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Transient Increase in Vimentin Phosphorylation and Vimentin-HSC70 Association in 9L Rat Brain Tumor Cells Experiencing Heat-Shock

Ting-Jen Cheng and Yiu-Kay Lai

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

Abstract Characteristic changes in vimentin were studied in 9L rat brain tumor cells treated at 45°C. During heat-shock treatment, vimentin molecules were rapidly phosphorylated and reorganized from a filamentous form into a perinuclear higher-order structure that was less extractable by nonionic detergent. These effects were found to be highly transient, peaked at 30 min after the onset of heat-shock treatment, and subsided thereafter. Simultaneously, the solubility of the constitutively expressed heat-shock protein70 (HSC70) was also temporarily decreased and the kinetics was identical to that of vimentin. The results indicated that HSC70 and vimentin were co-insolubilized during the heat-shock treatment. We propose that the reorganization of the intermediate filaments resulted from enhanced phosphorylation of vimentin leads to the concurrent association of HSC70 to the intermediate filaments. This process may play an essential role in regulating heat-shock genes.

Key words: heat-shock genes, HSC70, vimentin, protein phosphorylation, intermediate filaments

The heat-shock response in mammalian cells can be characterized by the reorganization of the cytoskeleton, alteration of protein phosphorylation, suppression of general protein synthesis, and induction of heat shock proteins (HSPs) synthesis [for recent review, see Welch, 1992]. Transactivation of heat-shock genes in mammalian cells requires the binding of a trans-acting heat-shock transcription factor (HSF) to the cis-acting heat-shock element (HSE) located in the promoters of heat-induced genes and the subsequent phosphorylation of HSF [for reviews, see Morimoto et al., 1990; Sorger, 1991]. Alteration of protein phosphorylation plays important roles in signal transducing pathways and may play a central role in the induction of heat-shock response [Duncan and Hershey, 1984, 1989; Hou et al., 1993]. In heat-shocked cells, enhanced phosphorylation levels of HSPs, HSF [Larson et al., 1988; Sorger and Pelham, 1988], and tyrosine phosphorylation of proteins with a wide range of molecular masses [Maher and Pasquale, 1989] have been reported. Most recently, enhanced phosphorylation of a 65 kDa protein is commonly observed in cells subjected to heat-shock and a variety of chemical stress [Lee et al., 1991; Lai et al., in press]. Although heat-shock may have direct effect on certain protein kinase(s) [Legagneux et al., 1990; Dubois and Bensaude, 1993], the exact molecular event underlining changes in protein phosphorylation and initiating heat-shock response is not fully understood.

Cytoskeleton, an extensive and intricate protein scaffold in the cytoplasm, consists of three major filamentous systems, microtubules, intermediate filaments, and microfilaments [Bershadsky and Vasiliev, 1988]. It appears to function in cell shape, cell motility, transportation of intracellular macromolecules, translation of mRNA into protein, and regulation of gene expression [Traub, 1985; Bershadsky and Vasiliev, 1988]. Among the major cytoskeletal components, intermediate filaments (IFs) appear to be the most stable fibrils of mammalian cells and the most widely distributed class of IFs in cultured cells are composed of a single subunit protein called vimentin. Vimentin IFs form a physical continuum from the nuclear envelope to the plasma membrane [Georgatos and Blobel, 1987], thus forming an elaborate system of nucleolemmal/plasmalemmal interactions [Geiger, 1987; Skalli and Goldman, 1991; Georgatos, 1993]. An inter-

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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.

esting feature of vimentin is the presence of its phosphorylated variant [Cabral and Gottesman, 1979; Nelson and Traub, 1982; Lee et al., 1993], and the site-specific phosphorylation of this molecule in the 9-kDa N-terminal region is related to its structural reorganization [see Lee et al., 1992]. It has also been reported that heat-shock can elicit the disruption of vimentin IFs [Biessmann et al., 1982; Thomas et al., 1982; Welch and Suhan, 1985]; however, the mechanism underlying this alteration remains to be elucidated.

In the present study, we have characterized the effects of heat-shock on vimentin, the only IF protein in 9L cells [Lai et al., 1993a]. Our results suggest that the modification of vimentin by heat-shock may play a role in the activation of HSF, thus the induction of the HSPs.

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). Other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Cell Culture, [^{32}P]Orthophosphate Labeling, and Heat Treatments

The 9L rat brain tumor (RBT) 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 cells was performed as previously described [Lee et al., 1992; Hou et al., 1993]. Prior to each experiment, stock cells were plated in 25-cm^2 flasks or six-well plates at a density of 4–6 × 10^4 cells per cm^2. Exponentially growing cells at 80–90% confluency were used. In the heating experiments, the flasks or plates were sealed with Parafilm and submerged in a waterbath preset at 45 ± 0.1°C. The designated temperature of the medium in the heating protocol was reached within 3 min and the time required for equilibrium was included in the treatment duration. For the studies of protein phosphorylation in heat-shocked cells, cells were prelabeled with 1 mCi of [^{32}P]orthophosphate for 1 h in 1 ml of phosphate-free medium and then heat-shocked in the presence of the isotope for various durations as indicated.

Gel Electrophoresis, Autoradiography, and Immunoblotting

SDS–PAGE was performed according to the method of Laemmli [1970]. Sample preparation and other related experimental procedures were carried out as previously described [Lee et al., 1991, 1992]. 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 10 min, and then immersed in a freshly made 1 M KOH at 55°C for 2 h. Subsequently, they were neutralized in fixing solution for 2 h with 3 changes of solution, dried, and processed for autoradiography. Bands of interest on the X-ray films (Fuji) were quantitated by densitometric scanning in 2-D mode (Molecular Dynamics). For immunoblotting analysis, cellular proteins resolved in SDS–PAGE gels were electrotransferred onto a nitrocellulose membrane by a semi-dry method and then processed into a color blot by using mAb to vimentin (Amersham, UK) as a probe [Lee et al., 1992].

Protein Extraction and Fractionation

Cells were prelabeled with 20 μCi of [^{35}S]methionine for 6 h in 1 ml of methionine-free culture medium and then treated with heat as described above. After treatments, the cells in 35-mm wells were washed with PBS and incubated with 0.2 ml of extraction buffer (1% Triton X-100 and 0.15 M NaCl in 50 mM Tris–HCl, pH 8.0) on ice for 30 min. Soluble proteins extracted under this condition were collected into microfuge tubes and the samples were centrifuged (Eppendorf, full speed) at 4°C for 10 min. Aliquots of the supernatants, referred to as the soluble fraction of cellular proteins, were added to equal amounts of double-strength sample buffer before electrophoresis. On the other hand, cellular proteins remained on the surface of the culture dishes were rinsed three times with ice-cold PBS and solubilized in 0.2 ml of sample buffer before electrophoresis. The samples thus collected were referred to as the insoluble fraction of the cellular proteins and

presumed to be the nuclear-cytoskeletal fraction as previously reported [Collier and Schlesinger, 1986; Lee et al., 1992, 1993]. Equal volumes of protein samples were then resolved by SDS–PAGE and the protein bands of interest were quantitated by densitometry as described above.

Indirect Immunofluorescence and Microscopy

For indirect immunofluorescence studies, cells were grown on chamber slides (Nunc) and heat-treated as described. After treatments, the slides were briefly washed with PBS and the cells were fixed and permeabilized for 10 min in −20°C methanol. After being rinsed in PBS, the fixed cells were incubated with monoclonal antibody against vimentin (Amersham, diluted 1:20 in PBS containing 3% BSA) at room temperature for 1 h. The cells were then washed in PBS and incubated with fluorescein-conjugated goat anti-mouse IgG (1:20 dilution) for 1 h. After another rinsing with PBS, the cells were mounted in glycerol and examined on a Nikon photomicroscope equipped with epifluorescence optics (Nikon Optiphot, Tokyo, Japan). Micrographs or the fluorescent images were then recorded.

RESULTS

Alterations of Protein Phosphorylation in 9L RBT Cells During Heat-Shock Treatment

Protein phosphorylation was analyzed after the cells were prelabeled with [^{32}P]orthophosphate for 1 h and then heated at 45°C for various durations in the presence of the isotope. Figure 1A shows that the levels of protein phosphorylation were generally enhanced as the labeling time increased. However, the phosphorylation levels of two proteins with molecular weights of 36 and 57 kDa were distinctly enhanced as compared to the unheated controls, as well as other phosphoproteins in general. Immunoblotting experiments revealed that the 57-kDa phosphoprotein is vimentin (Fig. 1B), the only known IF component in 9L RBT cells. During heat-shock at 45°C, the phosphorylation level of vimentin increased gradually in the first 20 min, then raised drastically for 10 min and rapidly subsided afterward (Fig. 1C). Similar but much delayed responses were observed in cells heat-shocked at lower temperature. The phosphorylation level of vimentin reached its optimum after 60 min of heating at 43°C (not shown), in contrast to peaking at 30 min at 45°C. Alteration of protein phosphorylation was also analyzed on alkaline-treated gels (Fig. 2). It was found that the phosphorylation levels of two proteins with molecular weights of 36 and 65 kDa were distinctly enhanced continuously during heat-shock, as compared to the other alkali-stable phosphoproteins in general. Quantitative analysis indicated that there was a 10-fold increase in phosphorylation level of the 65 kDa phosphoprotein (Fig. 2B). Interestingly, no significant dephosphorylation was observed.

Partition of Vimentin and HSC70 in the Detergent-Insoluble Fraction in Heat-Shocked 9L RBT Cells

Cells were metabolically labeled with [^{35}S]methionine for 6 h and changes of detergent-extractability of intracellular proteins during heat-shock were analyzed by extracting the cells with buffer containing 1% Triton X-100 and 0.15 M NaCl. At 45°C, large portion of several soluble proteins with molecular weights of 45, 57, 72, 90, and 110 (designated as p45, p57, p72, p90, and p110, respectively) were found to be partitioned into the detergent-nonextractable pool as the treatment time increased (Fig. 3A). For instance, p45, which was completely extractable in the control cells, redistributed into the insoluble fraction during the heat treatment. In addition, p57 and p72, identified as vimentin and HSC70, respectively, by immunoblot analysis, exhibited the similar kinetics of partition between the extractable and nonextractable fractions. In unheated cells, vimentin was mostly distributed in nonextractable, whereas most HSC70 was readily extractable. Upon heat treatment, the amount of vimentin and HSC70 in the nonextractable fractions increased, peaked at 30 min and then declined (Fig. 3B). Two other proteins, p90 and p110, presumed to be HSP90 and HSP110, simply repartitioned into the insoluble fraction to a higher degree during the heat-shock treatment in a time-dependent manner.

Effects of Heat-Shock on Cell Morphology and Reorganization of Vimentin Intermediate Filaments

To determine whether the biochemical changes of vimentin was accompanied by its reorganization, cell morphology as well as cytoskeletal organization were examined. Morphologically, the 9L cells rounded up slightly when treated at 45°C (Fig. 4A–C). Among the three major cytoskeletal systems, vimentin IFs exhib-

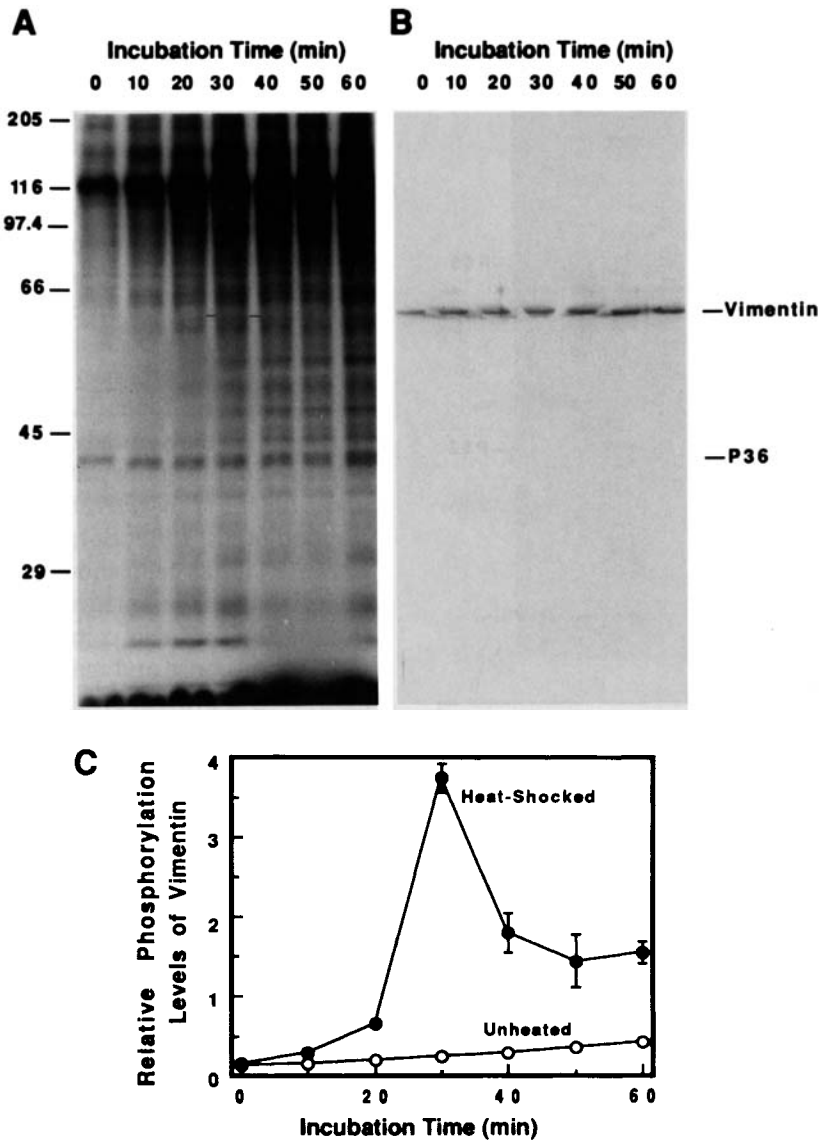

Fig. 1. Time-dependent phosphorylation of intracellular proteins during heat-shock in 9L RBT cells. Cells were prelabeled with [^{32}P]orthophosphate (1 mCi/ml) for 1 h and then heated at 45°C for various durations as indicated. The cells were then lysed with SDS sample buffer and the proteins were resolved by 10% SDS–PAGE. After electrophoresis, the gels were processed for autoradiography (**A**). Duplicate gels were electrotransferred onto a nitrocellulose membrane by a semi-dry method and then processed into a color blot byusing mAb to vimentin as a probe (**B**). Subsequently the relative phosphorylation levels of vimentin were determined by densitometry (**C**). Background levels of optical density were subtracted before calculations. The relative phosphorylation levels of vimentin were presented as peak volumes in the heat-shocked and unheated controls. The data are means ±SD from three independent experiments. Molecular weight standards are shown at the left (in kDa) and vimentin is marked at the right.

ited the most dramatic changes. In unheated cells, vimentin IF formed an elaborate lattice work in the cytoplasm (Fig. 4D). It was found to be redistributed into a cagelike form around the nucleus after 30 min at 45°C (Fig. 4E). Subsequently, the collapsed IFs were reorganized into filamentous form and exhibited a fine meshwork closely resembled the normal appearance (Fig. 4F). It is noteworthy that the kinetics of vimentin organization was strictly coincided to that of vimentin phosphorylation mentioned in the previous section.

DISCUSSION

We have demonstrated that the phosphorylation level and the *insolubility of vimentin are*

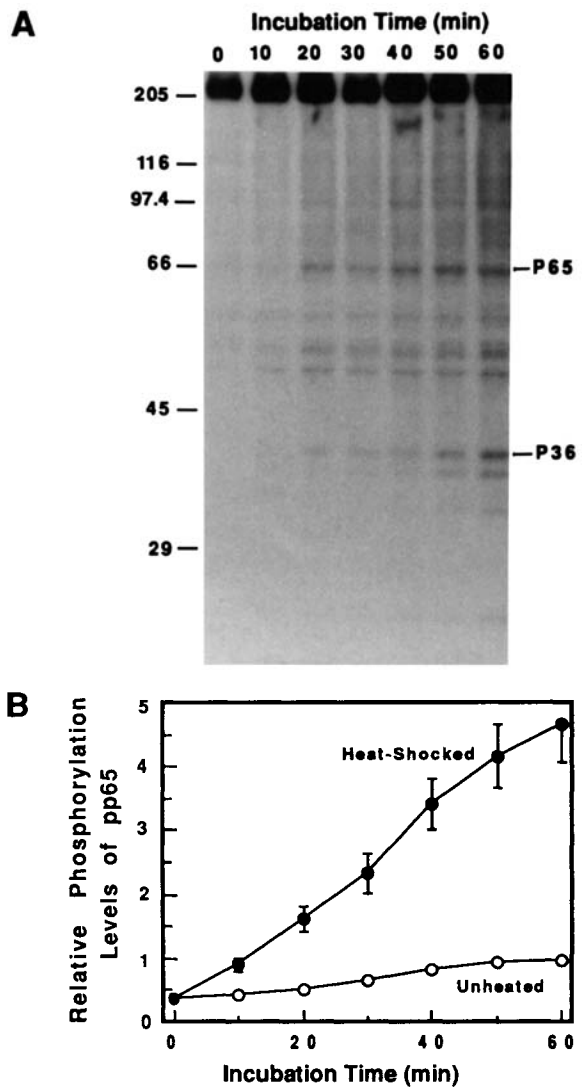

Fig. 2. Time-dependent phosphorylation of alkali-resistant phosphoproteins during heat-shock in 9L RBT cells. Cells were prelabeled with [^{32}P]orthophosphate (1 mCi/ml) for 1 h and then heated at 45°C for various durations as indicated. The cells were then lysed with SDS sample buffer and the proteins were resolved by 10% SDS–PAGE. After electrophoresis, the gels were heated at 55°C in the presence of 1 M KOH for 2 h, and then processed for autoradiography **(A)**, and the relative phosphorylation levels of pp65 were determined by densitometry **(B)**. Background levels of optical density were subtracted before calculations. The relative phosphorylation levels of pp65 were presented as peak volumes in the heat-shocked and unheated controls. The data are means ±SD from three independent experiments. Molecular weight standards are shown at the left (in kDa), and pp65 is marked at the right.

transiently elevated in 9L RBT cells during heat-shock, and that the above biochemical changes were coincided with transient reorganization of vimentin intermediate filaments and redistribution of HSC70 (also referred to as HSP72 in rodent cells) into the detergent-insoluble fraction. It has been known that many cellular activities are related to protein phosphorylation/dephosphorylation. Alteration of protein phosphorylation plays important roles in signal transducing pathways and a central role in exhibiting the heat-shock response [Hou et al., 1993]. For instance, enhanced phosphorylation of ribosomal protein L14 and eIF-2α as well as extensive dephosphorylation of ribosomal protein S6 and eIF-4E have been suggested to be responsible for the suppression of general protein synthesis and preferential synthesis of HSPs [Glover, 1982; Kennedy et al., 1984]. On the other hand, phosphorylation of HSF is a prerequisite for the transactivation of heat-shock genes [Larson et al., 1988; Sorger and Pelham, 1988]. Recently, it has been shown that enhanced phosphorylation of vimentin and resulted reorganization of IFs are also common features of drug-induced response and may be responsible for the alteration of protein synthesis as well as changes in cell morphology [Lee et al., 1991, 1992, 1993; Hou et al., 1993]. Furthermore, the enhanced phosphorylation of a 65 kDa protein during heat-shock further substantiate our previous finding that this protein may be involved in the initiation of heat-shock response since its enhanced phosphorylation was commonly observed in the heat-shock responses ellicited by a variety of chemical treatments [Lee et al., 1991; Lai et al., in press]. However, the identity and mode of action of this protein remains to be elucidated.

Enhanced phosphorylation of vimentin has been observed in cells treated with cAMP increasing agents [Gard and Lazarides, 1982; Lamb et al., 1989], phorbol ester PMA [Huang et al., 1988], chemotactic factors [Huang et al., 1984; Wyatt et al., 1991], and hormones [Spruill et al., 1983; Coca-Prados, 1985], as well as during mitosis [Celis et al., 1983; Franke et al., 1984; Norbury and Nurse, 1989; Chou et al., 1989, 1990]. These events have been suggested to be mediated by a number of protein kinases including PKA, PKC, PKG, and cdc2 kinase [see Lee et al., 1992]. On the other hand, phosphatases are also involved in regulating the phosphorylation level of vimentin IFs. Inhibition of type 1 serine/threonine phosphatase by the treatment of okadaic acid [Yatsunami et al., 1991; Lee et al., 1992], dinophysistoxin 1, and calyculin A [Eriksson et al., 1992] has been shown to cause increased phosphorylation of vimentin and

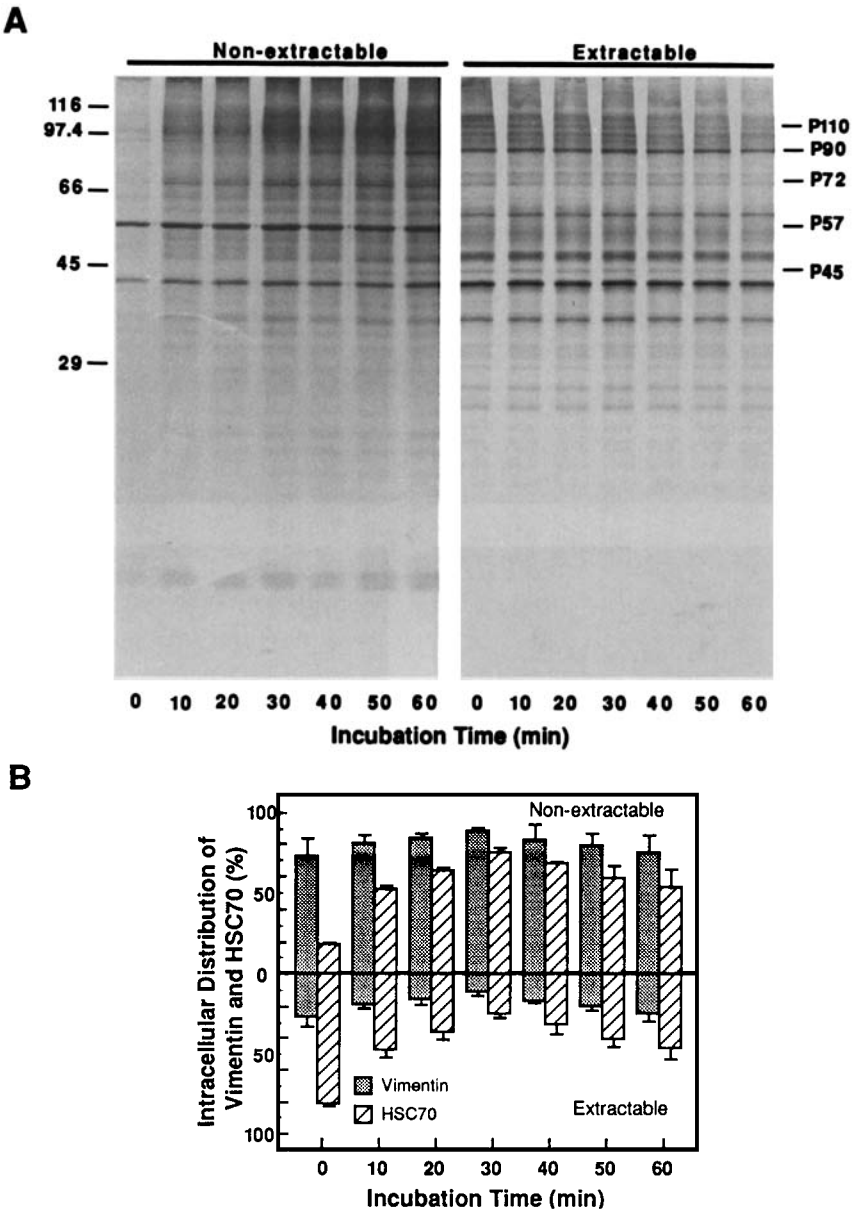

Fig. 3. Changes in detergent-extractability of vimentin and HSC70 in cells experiencing heat-shock. Cells were prelabeled with [^{35}S]methionine (20 μCi/ml) for 6 h before they were heated at 45°C for various durations as indicated. After treatment, the cells were lysed with extraction buffer containing 50 mM Tris-HCl, 1% Triton X-100 and 0.15 M NaCl for 30 min and the nonextractable fractions were collected. The samples were resolved by SDS–PAGE (**A**). Molecular weightstandards are shown at the left (in kDa). Notions at the right indicate proteins with stated molecular weights where p57 and p72 were respectively identified as vimentin and HSC70. The relative amounts of vimentin and HSC70 were determined by densitometry and presented as peak volumes (**B**). Background levels of optical density were subtracted before calculations. The data are means ± SD from three independent experiments.

rearrangement of IFs. A Ca^{2+}-stimulated phosphatase has also been suggested to be involved in the regulation of vimentin IFs phosphorylation [Evans, 1989]. As a result, the turnover of vimentin phosphate observed in intact cells was a reflection of both the changes of kinase and phosphatase activities. Taken together, the transient increase in vimentin phosphorylation in cells experiencing heat-shock may be due to an activation of kinases or inactivation of phosphatases. Alternatively, heat-shock may cause some conformational changes in vimentin and render this protein more susceptible to kinases or resistant to phosphatases.

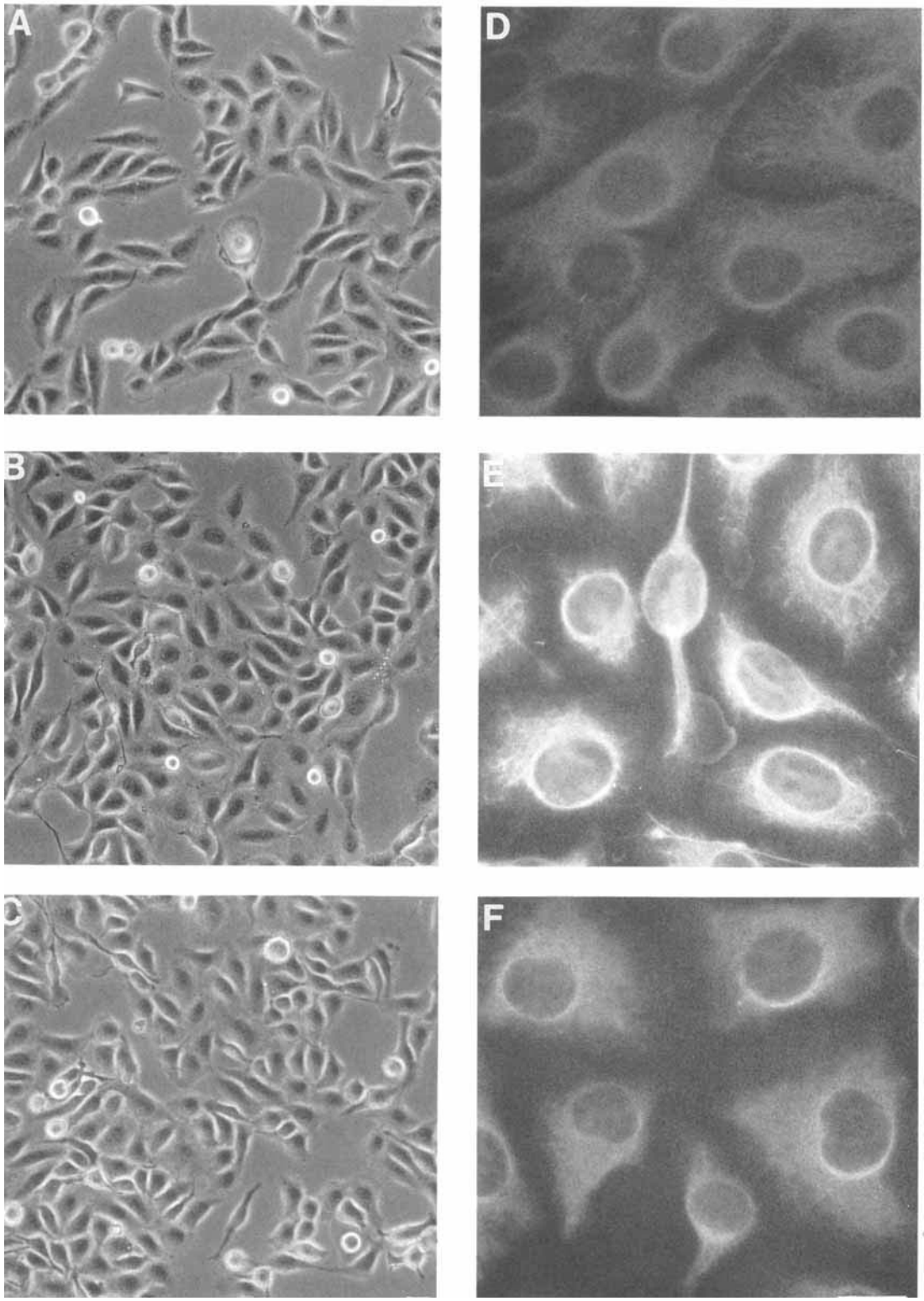

Fig. 4. Effect of heat-shock on morphology (**A–C**) and organization of vimentin IFs (**D–F**) in control and heat-shocked 9L cells. Cells were heat-shocked at 45°C. Organization of vimentin IFs was analysed by indirect immunofluorescence using mouse monoclonal antibodies against vimentin. **A–C:** Phase-contrast microscopies. **D–F:** Immunofluorsent microscopies. A,D: Untreated controls. B,E: cells heated at 45°C for 30 min. C,F: cells heated at 45°C for 60 min. Bar represents 10 μm.

It has been shown that several intracellular proteins, such as chromatin proteins, ribosomal proteins, and HSPs, become more insoluble in heat-shocked cells [Subjeck et al., 1983; Littlewood et al., 1987; Napolitano et al., 1987]. We have shown that larger portions of vimentin and HSC70 were transiently redistributed into the detergent-insoluble fraction, and that the kinetics of solubility changes of vimentin and HSC70 are well correlated. HSC70, the constitutively expressed member of the HSP70 family, has been shown to be multi-functional. It is an ATPase [Chappell et al., 1986], a clathrin uncoating protein [Rothman and Schmid, 1986], a protein unfoldase for denatured protein, and a chaperone for protein folding and translocation [Ellis and van der Vies, 1991]. It is now obvious that most cellular activities of HSC70 are mediated through ATP hydrolysis [Pelham, 1986; Palleros et al., 1991; Beckmann et al., 1992; Sadis and Hightower, 1992]. In addition, HSC70 has been shown to be associated with a number of cellular components, including plasma membrane proteins, microtubules, intermediate filaments, and microfilaments, nucleoli, nuclear matrix, as well as nuclear heterogeneous ribonucleoprotein particles [for review, see Burdon, 1986]. More recently, it has been suggested that HSC70 may complex with HSF and act as a heat sensor involved in the regulation of the heat-shock genes [Craig and Cross, 1991; Abravaya et al., 1992]. In the unheated cells, HSF is in an inactivated state by forming a complex with HSC70. Upon heat-shock, the emergence of denatured, unfolded and malfolded proteins creates a large pool of new binding substrates for HSC70 [Goff and Goldberg, 1985; Ananthan et al., 1986; Parsell and Sauer, 1989], and stimulate ATP hydrolysis [Sadis and Hightower, 1992]. Subsequently, HSC70 dissocciates from the HSC70–HSF complex and thus activates the HSF [Baler et al., 1992; Morimoto et al., 1992]. It appears that HSC70 is involved in a negative regulation of heat shock gene expression via an autoregulatory loop and its target is HSF [Morimoto, 1993].

Taken together, we suggest that the physiological role of vimentin in the initiation of heat-shock response is as follows. Heat-shock induces a conformational change in vimentin, exposes more hydrophobic residues and renders this protein more susceptible for the appropriate protein kinase(s) thus the increase in the phosphorylation level. Because of the exposure of hydrophobic regions, the denatured vimentin will compete with HSF for HSC70 binding. Simultaneously, phosphorylation of vimentin causes the collapse of this elaborate network and further aggravates the emergence of denatured proteins. All of the above events will lead to the dissociation of the HSC70–HSF complex. The released HSF oligomerizes, binds DNA, and acquires transcriptional activity which leads to the synthesis of HSPs. Since HSPs are involved in the refolding of denatured proteins and the restoration of cellular activities during recovery, the de novo synthesized HSPs will participate in the conversion of oligomerized HSF back to its monomeric state which then reassociates with HSC70 to form the HSC70–HSF complex as in un-heated cells. In summary, our data indicate that reorganization of IF resulted from enhanced phosphorylation of vimentin may play an essential role in regulating heat-shock genes.

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