION OF HSP 70 AND 28 FAMILIES IN CHINESE HAMSTER OVARY CELLS

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We observed that members of two HSP families (70 and 28 kDa) preferentially redistributed into the nucleus after heating at 45.5°C for 10 min. The rates of synthesis and redistribution of these proteins were different for each member of HSP families during incubation period at 37°C after heat shock. The maximum rates of synthesis of HSP 70 and HSP 28 families, except HSP 28c, were 6-9 hr after heat shock, whereas the maximum rates of redistribution were 3-6 hr after heat shock. These results suggest that the rates of redistribution of these proteins may be dependent on the amount of intracellular proteins as well as the alteration of binding affinity of nucleoproteins following heat shock. © 1990 Academic Press, Inc.

Several studies have shown an excellent correlation between heat-induced accumulation of proteins within the nucleus and heat-induced cytotoxicity (1,2). The accumulated proteins appear to be associated with the nuclear matrix (3,4). However, it is not known whether the accumulation of proteins onto the nuclear matrix results from an influx of proteins, an increase in binding affinity of nucleoproteins (5), or alteration of solubility of certain proteins (6). Immunofluorescence studies (7,8) have shown that certain proteins i.e. the 70 kDa heat shock protein (HSP-70), migrate from the cytoplasm into the nucleus after heat shock. Nevertheless, these techniques have some drawbacks: presently, it is difficult to distinguish between constitutive and inducible HSP 70 by immunofluorescence staining techniques due to difficulties in producing specific monoclonal antibodies against each member of the HSP 70 family without cross reactivity. We have employed a two-dimensional polyacrylamide gel electrophoresis technique which affords quantitative analysis of each member of the HSP families.

Several researchers have shown that heat shock proteins might play a role in thermal resistance (9-13). Lee and Dewey (13,14), and Laszlo (15) postulated that the redistribution of HSPs may also have an important role in development of thermotolerance. Heat induces five major HSPs (Mr 110,000, 87,000, 70,000, 28,000,

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and 8,500) in CHO cells (16). We observed that two HSP families (70 and 28 kDa) preferentially migrate into the nucleus. Furthermore, the rate of redistribution of these proteins in the nucleus varied according to each member of HSP family during incubation period at 37°C after heat shock.

Materials and Methods

Exponentially growing Chinese hamster ovary (CHO) were cultured in McCoy's 5a medium (Cellgro). The media were supplemented with 26 mM sodium bicarbonate and 10% iron-supplemented calf serum (HyClone). T-25 flasks containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. Cells were heated in T-25 flasks by total immersion in a circulating water bath (Heto) maintained within $\pm$ 0.05°C of the desired temperature.

Exponential phase cells were pulse-labeled with 50 μCi/ml [^{3}H]amino acid mixture (Amersham, 70-140 Ci/mmol) or [^{35}S]methionine (ICN, 1196 Ci/mmol) for 3 hr at 37°C various times after heating at 45.5°C for 10 min. Because HSP 28 in mammalian cells contains few methionine residues, [^{3}H]amino acid mixture was employed for HSP 28 labeling, while [^{35}S]methionine was used for HSP 70 labeling. After labeling, they were washed twice with Hanks' balanced salt solution (HBSS), trypsinized and pelleted by centrifugation at 4°C for 5 min at 90 X g. Pellets were resuspended with spinner salt solution (5.36 mM KCl, 0.83 mM $MgSO_4$, 116 mM NaCl, 26.2 mM $NaHCO_3$, 11.7 mM NaH_2PO_4, 5 mM D-glucose/liter, pH 7.0) and twice repelleted by centrifugation for 5 min at 300 X g.

For nuclear isolation, cells were resuspended in cold nuclear isolation buffer (50 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40 in 50 mM Tris-base:Tris-HCl, pH 7.4) and set on ice for 4-7 min for cell lysis. After lysis, the nuclei were pelleted by centrifugation for 10 min at 1000 X g, the supernatant was removed, and nuclei were resuspended in bicarbonate buffer (0.03 M NaCl, 0.03 M $NaHCO_3$, 0.1 mM PMSF, pH 8.1). Bicarbonate buffer washes were repeated twice by centrifugation.

Two-dimensional polyacrylamide gel electrophoresis (PAGE) was employed to analyze proteins. Isolated nuclei were treated with staphylococcal nuclease (50 μg/ml final concentration, Sigma Chemical Co.), and solubilized in 0.3% SDS and 1% β-mercaptoethanol, followed by a brief treatment with pancreatic DNase I (33 units/ml final concentration, Promega Corp.) and RNase-A (50 μg/ml final concentration, Sigma Chemical Co.). The nuclear sample was lyophilized and then dissolved in sample buffer containing 8 M urea, 1.7% NP-40, and 4.3% β-mercaptoethanol. Whole cells were directly dissolved in the sample buffer. Proteins were first separated in isoelectric focusing gels (pH 3.5-10). These gels are then laid across the top of an SDS gradient slab gel and the proteins were analyzed on 7.5-18% linear gradient SDS polyacrylamide gels. After electrophoresis, gels were fixed in 30% trichloracetic acid (TCA) for 30 min.

For gels containing a low energy β-emitting isotope like [^{3}H]amino acid mixture labeled proteins were fluorographed to enhance sensitivity, whereas those containing a high energy β-emitting isotope like [^{35}S]methionine labeled proteins were contact autoradiographed. The disadvantage of the fluorography is a loss of resolution. Thus, [^{35}S]methionine was employed for the study of HSP 70 family whose members locate closely in two-dimensional PAGE. For autoradiography, gels were dried in a slab gel dryer (Model 483, Bio-Rad, Richmond, CA) for 1.2 hr at 80°C and autoradiographed on Kodak SB-5 X-ray film. After exposure, autoradiographic film was developed with Kodak GBX developer and fixed with Kodak GBX fixer. For fluorography, gels were fixed in fixing solution (7% acetic acid, 20% methanol) for 1 hr and dehydrated by washing for 15 min in each of 25% acetic acid, 50% acetic acid, and glacial acetic acid consecutively. After fixation, the gel was placed in 125 ml PPO solution [20% (w/v) 2,5-diphenyloxazole in glacial acetic acid] for 2.5 hr. The PPO solution was removed, and the gel was shaken gently for 2 hr in distilled water, dried for 2.5 hr at 60°C, and then placed into a cassette with Kodak SB-5 X-ray film. The cassette was placed in the -70°C freezer. After exposure, fluorograph film was developed with Kodak GBX developer and fixed with Kodak GBX fixer. The films were scanned with a computerized laser scanning densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA). Quantitative measurement was performed with this instrument. Spots associated with each protein were quantitated by area integration of the optical density of the film.

Results and Discussion

To examine the kinetics of the synthesis of HSPs and their redistribution, cells were heated for 10 min at 45.5°C and then pulse-labeled with either [^{35}S]methionine or [^{3}H]amino acid mixture for 3 hr at 37°C immediately, 3 hr, 6 hr, 9 hr after heat. Nuclei were isolated immediately after labeling and analyzed. Five major heat shock proteins (HSPs; Mr 110,000, 87,000, 70,000, 28,000 and 8,500) were preferentially synthesized after heat shock (16). However, only two major HSP families (70 and 28 kDa) preferentially redistributed into the nucleus (data not shown).

Figure 1 shows an autoradiograph of two-dimensional gel electrophoresis of proteins from whole cells and isolated nuclei. Figure 2 shows quantitation of each member of HSP 70 family in two-dimensional gels. The ratios of the relative amount of the HSP 70 family to that of actin, which has been shown to be constant under these experimental conditions (10) were plotted. Data from whole cells (left panel in Fig. 2)

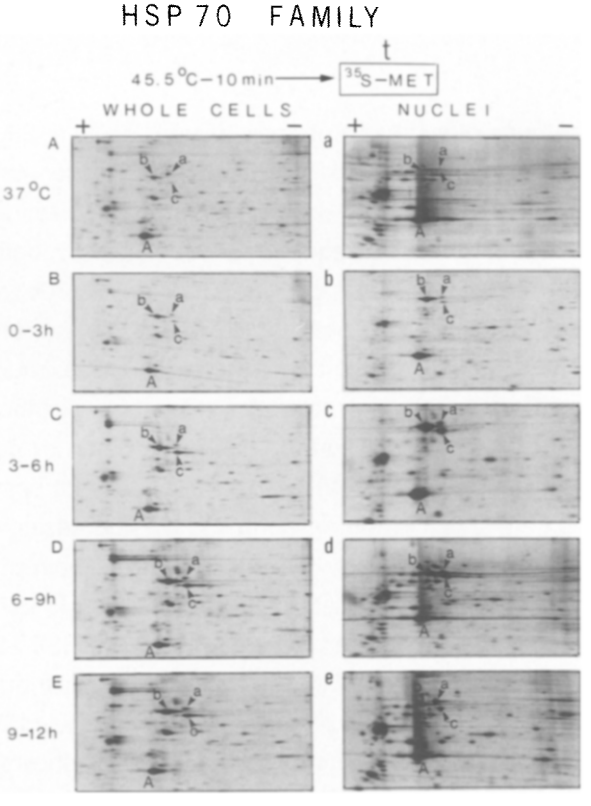

Figure 1. Autoradiograph of two-dimensional gel electrophoretic analysis of proteins synthesized in whole cells (panels A-E) and redistributed in nuclei (panels a-e). Cells were heated at 45.5°C for 10 min and pulse-labeled with [^{35}S]-methionine for 3 hr at 37°C immediately, 3 hr, 6 hr, 9 hr after heat. Nuclei (panels a-e) were isolated immediately after labeling. Only a section of each autoradiograph is shown. The locations of 70-kDa HSP-a (a), 70-kDa HSP-b (b), and 70-kDa HSP-c (c) and the location of actin (A) are identified.

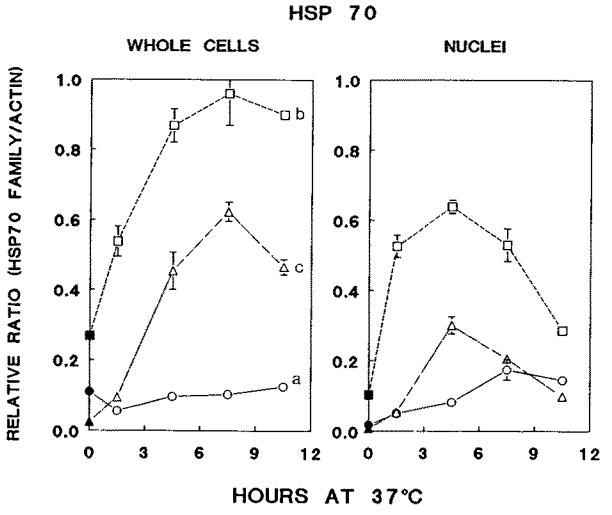

Figure 2. The relative rate of HSP 70 family synthesis in whole cells (left panel) and redistribution in nuclei (right panel). The ratio of the intensity of the 70-kDa HSP-a (○), 70-kDa HSP-b (□), 70 kDa HSP-c (△) spots to the intensity of actin is plotted as a function of various periods of incubation at 37°C after heat at 45.5°C for 10 min. Other details are as described for Fig. 1. (●,■,▲) Unheated control cells. Error bars represent one standard deviation of the data for each point.

demonstrated that the rates of synthesis of constitutive HSP 70 (HSP 70b: 70 kDa HSP-b) and inducible HSP 70 (HSP 70c: 70 kDa HSP-c) among three major HSP 70 family (11) gradually increased with increasing incubation time after heating at 45.5°C for 10 min. The maximum rate of synthesis required at least 6 hr incubation time after heat treatment. The relative rates of synthesis of HSP 70b and HSP 70c to that of actin were 0.96 and 0.62, respectively, 6-9 hr after heat shock. However, the rates of redistribution of both the HSP 70b and the HSP 70c in the nuclei reached maximum 3-6 hr after heat shock (right panel in Fig. 2). Furthermore, the rates of redistribution of these two polypeptides gradually decreased with further incubation time. In contrast, the rate of relocalization of HSP 70a (70 kDa HSP-a), whose level was not significantly altered in CHO cell line following heat shock gradually increased with increasing incubation time.

Figure 3 shows a fluorograph of two-dimensional gel electrophoresis of proteins from whole cells and isolated nuclei to determine the kinetics of HSP 28 synthesis and redistribution. Figure 4 shows quantitation of each member of HSP 28 family in the two-dimensional gels. The relative rate of synthesis and rate of redistribution of this protein were measured as described above. All four proteins of the HSP 28 family (HSP 28a,b,c,d) are related isoforms. The fluorograph of Figure 3 shows that the levels of HSP 28 family, unlike HSP 70 family, were low in unheated control cells. The left panel of Fig. 4 shows that the rates of synthesis of HSP 28a with two of the phosphorylated isoforms (HSP 28b and HSP 28c) (17) were increased with incubation time after heating at 45.5°C for 10 min. The presence of HSP 28d was not well observed in whole cells, due to the small number of molecules which are highly localized in the nucleus. The

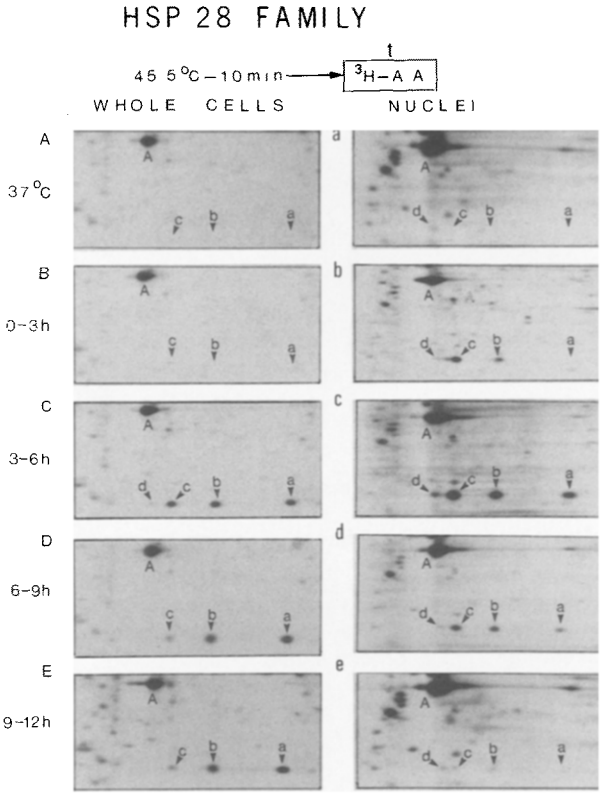

Figure 3. Fluorograph of two-dimensional gel electrophoretic analysis of proteins synthesized in whole cells (panels A-E) and redistributed in nuclei (panels a-e). Cells were heated at 45.5°C for 10 min and pulse-labeled with [^{3}H]-amino acid mixture for 3 hr at 37°C immediately, 3 hr, 6 hr, 9 hr after heat. Nuclei (panels a-e) were isolated immediately after labeling. Only a section of each fluorograph is shown. The locations of 28-kDa HSP-a (a), 28-kDa HSP-b (b), 28-kDa HSP-c (c), and 28-kDa HSP-d (d) and the location of actin (A) are identified.

maximum rates of synthesis of HSP 28a,b and HSP 28c required 6-9 hr and 3-6 hr incubation time, respectively, after heat shock. The relative rates of synthesis of these polypeptides to that of actin was probably underestimated 0-3 hr after heat shock due to underexposure of the fluorograph film. The maximum relative rates of synthesis of HSP 28a, HSP 28b and HSP 28c to that of actin were 0.34, 0.28, and 0.196, respectively. Nevertheless, the rates of redistribution of all HSP 28 family members into the nuclei reached a maximum 3-6 hr after heat shock (right panel in Fig. 4). Interestingly, phosphorylated forms (HSP 28b,c) preferentially relocalized in the nuclei. Furthermore, the rates of redistribution of these HSP 28 family members rapidly decreased with further incubation time suggesting that they redistribute back to the cytoplasm by 9 hr.

The entry of most small molecules or proteins into the nucleus is mediated by diffusion while, in contrast, migration of large proteins (>30 kDa) into the nucleus is facilitated by an active transport system involving two steps: binding and translocation (18). The first step, binding to the nuclear pore glycoproteins, is due to the specific

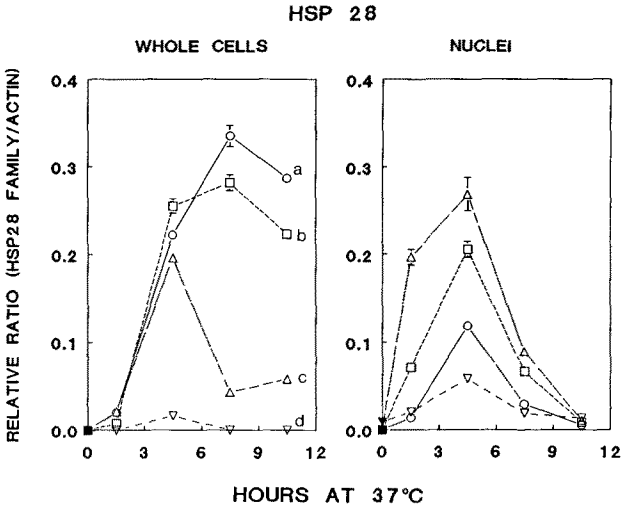

Figure 4. The relative rate of HSP 28 family synthesis in whole cells (left panel) and redistribution in nuclei (right panel). The ratio of the intensity of the 28-kDa HSP-a (○), 28-kDa HSP-b (□), 28-kDa HSP-c (△), 28-kDa HSP-d (▽) spots to the intensity of actin is plotted as a function of various periods of incubation at 37°C after heat at 45.5°C for 10 min. Other details are as described for Fig. 3. (●,■,▲,▼) Unheated control cells. Error bars represent one standard deviation of the data for each point.

recognition of the transport signals, known as nuclear targeting signals (19). The second step, translocation through the nuclear pore, is ATP-dependent (20). This active transport system is inhibited by either low temperature (21) or treatment with the lectin, wheat germ agglutinin (WGA) (22). It is also known that HSP-70 contains nuclear targeting sequences (19). This signal may be cryptic at physiologic temperatures, revealing itself under hyperthermic conditions to be recognized by nuclear pore proteins. This sequence of events may result in the accumulation of HSP-70 in the nucleus after heat shock. In contrast, HSP 28 which is phosphorylated during (23) and/or after heating (17) may enter the nucleus by diffusion.

As indicated above several studies have shown that heat induces protein accumulation on the nuclear matrix (3,4) which is known to be the most thermolabile nuclear component and becomes "sticky" during heating (24). This may be due to a heat-induced alteration in the distribution of hydrophilic and hydrophobic residues at the surface of the protein molecules which lessens gradually during the recovery period at 37°C. The consequently reduced binding affinity results in the lowered accumulation of these polypeptides in the nucleus accounting for the maximum rate of redistribution of HSP 70 and 28 families in the nucleus occurring earlier than the maximum rate of synthesis of these proteins. These results suggest that the rates of redistribution of HSP 70 and HSP 28 families are dependent on the amount of intracellular proteins as well as the alteration of binding affinity of nucleoproteins following heat shock.

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