**A Heat Shock-Resistant Variant of Chinese Hamster Cell Line Constitutively Expressing Heat Shock Protein of Mr 90,000 at High Level**

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**ABSTRACT.** A heat shock-resistant variant of Chinese hamster cell line (CHO) was isolated from ethane methane sulfonate-treated CHO cells through selection by repeated exposures to elevated temperature. The variant, designated HR-01, was one to two order of magnitudes more resistant to lethal heat shock (46.0°C) than the parental CHO strain (p-CHO). The heat shock resistance characteristic of this variant was stable. In addition, the HR-01 variant showed more elongated cell morphology, and was more adherent to substrate than p-CHO. When total proteins of p-CHO and HR-01 cells were compared in two-dimensional polyacrylamide gel electrophoresis, HSP90, a heat shock protein of Mr 90,000, was found to be the only protein that was expressed at a significantly higher level in HR-01 cells than in p-CHO cells. Because of the known intriguing molecular properties of HSP90, the HR-01 variant would be useful for further investigation of functions of HSP90 as well as the mechanism of acquiring heat shock resistance in mammalian cells.

Heat shock response is commonly induced in most living organisms, from bacteria to higher plants and mammals, when cells, tissues and bodies of these organisms are exposed to elevated temperatures and other environmental stresses (23, 24). The response is characterized by an induced expression of a set of proteins called heat shock proteins (HSPs), whose species and amino acid sequences have been highly conserved during evolution (1, 9). Severe heat shock causes eventual cell death though it induces synthesis of HSPs to some extent before cell death. When cells were pre-heated at moderately high temperatures so as to induce and accumulate HSPs and not to cause cell death, these cells became highly heat shock-resistant upon a following exposure to lethal heat shock (11, 16). These facts raised the possibility that HSPs function in protection of cells against lethal heat shock. In fact, cells treated with stressful agents such as ethanol (15) or arrested in the resting (G0) state (10) accumulated HSPs, and were also highly resistant to heat shock. There are several to a dozen different HSPs in each species (23). An important question is which HSP(s) does make cells heat shock-resistant.

By isolating a highly heat shock-resistant mutant of the yeast, *Saccharomyces cerevisiae*, we have previously made the new finding that the expression of HSP48,
a heat shock protein of Mr 48,000, but not of other HSPs is parallel to the level of heat shock-resistance (11). Furthermore, we have identified HSP48 as enolase 1, one of the two isoproteins of enolase expressed in this organism (12). However, only little is known about how mammalian cells do acquire the heat shock resistance property when exposed to moderately high temperatures. This study was designed to disclose this problem by isolating heat shock-resistant variants of a Chinese hamster cell line (CHO). We found that only HSP90 but not other HSPs was expressed to a higher degree in one of heat shock-resistant variants that we have isolated. The properties associated with this variant, designated HR-01, are described herein.

**MATERIALS AND METHODS**

**Cell line and culture medium.** The Chinese hamster cell line, CHO (GAT\(^+\)) (5) was provided by Dr. K. Onodera (University of Tokyo). CHO cells were cultured in α-MEM (with ribonucleosides and deoxyribonucleosides) (Gibco Laboratories, Inc., Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco Laboratories).

**Mutagenesis and Selection.** CHO cells were treated with 150 µg/ml ethane methane sulfonate (EMS) in the medium at 37°C for 18 h. The viability of EMS-treated cells as determined by colony formation decreased to approximately 45% of the untreated cells. The cells were trypsinized, and re-plated in flat glass bottles at the density of 6 × 10^4 cells/16 cm²/bottle. The bottles were incubated at 37°C for 2 days, and heated in a water bath at 46.0 ± 0.1°C for 18 to 20 min. The temperature of the medium as directly determined with a thermister reached the plateau, 46.0°C, within 80 sec. The bottles were rapidly cooled to room temperature. The colony forming unit decreased to 0.002–0.01% of the control by the heat treatment. Efficiency of colony formation was approximately 55% for control CHO (GAT\(^+\)) cells. The cells were trypsinized, re-plated in plastic bottles containing the medium, and incubated for several days. Surviving colonies appeared, after which cells were trypsinized and re-plated in glass bottles as described above. The heat selection was repeated. Finally, we isolated several surviving colonies from different bottles of the initial culture. Recloning was made for clones which appeared to possess the desired property. Properties of one (HR-01) of heat shock-resistant variants were examined in details as compared to the parental strain (p-CHO). The HR-01 variant was established three years ago, and maintained in culture for more than 450 generations without altering the heat shock resistance characteristic.

**Heat shock resistance as measured by colony formation.** A water bath was maintained at 46.0 ± 0.1°C throughout the heat treatment. Cells to be tested were cultured at 37°C in 20 to 30 flat glass bottles. Just prior to exposures to the elevated temperature, the bottles were filled with α-MEM-10% FBS pre-warmed at 37°C. All the bottles were placed in the above water bath at once. The temperature of medium in the bottles reached to 45.5°C within 50 sec and to 46.0°C within 80 sec. At the indicated times, the bottles were picked up, and placed in another water bath adjusted at 20°C. After completion of the heat treatment, cells were separately harvested from all the bottles by trypsinization. Cells were appropriately diluted, and plated in plastic dishes to determine colony formation. Decreases in colonies were determined as a function of time of the heat treatment. We have noted that heated cells for longer periods required longer incubations for their colony formation. Therefore, determinations of colony numbers were made after prolonged incubations for cells heated for longer periods. The colonies were fixed with methanol-acetic acid (3 : 1), and stained with Giemsa.
Heat shock resistance as measured by cell adhesion. Exponentially growing cultures of p-CHO and HR-01 strains were treated with 0.25% trypsin and 1 mM EDTA in PBS. Trypsinized cells were suspended in α-MEM-10% FBS at 1 x 10^5 cells/ml. Aliquots (1.0 ml) of the cell suspensions were added to siliconized glass tubes (10 x 80 mm). The tubes were sealed with parafilm before exposures to elevated temperature. The cell suspensions were incubated at 46.0 ± 0.1°C for the indicated periods, and cooled to room temperature as described above. The heated and control cells were plated in 35-mm plastic dish containing 1 ml of the medium, and incubated at 37°C for 6 h. Non-adherent cells were removed by gentle aspiration. The remaining adhered cells were treated with 0.25% trypsin and 1 mM EDTA, and re-suspended in 1 ml of PBS containing 4% FBS. The PBS-4% FBS should be filtrated through a Millipore filter with 0.45 µm pore size (Millipore Co., Bedford, MA, USA) before use. An 0.5 ml of each of the cell suspensions was mixed with 9.5 ml Isoton II (Coulter Diagnostic, Inc., Hialeah, Fla., USA), and subjected to determination of cell number with a Coulter Counter, model D (Coulter Electronics, Inc., Hialeah, Fla., USA). At least three determinations were made with each sample.

Analysis of total proteins by two-dimensional polyacrylamide gel electrophoresis. Two-dimensional polyacrylamide gel electrophoresis (2D-NEPHGE/SDS-PAGE) (18) was used for analysis of proteins in whole cell extracts. The detailed method was described elsewhere (10). Briefly, the cell extracts were firstly electrophoresed in 4% acrylamide/bisacrylamide gel, 9.2 M urea, 2% Nonidet P-40, and 2% Ampholine (pH 3.5–10, LKB, Bromma, Sweden), and secondly electrophoresed in 11% polyacrylamide gel containing SDS. The gels were stained with Coomassie brilliant blue. The quantitation of spots on two-dimensional gels was described elsewhere (10).

RESULTS

Isolation of heat shock-resistant variants. Heat shock-resistant variants have been reported to spontaneously occur in mammalian cell cultures without mutagenesis (7, 13, 21, 27). To obtain different types of heat shock-resistant variants or mutants, we used in this study ethane methane sulfonate-treated CHO cells as the source of the variants. Repeated exposures to 46.0°C were used for selection of heat shock-resistant variants because the relative viability of p-CHO cells as measured by colony formation was decreased to 1 x 10^-4 at 46.0°C in 20 min. Various surviving colonies were picked up and subjected to further characterizations on the heat shock sensitivity. Finally, individual variants with the desired property were cloned by the limiting dilution method. One (HR-01) of stable heat shock-resistant variants was used in this study. The heat shock resistance associated with HR-01 strain was not altered by repeated passages or during storage in liquid nitrogen.

Heat shock resistance property associated with HR-01. First, decreases in colony forming unit were determined for both p-CHO and HR-01 strains as a function of exposure time to 46.0°C. It should be noted that cells of cultures heated longer needed longer times to form colonies. Incubation for several days was sufficient for colony formation of unheated cells whereas cells heated at 46°C for 16 min or longer required a few weeks to form colonies with an equivalent size (φ=3 mm). For this reason, determination of surviving, colony-forming cells after an exposure to 46.0°C for a long period was more or less inaccurate.

The results showed that HR-01 is much more resistant to the heat treatment than p-CHO (Fig. 1A). Survival fractions of HR-01 cells heated at 46.0°C for 14 min were
Fig. 1. Heat shock-response curves of p-CHO strain and HR-01 variant. A. Cells cultured at 37°C or preheated at 38.5°C for 6 h were exposed to 46.0°C for the indicated periods on the abscissa. Decreases in colony forming units were determined as a function of exposure time for p-CHO cells (○), HR-01 cells (●), pre-heated p-CHO cells (△), and pre-heated HR-01 cells (▲). B. Trypsinized p-CHO (○) and HR-01 (●) cells were heated at 46.0°C for the indicated periods. Loss of the ability to adhere to substrate was determined as a function of exposure time.

approximately 40 times higher than those of p-CHO cells. When cells were preheated at 38.5°C for 6 h to induce and accumulate HSPs, the cells became more resistant to the lethal heat shock than unheated cells (Fig. 1A). The acquisition of the heat shock resistance property by the pre-treatment was remarkable for the both strains.

We have next examined the heat shock resistance characteristic associated with HR-01 by another means, determination of number of cells that retained the ability to adhere to substrate after exposures to heat shock. As was described above, trypsinized cells were exposed to 46.0°C for various periods, and plated in dishes. Loss of the adhering ability was determined as a function of exposure time (Fig. 1B). Since unheated HR-01 cells adhered to substrate much faster than unheated p-CHO cells, an incubation of shorter than 3 h was inadequate for comparison of the heat shock sensitivities of the two strains. Non-adherent cells were removed by gentle aspiration. Cells remaining on the bottom of plastic dishes were harvested in PBS-4% FBS. Cell numbers were then determined with a Coulter counter. The result showed that HR-01 cells are much more resistant to heat treatment than p-CHO cells (Fig. 1B). It should be noted that the number of cells maintaining the ability to adhere to substrate were larger than the number of those maintaining the ability to form colony. This difference is attributable to the fact that cell death eventually occurred 1–2 days after an exposure to lethal heat shock on some populations of the cells although they had retained the adhering ability at least up to 6 h after the heat
A Heat Shock-Resistant CHO Variant

Colonies of HR-01 were slightly but significantly larger than those of p-CHO when they were compared on day 7 (Fig. 2a). Since the doubling time was found to be 16.8±0.2 h for both p-CHO and HR-01 in the exponentially growing phase, the difference in colony size between the two strains could be due to difference in cell motility. In fact, HR-01 cells growing in margins of their colonies were more sparse than p-CHO cells were (Fig. 2b, c). In addition, HR-01 cells showed more elongated cell morphology, and were sometimes more flat than p-CHO cells. Cell volume was estimated to be 315 μm³ (mode) for p-CHO and 330 μm³ (mode) for HR-01.

Difference in proteins between p-CHO and HR-01 strains. The total cell extracts were prepared from exponentially growing cultures of the two strains, and analyzed by 2D-NEPHGE/SDS-PAGE. The major difference in the gel patterns between the two strains was found to occur in the spot corresponding to HSP90, a heat shock protein.

Fig. 2. Colony size and cell morphology of p-CHO and HR-01 strains. (a) Cells of p-CHO (left) and HR-01 (right) strains were plated in 60-mm plastic dishes. Colonies were fixed and stained on day 7. Marginal regions of colonies of p-CHO (b) and HR-01 (c) strains were observed by phase contrast microscopy. Magnification; × 180.
Fig. 3. Two-dimensional polyacrylamide gel electrophoresis (NEPHGE/SDS-PAGE) of total proteins of p-CHO (a) and HR-01 (b) strains. The gels were stained with Coomassie brilliant blue. Large arrowheads, small arrowheads and arrows indicate HSP90, HSP70 and actin, respectively.

The content of HSP90 was 5 to 10-fold increased in strain HR-01 compared to strain p-CHO although accurate estimation of the spot intensity was difficult because of spot trailing always seen with HSP90 (10). The synthesizing rate of HSP90 in HR-01 cells was also 5 to 10 times higher than that in p-CHO cells (data not shown). The result clearly indicated that the expression of HSP70, a major heat shock protein of Mr 70,000, did not differ between p-CHO and HR-01 strains (Fig. 3). Although there appeared to be minor differences in some proteins between the two strains, they were not reproducible or significant.

DISCUSSION

Degree of heat shock resistance may depend upon the amount of specific proteins expressed in cells. HSPs are candidates for such proteins because an accumulation of HSPs is positively correlated with the degree to which cells are resistant to high temperature (23, 24). The question we dealt with in this study is which HSP(s) is essential for the acquisition of heat shock resistance. We isolated a heat shock-resistant variant of CHO cell line which expressed HSP90 at an increased level. Inasmuch as other HSPs were not significantly overproduced in the variant, it seems that HSP90 is possibly involved in heat shock resistance in this case. There are several reports pertinent to this point, however. Firstly, Loomis and Wheeler have isolated a mutant of Dictyostelium that is defective in acquiring heat shock resistance, and have shown that this mutant specifically fails to synthesize a set of HSPs of Mr 26,000–32,000 (17). This suggests that these low molecular weight HSPs are involved in the acquisition of heat shock resistance in this organism. Secondly, Finkelstein and Strausberg have shown that over-expression of HSP90 in yeast cells by introducing a multicopy vector carrying the yeast HSP90 gene did not increase the degree of heat shock resistance (6). Thirdly, we have recently shown that HSP48...
is the indicator of heat shock resistance in the yeast (11). Furthermore, the yeast HSP48, identified as enolase 1, was shown to have partial homology in amino acid sequence to the HSP70 family (12). None of these results is compatible with the present finding if the unique HSP is the indicator of heat shock resistance, and is common in all species. Alternatively, more than one HSP species may be involved in heat shock resistance. In fact, pre-heated CHO cells that were induced to synthesize a full set of HSPs were more resistant to lethal heat shock than HR-01 cells (Fig. 1A). It is also possible that different HSPs are indicators in different species, particularly between the homeotherm and the heterotherm. In fact, the mutant in the yeast system is three order of magnitudes more resistant to lethal heat shock than the parental strain whereas the degree of heat shock resistance associated with the HR-01 variant of CHO cell line is considerably lower.

Several groups have isolated heat shock-resistant variants of cultured mammalian cell lines (7, 13, 21, 27). Among these variants, only one has been analyzed for its protein composition: That is a heat shock-resistant variant of Chinese hamster fibroblastic cell line that over-expresses HSP70 to a significant degree (13). The previous studies have shown that HSP70 translocates into nuclei upon exposure to heat shock (14, 25) and binds to nucleoli (20, 28). It was suggested, therefore, that HSP70 may function in nuclei. Differently from HSP70, HSP90 did not translocate into nuclei when cells were exposed to heat shock. HSP70 and HSP90 may co-operatively function in protection of cells against lethal heat shock although relatively weak, but significant heat shock resistance is given to cells by either one of the HSPs alone.

The HR-01 variant showed various altered properties as to morphology and cell adhesion to substrate in addition to the heat shock resistance. We have recently found that HSP90 is an actin binding protein, and is localized in ruffling membranes together with filamentous actin (S.K. et al., submitted for publication). This suggests that HSP90 interacts with actin filaments in vivo and might be involved in cell adhesion and spreading. It would be possible, therefore, that all the altered properties associated with HR-01 strain are attributed to the increased expression of HSP90. Differently from HSP70 and low molecular weight HSPs, the distribution of HSP90 was not dramatically altered when cells were exposed to heat shock (unpublished observations). HSP90 may protect intracellular architectures consisting of actin filaments against disintegration to be caused by heat shock. It has been observed, in fact, that cellular actin structures were disintegrated and actin molecules were translocated into the nucleus, and re-organized into paracrystal-like structures of actin filaments, called actin rods, when cells were exposed to heat shock (ref. 29, and Kazuko Iida et al., in press).

HSP90 has been shown to form tripartite complexes with pp60src, the product of v-src of avian sarcoma virus, and a phosphoprotein of Mr 50,000 in avian cells (2, 3, 19). Since pp60src is known to function after binding to the inner surface of the plasma membrane, the complexes are supposed to transport the viral product to the membrane across the cytoplasm (3). More recently, it was reported that HSP90 is a steroid hormone receptor binding protein (4, 8, 22, 26). In this case too, HSP90 may function as the common carrier protein of receptors for various steroid hormones (4, 8, 26). Although it is not clear whether these intriguing properties associated with HSP90 is related to involvement of this protein in heat shock resistance, HR-01 variant would be useful for investigation of the functions of HSP90.
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