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Enhanced Phosphorylation of a 65 kDa Protein Is Associated With Rapid Induction of Stress Proteins in 9L Rat Brain Tumor Cells

Yiu-Kay Lai, Chi-Hsiu Shen, Ting-Jen Cheng, Ming-Ching Hou, and Wen-Chuan Lee

Institute of Life Science, National Tsing Hua University, Hsinchu, Taiwan 30043, Republic of China

Abstract Induction of heat-shock proteins and glucose-regulated proteins in 9L rat brain tumor cells can be differentially elicited by sodium arsenite, cadmium chloride, zinc chloride, copper sulfate, sodium fluoride, and L-azetidine-2-carboxylic acid. The kinds of stress protein induced by the above chemicals varied considerably, mainly determined by the nature and the concentration of the chemicals, as well as the treatment protocols. In addition, at the concentrations where stress proteins can be induced, the above chemicals were able to suppress general protein synthesis and were cytotoxic. Enhanced phosphorylation of a protein with an apparent molecular weight of 65 kDa was detected during the induction of stress proteins except in azetidine treatments during which uptake of phosphate by the cells was impaired after prolonged incubation. The phosphate moiety on the 65 kDa phosphoprotein appeared to be alkaline-stable and two-dimensional gel electrophoresis revealed that the phosphoprotein resolved into four isoforms with isoelectric points ranging from 5.1 to 5.6. Enhanced phosphorylation of the same protein was also detected in heat-shocked and withangulatin A-treated 9L cells in which stress proteins were induced. It is suggested that this phosphoprotein may be a common target for heat stress response-stimulated phosphorylation and important in the further metabolic responses of the cell to stress.

Key words: heat-shock proteins, glucose-regulated proteins, protein phosphorylation, heat-shock response, stress response, brain tumor cells

Exposure of cultured cells to a variety of stresses leads to the synthesis of a small group of proteins collectively known as the stress proteins which are usually subdivided into the heat-shock proteins (HSPs) and the glucose-regulated proteins (GRPs) [for recent reviews, see Pardue et al., 1989; Schlesinger et al., 1990; Hightower, 1991]. The HSP families are highly conserved and usually identified by their apparent molecular weights. Four classes of major HSPs, i.e., HSP32, 70/72, 90, and 110, are commonly detected in mammalian cells after heat-shock treatments [for reviews, see Lindquist and Craig, 1988; Schlesinger, 1990]. Besides heat-shock, the induction of HSPs can be elicited by a number of chemicals, such as heavy metal ions [Levinson et al., 1980; Caltabiano et al., 1986], sodium arsenite [Levinson et al., 1980], amino acid analogues [Kelley and Schlesinger, 1978; Hightower, 1980; Li and Laszlo, 1985], and puromycin [Hightower, 1980; Lee and Dewey, 1987], compounds that are cytotoxic. The GRPs are constitutively expressed and localized in the ER and the Golgi apparatus in higher eucaryotic cells. They are first identified as proteins whose synthesis is greatly enhanced in cells that are grown in medium depleted of glucose and two major classes have been reported: GRP78 and 94 [Shiu et al., 1977]. Synthesis of the GRPs is also enhanced when cells in culture respond to other physiological stresses such as inhibition of protein glycosylation [Pouyssegur et al., 1977] and treatment with calcium ionophores [Wu et al., 1981; Welch et al., 1983; Hightower, 1991]. In mammalian cells, induction of HSPs is always accompanied by other changes in cellular metabolism. Among others, reorganization of cytoskeleton [Thomas et al., 1982; Shyy et al., 1989; Welch and Suhan, 1985; 1986], suppression of general protein synthesis [Duncan and Hershey, 1984, 1989], and alteration in protein phosphorylation [Glover, 1982; Duncan and Hershey, 1984, 1989; Kennedy

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Address reprint requests to Dr. Yiu-Kay Lai, Institute of Life Science, National Tsing Hua University, Hsinchu, Taiwan 30043, Republic of China.

et al., 1984; Maher and Pasquale, 1989] are usually detected. Together with induction of HSPs, the above processes are collectively termed heat-shock or stress responses. Interestingly, these processes seem to be interconnected in living cells [see Hou et al., 1992].

Transcriptional activation of heat-shock genes involves the binding of a trans-acting heat-shock transcription factor [Parker and Topol, 1984; Wu et al., 1987; Wiederrecht et al., 1988] to the cis-acting conserved sequences known as the heat-shock element [Pelham, 1982; Amin et al., 1988; Xiao and Lis, 1988] and it is suggested that activation of the heat-shock transcription factor (HSF) in mammalian cells requires at least two steps: a conformational change which induces binding to the heat-shock element followed by phosphorylation that activates transcription [Larson et al., 1988; Hensold et al., 1990; Zimarino et al., 1990]. Transcription activation of GRP genes is less well understood; nevertheless, it has been shown that the transactivation of *grp*78 is mediated through a proximal region containing a CCAAT motif which interacts with CTF/NF-I [Wooden et al., 1991]. The regulation of stress gene expression has been elucidated to a certain extent and several lines of evidence indicate that the abnormal proteins in the cytoplasm may be the trigger for HSP induction [Lee and Hahn, 1988; Hightower, 1991] and that the presence of malfolded and underglycosylated proteins in the endoplasmic reticulum is the primary signal for GRP induction [Kozutsumi et al., 1988; Liu et al., 1992]. However, the exact molecular event(s) linking the physiological stresses and the activation of the transcription factor(s) is still unknown.

Protein phosphorylation plays important roles in signal transducing pathways and may play a central role in the induction of heat-shock proteins [Duncan and Hershey, 1987, 1989]. Changes in protein phosphorylation have been analyzed in heat-shock response. For instance, enhanced phosphorylation levels of HSPs [Schlesinger et al., 1982; Duncan and Hershey, 1987], HSF [Sorger and Pelham, 1988; Larson et al., 1988], and tyrosine phosphorylation of proteins with a wide range of molecular masses [Maher and Pasquale, 1989] have been detected. However, specific correlation between the alteration of phosphorylation of a particular phosphoprotein and the induction of stress proteins has not been demonstrated.

We have recently reported that both heat-shock and withangulatin A (a topoisomerase inhibitor) could augment the phosphorylation of a 65 kDa protein (pp65). Enhanced phosphorylation of pp65 appears to be involved in the upstream regulation of the stress response and may be related to stress protein induction [Lee et al., 1991]. The present studies demonstrate that enhanced phosphorylation of pp65 is a common event that occurs prior to stress protein induction elicited by a variety of chemicals. The interconnection of alteration of protein phosphorylation and various aspects of the stress response is further discussed.

MATERIALS AND METHODS

Materials

All cultureware was purchased from Corning (Corning, NY) and culture medium components were purchased from Gibco Laboratories (Grand Island, NY). [^{35}S]methionine (specific activity $>$ 800 Ci/mmole) was obtained from Amersham (Buckinghamshire, England) and [^{32}P]orthophosphate (specific activity 8,500–9,120 Ci/mmole) was from New England Nuclear (Boston, MA). Chemicals for electrophoresis were purchased from BioRad (Richmond, CA). Sodium chloride, cadmium chloride, zinc chloride, and cupric sulfate were purchased from Merck (Darmstadt, Germany). Sodium floride and L-azetidine-2-carboxylic-acid were obtained from Sigma (St. Louis, MO).

Cell Culture and Chemical Treatments

The 9L rat brain tumor cells, originated from a rat gliosarcoma, were a generous gift from Dr. M.L. Rosenblum, University of California at San Francisco [Weizsaecker et al., 1981]. Routine maintenance of the cells was performed as previously described [Lee et al., 1992; Hou et al., 1992]. Prior to each experiment, stock cells were plated in 25 cm^2 flasks or six-well plates at a density of 4–6 $\times$ 10^4 cells per cm^2. Exponentially growing cells at 80–90% confluency were used. To study the induction of stress proteins in cells subjected to chemical treatments, the culture medium was removed and cells were incubated at 37°C in fresh medium supplemented with various concentration of chemicals for different durations as indicated in Table I and figure legends.

Metabolic Labeling and Gel Electrophoresis

Synthesis of stress proteins in the chemical-treated cells was revealed by [^{35}S]methionine labeling. After chemical treatments, the cells were washed twice with methionine-free medium and labeled immediately with 20 μCi of [^{35}S]methionine in 1 ml of medium for 1 h. Alternatively, the cells were washed and allowed to recover in fresh culture media for different durations before labeling. After labeling, the cells were washed with ice-cold PBS and lysed with 300 μl of sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% sodium dodecylsulfate, 5% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) [Laemmli, 1970] or 200 μl of lysis buffer (9.5 M urea, 2% nonident P-40, 2% ampholytes, and 5% β-mercaptoethanol) [O'Farrell, 1975], depending on the electrophoresis system employed.

One-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [1970]. The samples for SDS-PAGE were heated in boiling water for 5 min and then microfuged (Eppendorf, full speed) for 3 min before loading. They were applied to 10% SDS-polyacrylamide gels on the basis of equal amounts of cell lysate. Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), and egg albumin (45 kDa) were used for molecular weight calibration. Two-dimensional PAGE was performed according to the method of O'Farrell [1975]. Equal amounts of cell lysate were loaded onto the pre-run isoelectrofocusing (IEF) gels and run for 16 h at 400 V and then 1 h at 800 V. Subsequently, the IEF gels were loaded onto 10% SDS-polyacrylamide slab gels with a 4.75% stacking gel for electrophoresis in the second dimension. After electrophoresis, the gels were stained, destained, and processed for autoradiography as described [Lee et al., 1991].

In Vivo [^{32}P]Phosphate Labeling and Alkaline Digestion of Phosphoproteins

Before treatment, cells were labeled with 1 mCi of [^{32}P]orthophosphate for 1 h in 1 ml labeling medium (phosphate-free DMEM containing 10% FBS). Various concentrations of drugs were then added to the labeling medium and the cells were further incubated for 2 h. After treatments, the cells were washed, lysed, and subjected to SDS-PAGE. For the visualization of ^{32}P-labeled proteins, the gels were fixed, dried, and directly processed for autoradiography. For the detection of alkali-resistant phosphoproteins, the gels were subjected to alkaline digestion as described by Cooper et al. [1983]. After electrophoresis, the gels were fixed in 5% acetic acid and 7% methanol for 1 h, rinsed in water for 15 min, and then immersed in a freshly made 1 M KOH at 55°C for 2 h with occasional gentle agitation. Subsequently, they were neutralized in fixing solution for 2 h with 3 changes of solution, dried, and processed for autoradiography.

Determination of Rate of Protein Synthesis and Cell Survival

For determinations of the rate of protein synthesis in the presence of chemicals, cells were washed and incubated for 2 h in 1 ml of labeling medium containing 20 μCi of [^{35}S]methionine and chemicals at various concentrations as indicated. After labeling, the cells were washed three times with ice-cold PBS and lysed with 300 μl of sample buffer. Incorporation of [^{35}S]methionine in acid-insoluble fractions was then determined by filter collection and scintillation counting as described [Lai et al., 1988]. The rate of protein synthesis in the treated cells was referred to as the fraction of [^{35}S]methionine incorporation relative to that of untreated controls. Cell survival was determined by colony formation technique. After treatment, the cells were trypsinized, serially diluted, and counted with a hemocytometer. The plating efficiency of 9L cells was determined by seeding them in duplicated dishes at an appropriate density of cells per 60 mm dish containing 4 ml of culture medium. The plated cells were then incubated at 37°C for 8 to 10 days. Subsequently, the samples were rinsed with phosphate-buffered saline (PBS), stained with 1.5% methylene blue in PBS, drained, and rinsed gently under running water. The colonies formed with more than 50 cells were scored. The plating efficiency of 9L cells was 60 to 90%. The surviving fraction of the treated cells was referred to as the fraction of plating efficiency relative to that of untreated controls.

RESULTS

Chemical Induction of Stress Proteins in 9L Cells

Chemically induced stress protein synthesis is known to be dose-dependent and the kinetics of

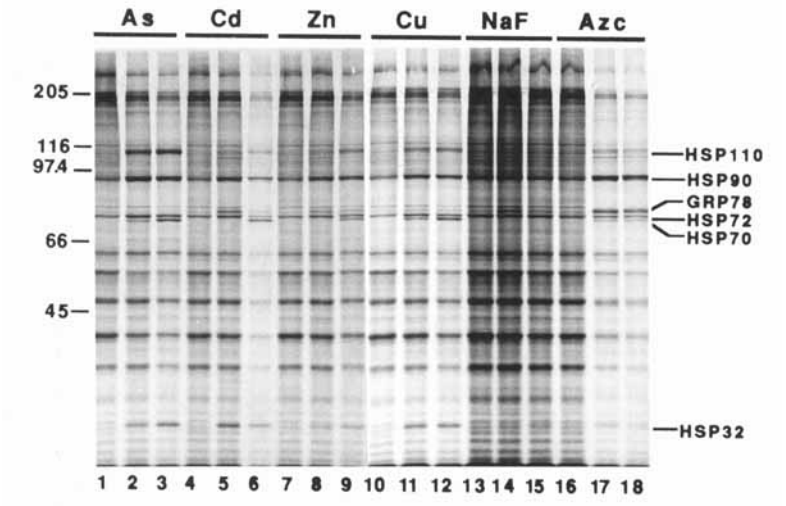

Fig. 1. Induced synthesis of stress proteins in 9L cells treated with $NaAsO_2$, $CdCl_2$, $ZnCl_2$, $CuSO_4$, NaF, and azetidine (Azc). Cells were treated with the chemicals at the concentrations described below at 37°C. The incubation time is 15 h for Azc and 2 h for the other chemicals. After treatment, the cells were allowed to recover under normal culture conditions from 0 to 8 h before being labeled with [^{35}S]methionine for 2 h. The cells were then lysed and the cell lysates were resolved by SDS-PAGE. After electrophoresis, the gels were processed for autoradiography and the autoradiograms are shown. The descriptions following each lane sequentially indicate chemicals used, treatment concentrations, treatment time, and recover durations in hours. **Lanes 1, 4, 7, 10, 13, 16:** Untreated controls for corresponding treatments. **Lane 2:** $NaAsO_2$, 200 μM, 2 h, R8. **Lane 3:** $NaAsO_2$, 300 μM, 2 h, R8. **Lane 5:** $CdCl_2$, 80 μM, 2 h, R4. **Lane 6:** $CdCl_2$, 100 μM, 2 h, R4. **Lane 8:** $ZnCl_2$, 225 μM, 2 h, R2. **Lane 9:** $ZnCl_2$, 250 μM, 2 h, R2. **Lane 11:** $CuSO_4$, 1.0 mM, 2 h, R2. **Lane 12:** $CuSO_4$, 1.2 mM, 2 h, R2. **Lane 14:** NaF, 5 mM, 2 h, R4. **Lane 15:** NaF, 10 mM, 2 h, R4. **Lane 17:** Azc, 5 mM, 15 h, R0. **Lane 18:** Azc, 10 mM, 15 h, R0. Molecular weight standards are shown at the left (in kDa) and stress proteins induced by the chemicals are marked at the right.

induction varies greatly among different chemicals. In the present studies, cells were incubated under different concentrations of various chemicals for different durations. Metabolic labeling by [^{35}S]methionine was done immediately after the chemical treatments or after the cells were allowed to recover under normal conditions for a period of time. Numerous cytotoxic chemicals were used in the early screening experiments and only the positive results are shown.

De novo synthesized proteins in chemical-treated 9L cells were shown in Figure 1. The data indicate that treatment of cells with arsenite, cadmium, zinc, copper, fluoride, and azetidine led to the synthesis of a wide range of stress proteins, comprising HSP32, 70, 72, 90, and 110 as well as GRP78 (Fig. 1). Except azetidine, the induction of stress proteins by different chemicals occurred after the cells were treated for 2 h and allowed to recover for 2 to 8 h after treatment. Further examination of the data revealed that the induction of stress proteins by heavy metal ions varied greatly for the species of stress protein induced and for the kinetics of induction, depending on chemical concentrations. For instance, optimal synthesis of GRP78 was detected 4 h after the cells were treated with 80 μM of $CdCl_2$ for 2 h. Interestingly, when the concentration of cadmium was increased to 100 μM, HSP70 became the major stress protein induced under exactly the same treatment protocol (Fig. 1, lanes 4–6). Similar results were obtained in cells treated with zinc. GRP78 and HSP70 were, respectively, induced by relative low and high concentration of the chemical (Fig. 1, lanes 7–9). Both arsenite and copper induced the synthesis of all of the HSPs but not the GRP78 (Fig. 1, lanes 1–3, 10–12); by contrast, sodium fluoride specifically enhanced the synthesis of GRP78 but none of the HSPs was induced (Fig. 1, lanes 13–15). On the other hand, treatment with azetidine for 2 h resulted in a slight induction of GRP78 (data not shown), and the enhanced synthesis of HSPs was only detected after 15 h of continuous incubation (Fig. 1, lanes 16–18). Treatment protocols and resulting stress protein inductions are summarized in Table I.

Effects of Chemicals on Protein Phosphorylation

Concomitant to the induction of stress proteins, the effects on protein phosphorylation in the chemical-treated cells were studied. Cells were treated with the above chemicals at the stated concentrations for 2 h and [^{32}P]orthophos-

TABLE I. Chemical Induction of Stress Proteins in 9L Cells*

Chemicals	Chemical concentrations	Treatment duration	Recovery duration	Stress proteins induced					
				HSP32	HSP70	HSP72	GRP78	HSP90	HSP110
$NaAsO_2$	200 μM	2 h	8 h	+	+	+	−	++	++
	300 μM	2 h	8 h	++	++	+	−	++	+++
$CdCl_2$	80 μM	2 h	4 h	++	−	+	++	+	+
	100 μM	2 h	4 h	++	+++	+	++	+	++
$ZnCl_2$	225 μM	2 h	2 h	+	−	+	+	+	+
	250 μM	2 h	2 h	++	++	+	+	+	+
$CuSO_4$	1.0 mM	2 h	2 h	++	+	+	−	+	+
	1.2 mM	2 h	2 h	++	++	+	−	+	+
NaF	5 mM	2 h	4 h	−	−	−	+	−	−
	10 mM	2 h	4 h	−	−	−	+	−	−
Azetidine	5 mM	2 h	−	−	−	−	+	−	−
	10 mM	2 h	−	−	−	−	+	−	−
	5 mM	15 h	−	−	+	+	+++	++	+
	10 mM	15 h	−	−	+	+	+++	++	+

*Gels as shown in Figure 1 were quantified by laser densitometry. The change in band intensity is matched against its respective control and scored: +, less than 2-fold increase; ++, 2- to 5-fold increase, +++, > 5-fold increase in protein induction.

phate was added to the medium 1 h prior to the chemical treatment. Cellular phosphoproteins were resolved by gel electrophoresis and visualized by autoradiography. It was found that only vimentin was specifically phosphorylated after the cells were treated with all of the above chemicals except azetidine and that other changes in the levels of phosphorylation were insignificant (Fig. 2A). After prolonged treatment with azetidine, incorporation of [^{32}P]orthophosphate into cellular phosphoproteins appeared to be greatly impaired. Thus direct correlation of stress protein induction and alteration of phosphoprotein patterns was not possible for this chemical.

The patterns of phosphoproteins were further investigated. Figure 2B shows the alkaline-resistant phosphoproteins in chemical-treated cells. The autoradiogram shows that the phosphorylation level of a phosphoprotein with a molecular weight of 65 kDa (pp65) was significantly enhanced in cells treated with all chemicals but azetidine. In addition, enhanced alkaline-resistant phosphorylation was detected on a phosphoprotein with a molecular weight of 28 kDa in zinc- and fluoride-treated cells (Fig. 2B). Alkaline-resistant phosphoproteins in the chemical-treated cells were also analyzed by two-dimensional gel electrophoresis. It was found that pp65 was resolved into 4 isoforms with isoelectric points ranging from 5.1 to 5.6. It was also found that the phosphorylation level of different isoforms was different (as indicated by four arrows in Fig. 3). Again, phosphorylation of pp65 was not detected in azetidine-treated cells (data not shown). The data clearly indicated that enhanced phosphorylation of pp65 is a common phenomenon in chemical-treated cells in which stress proteins were rapidly induced.

Effects of Chemicals on Cell Survival and Rate of Protein Synthesis

All chemicals that are known to be able to elicit stress protein synthesis are cytotoxic. The cell survival after treatment with the above chemicals at their effective dose for stress protein induction was determined by colony formation assay. The results clearly showed that the reductions of cell survival by the chemicals were dose-dependent and that chemicals at the concentrations used in the induction of stress proteins were cytotoxic. However, the survival curves obtained from different chemicals varied considerably in terms of rates of decline (Fig. 4). In concomitant experiments, the rates of protein synthesis by the chemical-treated cells were monitored by measuring [^{35}S]methionine incorporation. It was found that the rates of protein synthesis were suppressed in all of the treated cells, but again the trends were different among treatments (Fig. 4). Therefore, the chemicals at the concentrations for stress protein induction were both cytotoxic and able to suppress protein synthesis which are typical characteristics of the stress response.

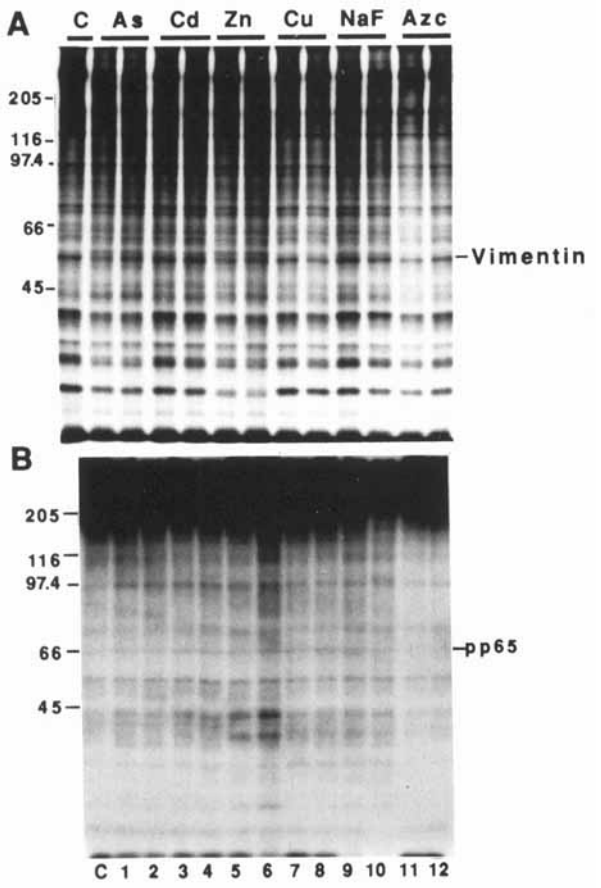

Fig. 2. Effects of $NaAsO_2$, $CdCl_2$, $ZnCl_2$, $CuSO_4$, NaF, and azetidine (Azc) on protein phosphorylation in 9L cells. Cells were pre-labeled with [^{32}P]orthophosphate (1 mCi/ml) for 1 h and further labeled in the presence of various chemicals for 2 h at the concentrations indicated. The cells were then lysed and the proteins were resolved by SDS-PAGE. For the visualization of total phosphoproteins, the gel was directly processed for autoradiography (**A**). Duplicate gel was heated at 55°C in the presence of 1 M KOH for 2 h, and then processed for autoradiography to detect the alkaline-resistant phosphoproteins (**B**). Exposure time was 12 h for A and 96 h for B. **Lane C:** control. **Lane 1:** $NaAsO_2$, 200 μM. **Lane 2:** $NaAsO_2$, 300 μM. **Lane 3:** $CdCl_2$, 80 μM. **Lane 4:** $CdCl_2$, 100 μM. **Lane 5:** $ZnCl_2$, 225 μM. **Lane 6:** $ZnCl_2$, 250 μM. **Lane 7:** $CuSO_4$, 1 mM. **Lane 8:** $CuSO_4$, 1.2 mM. **Lane 9:** NaF, 5 mM. **Lane 10:** NaF, 10 mM. **Lane 11:** Azc, 5 mM. **Lane 12:** Azc, 10 mM. Molecular weight standards are shown at the left (in kDa). Numbers at the right indicate the phosphoproteins generated by different chemicals (in kDa).

DISCUSSION

Chemical induction of stress proteins has been extensively studied in mammalian cells (see Table II). The present studies demonstrate that enhanced phosphorylation of pp65 is a common phenomenon prior to the synthesis of the stress proteins during the induction of the stress response by a variety of chemicals including arsenite, cadmium, zinc, copper, and fluoride. All of the above chemicals are cytotoxic and able to suppress protein synthesis at the concentrations that are able to elicit stress protein synthesis.

The kinds of stress protein induced by different chemicals vary considerably, primarily depending on the nature and concentration of a particular chemical, and also the duration of incubation (Tables I, II). In the early stage of the present experiments, a wide list of chemicals was screened for their ability to induce stress proteins in 9L cells. It was found that several well-known HSP inducers, including ethanol [Li, 1983] and puromycin [Lee and Dewey, 1987], fail to stimulate HSP synthesis in 9L cells. In addition, other cytotoxic drugs such as cycloheximide, vincristine, and VM-26 were also unable to induce stress protein synthesis (data not shown). We have also shown that the species of stress protein induced by the same chemical, as in the cases of cadmium and zinc, can be different when the cells are treated with different concentrations under identical treatment protocols. Taken together, it is apparent that the variations in the induction of stress protein(s) can be due to the differences in cell type, nature of the chemical, treatment protocol, and even concentrations of the same chemical. The process appears to be far more complicated than was previously realized.

The stress responses in mammalian cells usually comprise suppression of protein synthesis, alteration in protein turnover, and reorganization of the cytoskeleton. Nonetheless, all of these cellular activities are known to be related to protein phosphorylation [see Hou et al., 1992]. It has been shown that vimentin is a major target for phosphorylation under the chemical treatments and it is conceivable that the organization of intermediate filaments is altered in the treated cells. In fact, altered morphology was detected in the cells treated with the above chemicals (unpublished data). Recently, we have shown that phosphorylation of vimentin leads to the reorganization of the vimentin intermediate filaments, and the process may be related to the initiation of the stress response [Lee et al., 1992; Hou et al., 1992].

Since stress proteins can be elicited by a variety of chemicals and physiological stresses that are highly diversified, it has long been questioned whether there is a common factor (pathway) upon which the various inducers converged. It has been demonstrated that the

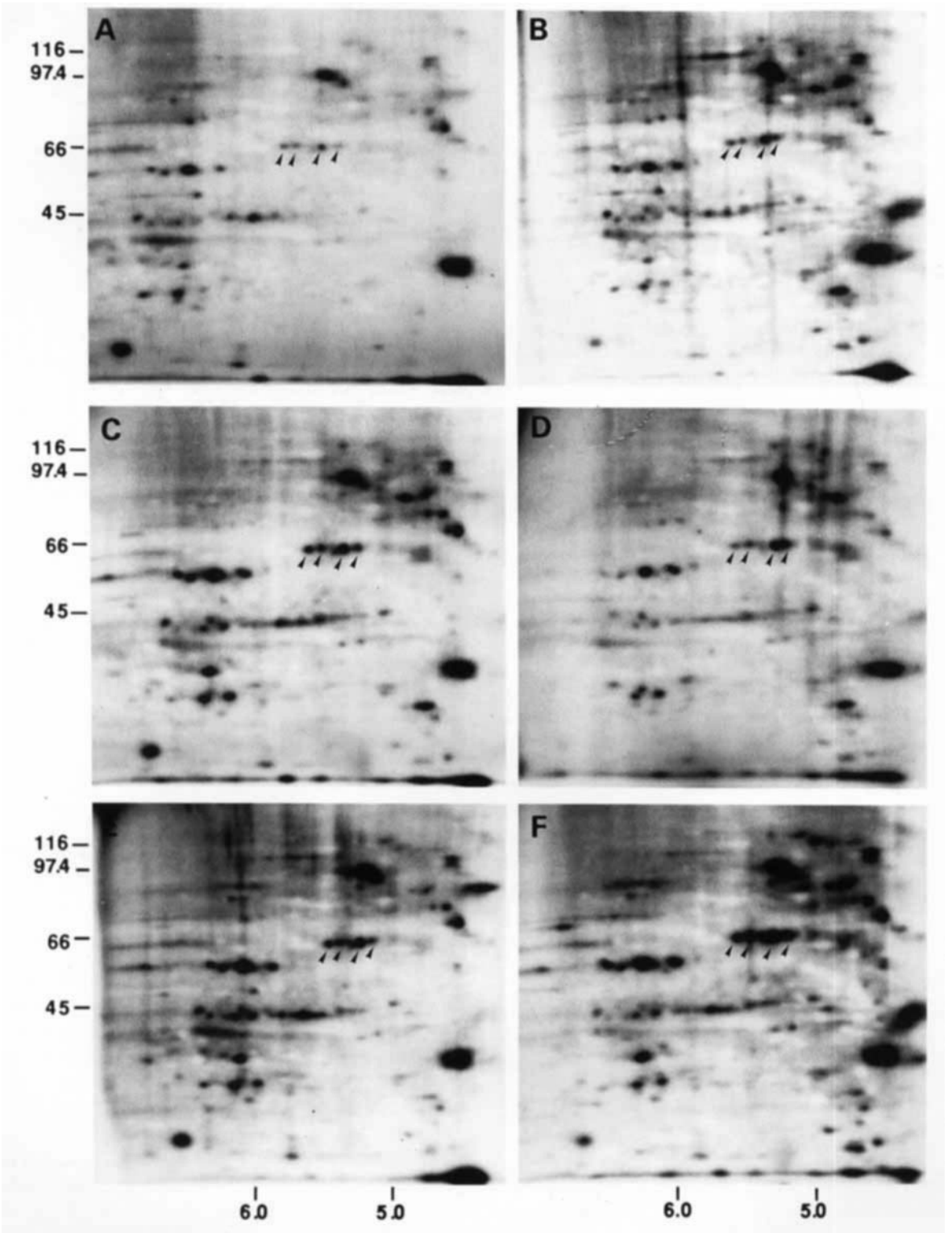

Fig 3 Two dimensional gel analyses of alkali resistant phosphoproteins of 9L cells treated with $NaAsO_2$ $CdCl_2$ $ZnCl_2$ $CuSO_4$ and NaF Cells were pre labeled with [^{32}P]orthophosphate and then treated with various chemicals as listed in Figure 2 The cell lysates were resolved by two dimensional electrophoresis and the gels were processed as described in Figure 2B Exposure time 7 days **A** Control **B** $NaAsO_2$ **C** $CdCl_2$ **D** $ZnCl_2$ **E** $CuSO_4$ **F** NaF Arrows mark the positions of the 65 kDa phosphoproteins

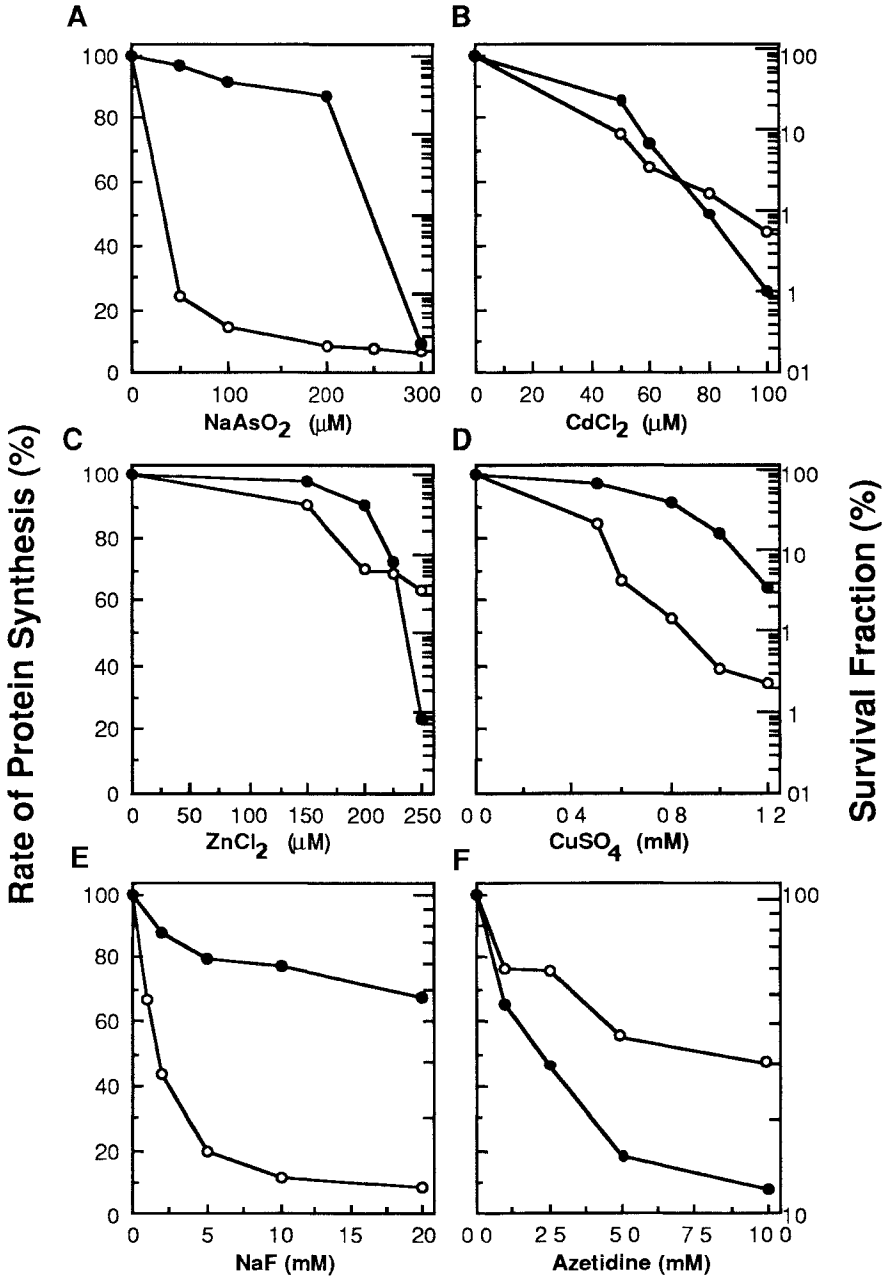

Fig. 4. Rate of protein synthesis and cell survival of 9L cells treated with $NaAsO_2$, $CdCl_2$, $ZnCl_2$, $CuSO_4$, NaF, and azetidine (Azc) Cells were incubated with various concentrations of chemicals at 37°C for different durations After chemical treatments, changes in cell survival (solid circle) and rate of protein synthesis (open circle) were monitored by colony formation technique and [^{35}S]methionine incorporation **A** $NaAsO_2$, 2 h **B** $CdCl_2$, 2 h **C** $ZnCl_2$, 2 h **D** $CuSO_4$, 2 h **E** NaF, 2 h **F** Azc, 15 h

activation of heat-shock genes is carried out by HSF and that phosphorylation of HSF is a prerequisite for transcription [Larson et al., 1988; Wiederrecht et al., 1988; Hensold et al., 1990; Zimarino et al., 1990]. It has recently been proposed that a sensor complex may be involved in the activation of HSF. According to this model, HSF and one or more of the constitutive stress proteins (HSP70 or 90) are major components of a sensor complex, which, literally, could sense and transduce a stress signal [Craig and Gross, 1991; Sorger, 1991]. Nevertheless, the molecular event(s) prior to the activation of HSF remains elusive. Judging from the diversity of the stress protein inducers and wide ranges of modes of stress response, it is not inconceivable that

TABLE II. Chemical Inducers of Stress Proteins in Cultured Mammalian Cells

Inducers[a]	Treatment protocols[b]	Cell origins[c]	Stress protein induced: HSP 32/34	HSP 70	HSP 72	HSP 87/90	HSP 110	GRP 78	GRP 94	References
$NaAsO_2$	25 μM, 1 h, 4h	CHO HA-1	−	+	−	+	−	−	−	Li [1983]
	75 μM, 8h	HeLa	−	+	−	+	−	−	−	Duncan and Hershey [1987]
	6–96 μM, 8h	Human A375	+	+	−	+	+	−	−	Caltabiano et al [1986]
	6–96 μM, 8 h	Mouse B16-F10	+	+	−	+	+	−	−	Caltabiano et al [1986]
Cadmium	100 μM, 2 h	Mouse 3T3	+	+	−	+	−	−	−	Hiwasa and Sakiyama [1986]
	10 μM, 4 h	HeLa	+	+	−	−	−	+	−	Watowich and Morimoto [1988]
Zinc	100 μM, 2 h	Mouse 3T3	+	+	−	+	+	−	−	Hiwasa and Sakiyama [1986]
	25 μM, 10 min, 3 h	HeLa	−	+	−	+	−	−	−	Duncan and Hershey [1987]
Copper	0 5 mM, 4 h	HFC	+	+	−	−	−	−	−	Levinson et al [1980]
BCNU	300 μM	Human HT-29	−	+	−	+	−	−	−	Schaefer et al [1988]
	200 μM	Human BE	−	+	−	+	−	−	−	Schaefer et al [1988]
Diamide	0 1–0 4 mM, 1 h, 4–8 h	CHO HA-1	−	+	−	+	+	−	−	Lee and Hahn [1988]
	500 μM, 30 min, 2 h	HeLa	−	+	−	+	+	−	−	Duncan and Hershey [1987]
Puromycin	20 μg/ml, 4 h	C H O	−	+	−	+	+	−	−	Lee and Dewey [1987]
	500 mM, 10 min, 3 h	HeLa	−	+	+	+	−	−	−	Duncan and Hershey [1987]
Ethanol	6%, 1 h, 1–7 5 h	CHO HA-1	−	+	−	+	−	−	−	Li [1983]
Azetidine	2 5 mM, 2–24 h,	CHO HA-1	−	+	−	+	−	−	−	Li and Laszlo [1985]
	10 mM, 6 h	HeLa	−	+	−	+	−	−	−	Duncan and Hershey [1987]
	5 mM, 8 h	HeLa	−	+	−	+	−	+	−	Watowich and Morimoto [1988]
	5 mM, 10 h, 1–7 h	REF-52	−	+	+	+	+	+	+	Welch and Suhan [1986]
Canavanine	0 6 mM, 3 h	Mouse 3T3	−	+	+	+	+	−	−	Hightower [1980]
	0 6 mM, 3 h	Human FS-4	−	+	+	+	+	−	−	Hightower [1980]
	0 6 mM, 2–24 h	CHO HA-1	−	+	−	+	+	−	−	Li and Laszlo [1985]
Nicotine	80–100 mM, 1 h	CHO HA-1	−	+	−	−	−	−	−	Hahn et al [1991]
Herbimycin A	0 1–0 25 μg/ml, 8 h	HeLa	−	+	−	+	−	−	−	Murakami et al [1991]
	1 0 μg/ml, 1–8 h	A431	−	+	−	+	−	−	−	Murakami et al [1991]
WA	15 μM, 1 h, 8 h	9L RBT	+	+	+	+	+	+	−	Lee et al [1991]
A23187	7 μM, 4 h	Rat-1 Cells	−	−	−	−	−	+	+	Welch et al [1983]
	7 μM, 16 h	Hamster K12	−	−	−	−	−	+	+	Lee [1987]
	7 μM, 12 h	HeLa	−	−	−	−	−	+	−	Watowich and Morimoto [1988]
2-DG	10 mM, 8–12 h	HeLa	−	−	−	−	−	+	−	Watowich and Morimoto [1988]
Tunicamycin	0 5 μg/ml, 12 h	HeLa	−	−	−	−	−	+	−	Watowich and Morimoto [1988]
	0 5 μg/ml, 8 h	A431	−	−	−	−	−	+	−	Murakami et al [1991]
Glucosamine	5 mM, 8 h	HeLa	−	−	−	−	−	+	−	Watowich and Morimoto [1988]
Okadaate	200 nM, 7 h	9L RBT	−	−	−	−	−	+	−	Hou et al [1992]

[a]BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea, WA, withangulatin A, 2-DG, 2-deoxyglucose
[b]Parameters sequentially indicate chemical concentration, treatment duration, and recovery duration
[c]CHO, chinese hamster ovary cells, REF, rat embryo fibroblasts, HFC, human foreskin cells, RBT, rat brain tumor cells

more than one type of HSF or "sensor complex" exists in mammalian cells and that pp65 may be a component of the sensor complex.

The present study shows that phosphorylation of pp65 is associated with stress protein induction in 9L cells but the mechanism for the enhanced phosphorylation is unknown. It was found that the increase in the pp65 phosphorylation level is not due to the increased amount of the protein per se, since the phosphorylation event is not affected by the presence of actinomycin D or cycloheximide in the stressed cells (unpublished data). When the protein level remains constant, increased phosphorylation of specific proteins can be achieved through the activation of protein kinases or the inhibition of protein phosphatases. Alternatively, the changes can occur in the protein molecule itself, i.e., an induced conformational change that increases/decreases its accessibility as a kinase/phosphatase substrate. One of the stress protein inducers used in the present experiments, fluoride, is a well-known general protein phosphatase inhibitor. The data suggest that protein phosphatase(s) is involved in the regulation of phosphorylation of pp65. This hypothesis is supported by the observation that okadaic acid, another protein phosphatase inhibitor, can also enhance the phosphorylation level of pp65 (not shown). Further dissection of the phosphorylation site(s) of pp65 and the kinases/phosphatase involved in the system is therefore warranted.

The identity of pp65 remains to be elucidated. Unexpectedly, and not elaborated by the investigators, the phosphorylation levels of pp65 isoforms were also augmented in cells treated with epidermal growth factor [Guy et al., 1991] as well as PDGF and bombesin [Kohno et al., 1990]. Since all these compounds are known to transactivate a number of genes, it is not unlikely that pp65 is also involved in some specific gene activation pathways. In our system, quantitative correlation between the phosphorylation level of pp65 and stress protein induction is not evident, despite the fact that enhanced phosphorylation of pp65 may be a common event associated with the induction of stress proteins. Therefore, the phosphorylation process may be a sufficient but not a necessary factor for the transactivation of stress genes. The elucidation of the nature and function of this protein may be important for the understanding of the signal transduction pathway(s) of the stress response.

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