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Modulation of Stress Protein Gene Expression by Environmental Stress and pH in the Mouse Fibroblasts and SCK Tumor Cells*

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생쥐의 纖維芽細胞와 SCK 腫瘍細胞에서 Stress와 pH에 의한 Stress Protein 遺傳子發現의 調節

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요 약

Stress protein (SP) 遺傳子發現의 調節機構를 밝히기 위한 한가지 방편으로 환경의 stress와 pH가 SP의 合成誘導에 어떤 作用을 하는지를 SDS-PAGE를 이용해서 分析하였다.

蛋白質合成의 전반적 양상은 MEF와 SCK 세포에서 달랐으나 SP의 양상은 동일하였다. 그중에서 SP70의 誘導와 減衰의 kinetics는 특히 흥미로왔다. SP70의 kinetics는 酸性 pH와 正常 pH에서 類似하였으나 最大量의 SP 合成에 필요한 溫度와 그 處理時間은 pH에 의해 달리 나타나서, 酸性 pH에서는 낮은 溫度와 짧은 處理時間에서 나타나고 더욱 오래 지속되는 경향을 보였다.

SP의 合成誘導와 SP mRNA의 축적은 actinomycin D에 의해 阻止되는 사실로 미루어 SP의 合成이 誘導되기 위해서는 새로운 mRNA의 合成이 필요함을 알수 있었고, cycloheximide 처리의 결과는 SP의 合成誘導에 앞서서 어떤 特異한 蛋白質의 合成은 일어나지는 않음을 알수 있었다.

이상과 같은 몇가지 實驗結果는 MEF와 SCK 세포에서 SP의 合成誘導는 일차적으로 轉寫水準에서 調節되며, SP70의 合成은 自動調節됨과 아울러 SP의 水準은 세포의 stress 상태와 相關關係가 있는 것으로 推論할수 있음을 보여주었다.

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INTRODUCTION

The effect of hyperthermia on protein synthesis in *Drosophila* is quite dramatic. Among the first detectable changes is the disintegration of polysomes engaged in mRNA translation, and this occurs prior to the onset of heat-shock mRNA translation. It was also found that intracellular protein rearrangements accompany these changes (Falkner and Biessmann, 1980).

Exposure of mammalian tissue culture cells to elevated growth temperature results in specific changes in the patterns of proteins being synthesized. (Slater and Cato, 1980; Tsukeda *et al.*, 1981; Burdon, 1982; Hicky and Weber, 1982; Welch *et al.*, 1982; Omar and Lanks, 1984) Similar to that which has been described for *Drosophila melanogaster* (reviewed by Ashburner and Bonner, 1979), heat-shock treatment of mammalian cells is characterized by the synthesis and accumulation of a small number of polypeptides concomitant with a decreased production of the normal complement of other cellular proteins.

It is now recognized that various other treatments besides heat shock give rise to the same response in eukaryotic cells. Hence, this generalized response to perturbation in the normal growth environment of cell is more aptly referred to as the "stress response" and accordingly the proteins induced termed the "stress proteins" (SP).

Despite the fact that the induction of SPs as a common response to environmental stress has been demonstrated in a wide range of organisms, the details of molecular mechanisms whereby this is achieved vary slightly from organism to organism. For instance, the initial induction appears to be regulated primarily at the level of transcription although some translation control is evident, and translation control mechanisms seem less important in the HeLa cell response than *Drosophila*. Not surprisingly, there has been considerable speculation with regard to the physiological significance of SPs. It has been suggested that they might have a role in the recovery of cellular homeostasis after stress (Burdon, 1982).

Therefore, we aimed at determining the changes in the induction and maintenance of stress proteins following stress treatment, as a parameter for the degree of heat susceptibility in mouse fibroblasts and SCK tumor cells under acidic or normal pH. Specifically, experiments presented here were directed toward understanding how SP synthesis is modulated during hyperthermia and recovery, together with the possible means for elucidating whether the correlation exist between stress protein and heat susceptibility in MEF and SCK cells in acidic or normal medium.

MATERIALS AND METHODS

Cell Culture

The SCK tumor cells used in the present experiments were originally from a carcinoma

of female A/J mice, which arose spontaneously in 1974 and was adapted to grow *in vitro* and *in vivo* (Kang *et al.*, 1980).

The cells were cultured in T-flasks with RPMI 1640 medium (GIBCO Co.) supplemented with 2 mg/ml sodium bicarbonate, 5 mM N-2-hydroxyethyl-piperazine-N'-ethane-sulfonic acid(HEPES), 100 units/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml fungizone and were maintained in a humidified CO_2 incubator at 37°C. Exponentially growing cells were subcultured with 0.25% trypsin and inoculated at appropriate number of cells every 3 or 4 days. Under these conditions, the population-doubling time was about 12 hr during the exponentially growing phase.

To obtain primary cultures of MEF cells, 2 week-old embryos were removed surgically from A/J mice. The embryos were rinsed with RPMI 1640 medium prewarmed to 37°C, and decapitated, and legs, tails, intestines, spines, bones, and skin were removed. The remainder was finely minced with scissors and incubated in 0.1% trypsin and 0.002% DNase for 20 min at 37°C. The cells were centrifuged, and were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, and were filtered through 4-fold lens papers. The cells obtained were seeded at 2×10^6 cells per ml of medium and incubated under standard condition until confluent. The confluent cells were subcultured with 0.1% trypsin every 2 days. The cells after third subculture were used, since among various mouse embryo cell types, only fibroblasts survived to that stage. The population-doubling time of MEF cells during the exponentially growing phase was about 24 hr.

pH Maintenance

The pH adjustment was made essentially by the method of Hahn *et al.* (1983). Normal pH of 7.4 and acidic pH of 6.7 were obtained by adjusting sodium bicarbonate dissolved in the media. The adjusted medium was then maintained by continuous gassing with 5% CO_2-air. The pH levels were reproducible from experiment to experiment.

Hyperthermia of the Cells

The SCK and MEF cells were seeded at the density of 8×10^5 and 1×10^6 in T-flasks, respectively. After 2-day culture, culture flasks inserted in plastic shelves were immersed horizontally in a constant temperature circulating water bath. Prior to and immediately after hyperthermia, the fresh media adjusted to normal or acidic pH were replaced. Following the hyperthermia the cells were incubated at 37°C for 4 hr, and then trypsinized, counted using the trypan blue exclusion and seeded at a density of 3×10^5 cells per 60 mm dish containing 4 ml of normal pH medium. The cells were daily trypsinized with 0.25% trypsin and 0.2% EDTA and were counted.

Incorporation of [^{35}S] Methionine

After hyperthermia under normal or acidic pH, the cultures were incubated for 4 hr at 37°C. At the end of incubation, media were discarded and the cells were incubated in methionine-free RPMI 1640 media containing [^{35}S] methionine (5 μCi/dish for SCK; 15 μCi/dish for MEF) at normal pH for 1 hr. Labeled cells were harvested by removing the

medium, washing 3 times with Ca^{2+}, Mg^{2+}-free phosphate buffered saline, and adding 200 μl of SDS-PAGE sample buffer containing 625 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue to each dish. Samples were stored at -20°C until used.

Gel Electrophoresis, Autoradiography and Fluorography

Labeled samples were completely dissociated by heating in boiling water for 3-5 min and were applied to slab gels containing 7% acrylamide using the discontinuous system of Laemmli (1970). Electrophoresis was carried out with a constant current of 20-30 mA for 3-5 hr. After electrophoresis, the gels were stained with 0.2% Commassie brilliant blue dissolved in 40% methanol plus 10% acetic acid for 2 hr and destained with destaining solution containing 35% methanol and 7% acetic acid until the background became clear. Destained gels were dried under gentle vacuum, and autoradiograms were prepared by exposing the dried gels on X-ray films.

Two-dimensional polyacrylamide gel electrophoresis was performed according to the method of O'Farrell (1975) with minor modifications. In the isoelectric focusing step, 0.32 ×15 cm cylindrical gels of polymerized 4% acrylamide and 0.2% bisacrylamide containing a mixture of 1.6% pH 4 to 6 and 0.4% pH 3 to 10 ampholyte with Nonidet P-40 were used. Electrophoresis was run for 16-20 hr at 400 V followed by 1 hr at 800 V. The gels were removed from the tube and each was equilibrated with 20 ml of 2% SDS sample buffer for 90 min. Gels were placed on top of the usual slab gels, overlaid with 1% agarose, and electrophoresed at 20-30 mA.

For fluorography, the gels were soaked in 20-volume of dimethylsulfoxide (DMSO) for 30 min. After a repeat of this step, the gels were immersed in 4-volume of 20% (W/V) PPO in DMSO for 3 hr and immersed in 20-volume of water for 1 hr. The gels were then dried under gentle vacuum and exposed on X-ray films for 12-24 hr at -60°C. After development, X-ray films were scanned in a densitometer (Helena Lab.) at 525 nm.

RESULTS

Protein Synthesis under Normal and Hyperthermic Conditions

The pattern of protein synthesis under normal conditions is different between MEF and SCK tumor cells. Fig. 1 shows that the LETS protein is apparent in MEF cells, whereas it is hardly discernible in SCK cells, and that the magnitude of the actin band appears most broad in MEF cells, while in SCK cells the same band appears much reduced in magnitude. Besides, a close examination of the autoradiograms reveals a number of minor proteins which distinguish between these two cells (Figs. 1 and 2).

In contrast, the cells exposed to different temperatures or to varying durations show the increased synthesis of three size classes of stress proteins, namely SP110, SP87 and SP70. Among these, the SP70 exhibits the most interesting characteristics of induction and decay

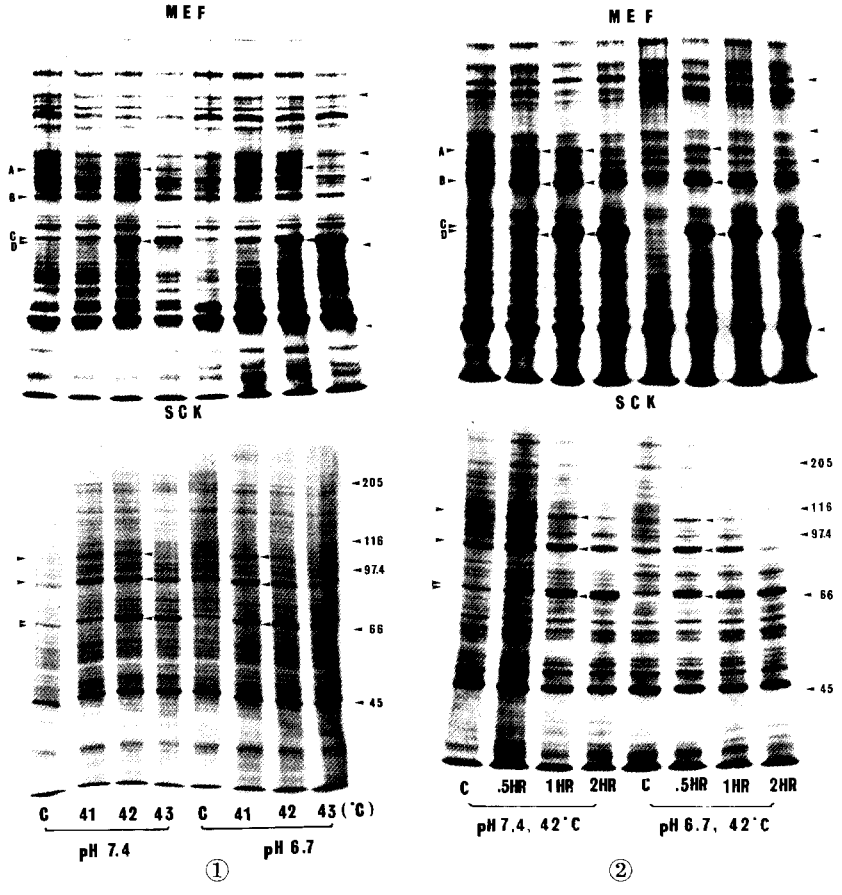


Fig. 1. Fluorogram (MEF) and autoradiogram (SCK) of SDS-polyacrylamide slab gels of [^{35}S] methionine labeled proteins from cells exposed to 41~43°C for 1 hr under normal or acidic pH. After hyperthermia the cells were returned to acidic or normal medium at 37°C for 4 hr prior to labeling for 1 hr. The arrows A,B,C, and D refer to the stress protein bands in the Mr regions of 110, 87, 70 and 68 kD. Molecular weight markers are indicated by arrows on the right of the figure. The MEF cells heated induced maximum amounts of stress protein at 42°C regardless of pH conditions. In contrast, the SCK cells heated at normal pH induced maximum amounts of stress protein at 42°C, whereas those at acidic pH induced at 41°C, indicating pH-sensitization in acidic pH.

Fig. 2. Fluorograms of SDS-polyacrylamide slab gels of [^{35}S] methionine labeled proteins from MEF and SCK cells exposed to 0.5~2 hr at 42°C under normal or acidic pH. After hyperthermia the cells were returned to normal or acidic medium at 37°C for 4 hr prior to labeling for 1 hr. The arrows A,B,C, and D refer to the stress protein bands in the Mr regions of 110, 87, 70 and 68 kD. Molecular weight markers are indicated by arrows on right of the figure. The MEF cells heated induced maximum amounts of stress protein at nearly same duration of treatment regardless of pH conditions. In contrast, the SCK cells heated at normal pH induced maximum amounts of stress protein at 1 hr duration, whereas those at acidic pH induced at 0.5 hr duration, indicating pH-sensitization in acidic pH.

kinetics (Fig. 3), as revealed by electrophoresis followed by autoradiography and densitometric scan. The scanning pattern clearly shows the kinetics of rise and fall of the two bands (Mr's 70 and 68kD) which separate out from SP70.

Since each of these stress protein bands could well be resulted from a mixture of different polypeptides of the same or similar molecular weights, two-dimensional gel analysis was carried out. As is evident from Fig. 5, SP70 band is found to be composed of 70 kD and 68 kD bands, and the former is again resolved into at least seven polypeptide spots and the latter at least three. Likewise, SP110 and SP87 are resolved into at least three and two polypeptide spots, respectively.

Stress Protein Synthesis under Normal or Acidic pH

The effect of pH during and after hyperthermia on stress protein synthesis is illustrated for temperatures ranging from 41 to 43°C for 1 hr (Fig. 1) and for varying durations at 42°C (Fig. 2). An apparent tendency in stress protein synthesis as affected by pH is that

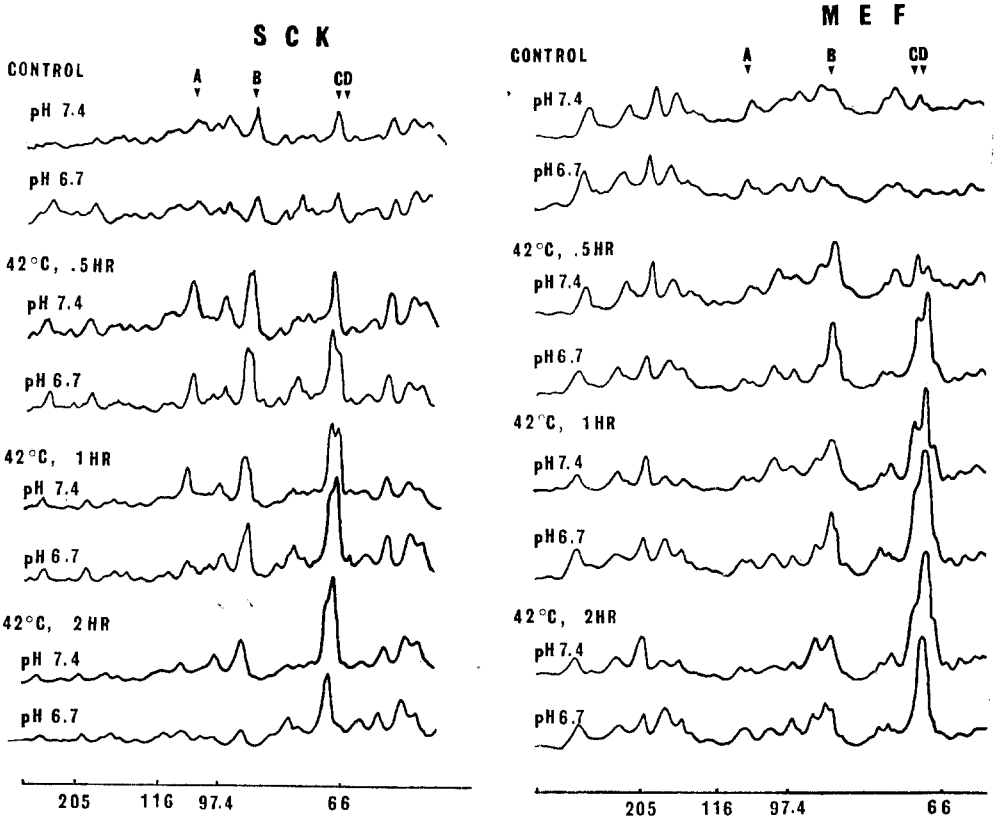


Fig. 3. Densitometric scans of fluorgraphic pattern of SDS-polyacrylamide slab gels of [^{35}S] methionine labeled proteins from MEF and SCK cells exposed to 0.5~2 hr at 42°C under normal or acidic pH. The arrows A,B,C, and D refer to the stress protein bands and molecular weight markers are indicated as numbers in kD on the bottom of the figure.

the stress proteins are produced at lower temperatures as well as shorter durations of heating under acidic pH compared to those under normal pH. As shown in Fig. 1, at acidic pH SCK cells produce maximum amounts of three stress proteins at 41°C, while at normal pH the same cells produce them at 42°C. The same is true for the duration of hyperthermia at 42°C in that cells produce maximum amounts of stress proteins earlier in acidic pH than in normal pH. These results together with the survival study (Fig. 4) indicate that the environmental pH levels affect thermal sensitivity of a cell.

A remarkable fact to be noted is that pH effect is more profound in SCK cells than in MEF cells. In other words, pH-sensitizing effect is clearly visible in SCK cells, whereas it is hardly distinguishable in MEF cells. These results led to the examination of cell survival in different pHs during and after hyperthermia. The results clearly indicate that the pH-sensitizing effect is profound only in the cells exposed to hyperthermia and is 4 times more significant in SCK cells than in MEF cells (Fig. 4).

Stress Protein Synthesis by Agents other than Hyperthermia

The effects of sodium arsenite and ethanol, at the concentration of 100 μM and 6%, respectively, on stress protein synthesis were examined in both cells under normal conditions. As is evident from Fig. 6, sodium arsenite induces an increase in the synthesis of SP70 in both cells, whereas ethanol induces the increased synthesis of SP70 in MEF cells but not in SCK cells. In addition, sodium arsenite enhances the synthesis of 25 kD protein in both cells. The densitogram shows the labeled protein profile more clearly (not shown).

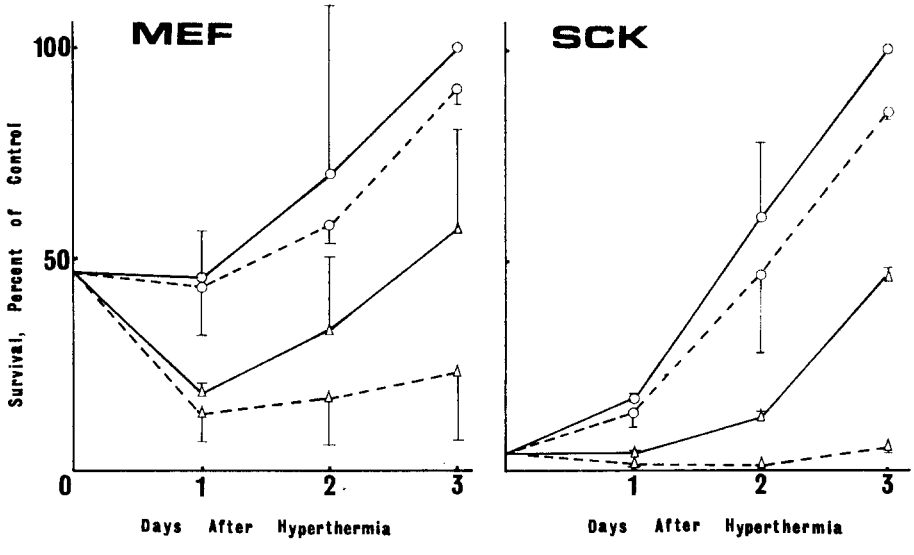
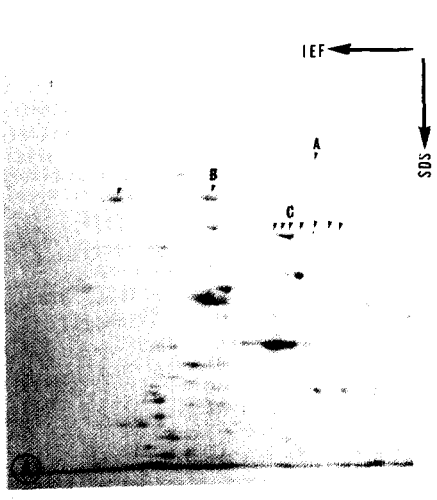
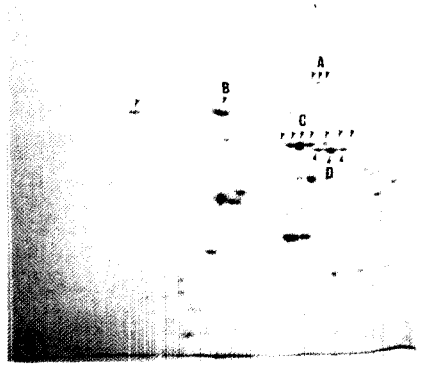


Fig. 4. Survival of MEF and SCK cells following hyerthermia at 42°C for 2 hr under normal or acidic pH. The sensitizing effect of acidic pH is predominate in SCK cells over in MEF cells.





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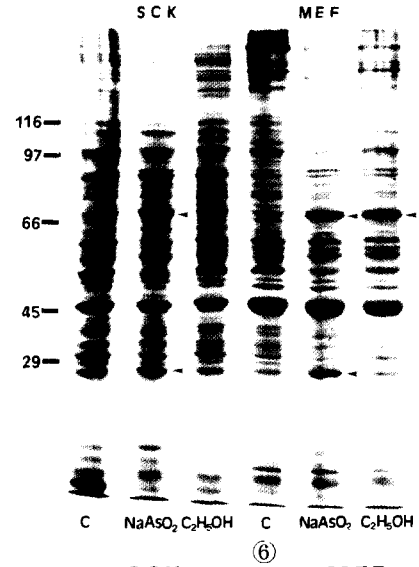

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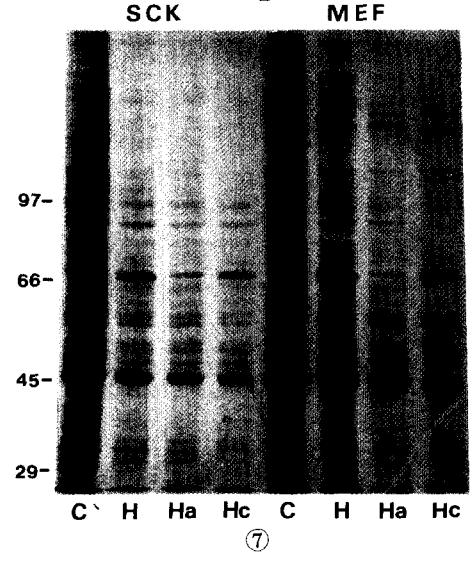

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Fig. 5. Autoradiograms of two-dimensional polyacrylamide gels of [^{35}S]methionine-labeled proteins from normal and heated SCK cells. A. Proteins from cells cultured at 37°C and labeled for 1 hr at 37°C; B. Proteins from cells exposed to hyperthermia at 42°C for 1 hr. The arrows A,B,C, and D refer to 110, 87, 70 and 68 kD proteins, respectively.

Fig. 6. Autoradiogram of [^{35}S]methionine-labeled proteins in the cells treated with sodium arsenite or ethanol. Sodium arsenite induced SP70 in both cells, whereas ethanol induced SP70 in MEF cells but not in SCK cells. In addition, SP25 appeared to increase in both cells treated with sodium arsenite but not with ethanol.

Fig. 7. Fluorogram of [^{35}S]methionine-labeled proteins in heated cells treated with actinomycin D or cycloheximide. C, unheated control; H, heated at 43°C for 1 hr and returned to 37°C for 2 hr prior to lebeling for 2 hr; H_a and H_c, treated with either actinomycin *D* or cycloheximide throughout the hyperthermic and subsequent incubation periods.

Susceptibility of Stress Protein Synthesis to Actinomycin D and not to Cycloheximide

In Fig. 7 is shown the effect of actinomycin D (1 μg/ml) or cycloheximide (25 μg/ml) during hyperthermia and subsequent incubation periods of 2 hr at 37°C on the stress protein synthesis. When actinomycin D was treated, no induction of SP70 was noticeable. This result suggests that hyperthermia-induced SP70 appears to be synthesized from the newly transcribed mRNA following hyperthermia. On the contrary, when cycloheximide is treated during the same period the induction of SP70 is not affected, providing the inhibitor is removed prior to addition of label. Referring to the fact that cycloheximide blocks the elongation of protein chains (Baliga *et al.*, 1970), the induction of SP70 synthesis after hyperthermia does not appear to require specific protein synthesis, at least during the actual hyperthermia and subsequent incubation periods.

DISCUSSION

Recently, numerous reports have been emerging that when confronted with environmental insults eukaryotic cells elicit a common and seemingly conserved stress response. Generally, the stress response in eukaryotes results in the induction of a limited number of proteins. The cellular response to hyperthermia and subsequent synthesis of stress protein have been investigated extensively in *Drosophila* (Ashburner and Bonner, 1979), in avian cells (Kelley and Schlesinger, 1978) and in mammalian cells (Slater *et al.*, 1981).

The present experiments show that SP70 is a noble protein, whereas SP110 and SP87 are revealed as the specific enhancement of existing proteins both in MEF and SCK cells. These proteins are idential or similar to those reported by other workers. Presumably, as pointed out by Hicky and Weber (1982), variations in the Mr reported are a consequence of the electrophoretic systems and molecular weight markers used. Apparently, the size of the major stress proteins is highly conserved among vertebrates.

The mRNA for the SP70 is considered to be synthesized *de novo* upon hyperthermia because actinomycin D inhibits the induction of the protein synthesis even if it is removed before the labeled precursor is added. This alone, however, is insufficient evidence that synthesis of stress protein is under transcriptional control, since we can not exclude the possibility that RNA processing or stability of stress protein mRNAs are the steps in gene expression affected by hyperthermia. Further experiments are required to determine whether transcription of DNA sequences coding for stress protein are stimulated at elevated temperatures in SCK and MEF cells. Cycloheximide, treated during hyperthermia and subsequent incubation periods, appears not to affect the increased induction of SP70. This is indicative of the fact that induction of SP70 after hyperthermia does not require specific protein synthesis which may play a role in the process of stress protein induction, as suggested by Slator *et al.* (1981) who treated cycloheximide only during the actual heat-shock period.

The effects of environmental pH on the increased induction of stress proteins are expressed in a quantitative manner. For example, the synthesis of SP70 is induced earlier and maintained longer in SCK cells under acidic pH than those under normal pH, whereas in the MEF cells pH effect is not so apparent. The exact mechanisms of low pH sensitization are not known. Overgaard (1976) suggested that reduction in pH at the time of hyperthermia increases lysosomal enzyme mediated cell destruction. It was also reported that the lysosomal enzyme activity appeared to be accelerated at a decreasing pH levels (Deduve and Wattiaux, 1966). Although this reaction is primarily intracellular, it may be involved because evidence supports the assumption that an extracellular acidity will cause a decrease in the intracellular pH (Poole *et al.*, 1964).

In the present experiments, it is shown that SCK cells are slightly more heat-susceptible than are MEF cells, and that the heat susceptibility is more profound under acidic pH, especially in SCK cells. It is well known that cancer cells experience many changes in the cell surface through transformation process. Among these changes are alterations in surface charge density as well as surface ion density. Nolan *et al.* (1981) reported that pH's less than 6.8 should sensitize cells to heat-induced cytotoxicity primarily through the influence on the polar portion of membranes. The differences between normal and cancer cell surfaces seem to make cancer cell to obtain different heat susceptibility compared to normal cells following hyperthermia at low pH.

The function of stress protein is not fully understood. It is suggested, however, that stress proteins may perform a function which is essential to cell survival following exposure to heat (Guttman *et al.*, 1980). On the relationship between stress protein synthesis and the differential heat susceptibility of normal and malignant cells, Tsukeda *et al.* (1981) reported that the modulation of stress protein synthesis after heat treatment reflects exactly the heat susceptibility of the malignant or normal human lung cells. In disagreement with this is the report by Omar and Lanks (1984) that the higher intrinsic resistance of the normal cells to killing by hyperthermia compared with transformed cells is not directly related to basal levels of stress protein or to the degree of stress protein synthesis induced following hyperthermia.

The present study as well as many reported in the literature indicates that a low environmental pH level at the time of hyperhermic treatment sensitizes mammalian cells to heat killing. The present data show that the pH effect seems to be cell-type dependent, being more heat sensitive in SCK cells than are in MEF cells. In this context, it is worth noting that synthesis of stress protein is likewise pH-dependent, when the cells are exposed to different temperatures and to varying durations of hyperthermia.

Based on our preliminary findings, we postulate that the pH dependence of heat susceptibility of MEF and SCK cells could possibly be correlated with the kinetics of stress protein synthesis. Thus, a differential kinetics for the synthesis of SP70 under different environmental pH's leads to a possible interpretation as follows: The low pH sensitizes the

cell to heat. The sensitized cells start to produce stress proteins rapidly as a stress response, and at this condition the response does last longer compared to those under normal pH conditions. However, in view of the fact that hyperthermic cell killing as well as hyperthermic target inactivation mechanism is different at low or high temperature, extensive studies are needed to elucidate whether correlation exist between stress protein induction and heat susceptibility as exemplified by intrinsic properties of the cell or by pH sensitization.

ABSTRACT

Aimed at elucidating the modulation of stress protein gene expression, the effect of environmental stress and pH on the induction of stress protein synthesis has been analyzed using SDS-polyacrylamide gel electrophoresis.

Although the general patterns of protein synthesis in MEF and SCK cells are different, stress protein patterns are identical in both cells. Among three stress proteins, the SP70 exhibits an interesting kinetics of induction and decay. The kinetics of SP70 under acidic or normal pH appears to be similar, but the degree of hyperthermia and duration of treatment required for maximum induction are found to be different, being lower temperatures and shorter durations under acidic pH compared to those under normal pH.

Induction of stress protein and the accumulation of mRNA coding for stress proteins are blocked with actinomycin D, indicating that new RNA transcription is required for stress protein induction. Treatment of cycloheximide during and after hyperthermia indicates that no specific protein is required for the induction of stress protein synthesis.

Based on our preliminary data, we postulate that induction of stress protein synthesis in MEF and SCK cells is regulated primarily at the level of transcription and that SP70 autoregulates its synthesis and levels of this protein are correlated with the stressed state of a cell.

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