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Reversible Hyperphosphorylation and Reorganization of Vimentin Intermediate Filaments by Okadaic Acid in 9L Rat Brain Tumor Cells

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Abstract Okadaic acid (OA), a protein phosphatase inhibitor, was found to induce hyperphosphorylation and reorganization of vimentin intermediate filaments in 9L rat brain tumor cells. The process was dose dependent. Vimentin phosphorylation was initially enhanced by 400 nM OA in 30 min and reached maximal level (about 26-fold) when cells were treated with 400 nM OA for 90 min. Upon removal of OA, dephosphorylation of the hyperphosphorylated vimentin was observed and the levels of phosphorylation returned to that of the controls after the cells recovered under normal growing conditions for 11 h. The phosphorylation and dephosphorylation of vimentin induced by OA concomitantly resulted in reversible reorganization of vimentin filaments and alteration of cell morphology. Cells rounded up as they were entering mitosis in the presence of OA and returned to normal appearance after 11 h of recovery. Immuno-staining with anti-vimentin antibody revealed that vimentin filaments were disassembled and clustered around the nucleus when the cells were treated with OA but subsequently returned to the filamentous states when OA was removed. Two-dimensional electrophoresis analysis further revealed that hyperphosphorylation of vimentin generated at least seven isoforms having different isoelectric points. Furthermore, the enhanced vimentin phosphorylation was accompanied by changes in the detergent-solubility of the protein. In untreated cells, the detergent-soluble and -insoluble vimentins were of equal amounts but the solubility could be increased when vimentins were hyperphosphorylated in the presence of OA. Taken together, the results indicated that OA could be involved in reversible hyperphosphorylation and reorganization of vimentin intermediate filaments, which may play an important role in the structure-function regulation of cytoskeleton in the cell.

Key words: okadaic acid, isoforms of vimentin, hyperphosphorylation, dephosphorylation, intermediate filaments, brain tumor cells

The cytoskeleton is an extensive and intricate scaffold composed of several filamentous proteins and its organization is vital for cellular activities [Bershadsky and Vasiliev, 1988]. Among the major cytoskeletal components, intermediate filaments (IFs) appear to be the most stable fibrils of mammalian cells and there are at least five distinct classes of subunit proteins making up the various IFs in different cell types [for reviews, see Steinert and Roop, 1988; Bloemendal and Pieper, 1989]. The most widely distributed class of IFs is composed of a single subunit protein called vimentin in cells of mesenchymal origin and most cells in culture. Vimentin-containing IFs form a network which makes contact with the nuclear envelope and the plasma membrane [Georgatos and Blobel, 1987], thus forming an elaborate system of nucleolemmal/plasmalemmal interactions [Geiger, 1987; Skalli and Goldman, 1991]. An interesting feature of vimentin is the presence of a small proportion as a phosphorylated variant [Cabral and Gottesman, 1979; Nelson and Traub, 1983]. Two-dimensional gel analysis of proteins from ^{32}P-labeled cells has revealed that phosphorylated vimentin is among the most prominent phosphoproteins in the cytoplasm [O'Connor et al., 1981; Spruill et al., 1983; Tsuda et al., 1988]. The phosphorylation of vimentin has been demonstrated to be site specific and restricted to the

Abbreviations used: IF, intermediate filament; OA, okadaic acid; PKA, cAMP-dependent protein kinase; PKC, diacylglycerol regulated protein kinase (protein kinase C); PKG, cGMP-dependent protein kinase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A.

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9 kDa N-terminal domain [Inagaki et al., 1987; Geisler et al., 1989; Ando et al., 1989] and the process appears to be involved in the reorganization of the vimentin filaments [Inagaki et al., 1987; Lamb et al., 1989; Chou et al., 1989; Geisler et al., 1989] which has been shown to be altered dramatically during mitosis [Celis et al., 1983; Franke et al., 1984; Chou et al., 1989], hormone treatments [Spruill et al., 1983; Coca-Prados, 1985], and heat-shock [Thomas et al., 1982; Welch and Suhan, 1985; Welch et al., 1985].

Okadaic acid (OA), a 38 carbon polyether monocarboxylic acid, is a toxin isolated from a variety of marine species [Tachibana et al., 1981]. It is further identified as a potent inhibitor of a number of protein phosphatases including type 1 and type 2A protein phosphatases [Bialojan and Takai, 1988]. The inhibitory effect blocks the dephosphorylation of proteins that are substrates for multiple protein kinases [Suganuma et al., 1989], resulting in the apparent "activation" of the kinases [Sassa et al., 1989] and subsequently induces a variety of cellular responses that are modulated by protein phosphorylation-dephosphorylation. For instance, OA "activates" the kinases and thus increases the phosphorylation states of the regulatory enzymes of glycogen and lipid metabolism, glycolysis, and gluconeogenesis in hepatocytes and adipocytes [Haystead et al., 1989]. It can also "activate" the cdc2/histone H1 kinase and transiently induce premature mitosis-like state in BHK21 cells [Yamashita et al., 1990]. In 9L rat brain tumor cells, vimentin is the major component of the IF and is one of the major cellular proteins. In the present study, we found that the phosphorylation level of vimentin could be drastically elevated by OA in a reversible manner. Furthermore, the reversible hyperphosphorylation of vimentin could result in the reorganization of the vimentin IFs.

MATERIALS AND METHODS

Materials

Okadaic acid was purchased from Boehringer-Mannheim (Mannheim, Germany), dissolved in dimethyl sulfoxide at a concentration of 0.1 mM, and stored in the dark at −20°C. The stock solution was diluted to appropriate concentrations with culture medium before use. All cultureware was obtained from Corning (Corning, NY) and culture medium components were purchased from Gibco Laboratories (Grand Island, NY). [^{32}P]orthophosphate (specific activity 8,500–9,120 Ci/mmole) was obtained from New England Nuclear (Boston, MA). Supplies for immuno-staining were purchased from Amersham (Buckinghamshire, England). Chemicals for electrophoresis were obtained from BioRad (Richmond, CA). General chemicals were from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany).

Cell Culture

The 9L brain tumor cells, originated from rat gliosarcoma, were a generous gift from Dr. M.L. Rosenblum, University of California at San Francisco [Weizsaecker et al., 1981]. Cells were maintained in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 μg/ml streptomycin. The cells were kept in a humidified 37°C incubator with a mixture of 5% CO_2 and 95% air in 75 cm^2 flasks. Stock cells were plated in 6-well plates at a density of 4–6 × 10^4 cells per cm^2 for all experiments. Exponentially growing cells at 80–90% confluency were used.

[^{32}P]phosphate Labeling and Drug Treatments

For the studies of drug effects, cells were pre-labeled with 1 mCi of [^{32}P]orthophosphate for 1 h in 1 ml of phosphate-free medium and treated with various concentrations of OA at 37°C in the presence of [^{32}P]orthophosphate for various durations as indicated. In some experiments, the labeled and drug treated cells were further incubated under normal growing conditions for various durations for the studies of protein dephosphorylation.

Protein Extraction and Fractionation

After treatments, the cells were briefly washed with ice-cold phosphate buffered saline (PBS) and lyzed 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) or 200 μl lysis buffer (9.5 M urea, 2% nonident P-40, 2% ampholytes, and 5% β-mercaptoethanol), depending on the electrophoresis system employed. Alternatively, cellular proteins were separated into soluble and insoluble fractions before they were subjected to gel electrophoresis described as follows. The treated cells were washed with PBS and incubated with 0.2 ml of extraction buffer (1% nonident P-40 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 remaining 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]. Equal volumes of protein samples were then resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands of interest were quantitated by densitometry as described in the following.

Gel Electrophoresis

One-dimensional 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 amount of cell lysate. For molecular weight calibration, a subset of the following standards (Sigma) was included in each gel: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa). After electrophoresis, the gels were stained for 1 h in staining solution (0.1% Coomassie brilliant blue R250 in 10% acetic acid and 50% methanol), destained, and dried under vacuum. Autoradiography was performed at room temperature using Fuji RX x-ray film in the presence of an intensifying screen. The optical densities of the protein bands of interest on the stained gels or autoradiographs were quantified by scanning the resulting autoradiographs on an Ultroscan laser densitometer (LKB, GSXL software). Background levels of optical density were subtracted to give the values presented. Alternatively, the gels were processed for immunoblot analysis (see below). Two-dimensional PAGE was performed according to the method of O'Farrell [1975]. Equal amount of cell lysates 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 gel slabs were processed for Coomassie blue staining and autoradiography as described. The pH gradient formed was measured from slices obtained from replicate IEF gels.

Immunoblot Analysis

Western blotting analysis was performed as previously described [Lee et al., 1991]. After electrophoresis, the gel was soaked in transfer buffer (50 mM Tris-borate, pH 8.3, 1 mM EDTA) for 10 min. Resolved proteins were then electrotransferred onto a nitrocellulose membrane (Hybond-C extra, Amersham) by a semi-dry method (OWL Scientific Plastics Inc., Cambridge, MA). The membrane was incubated for 1 h with 3% gelatin in Tween 20 containing Tris buffered saline (TTBS: 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.05% Tween 20) and then rinsed with TTBS briefly. Subsequently, the membrane was incubated with monoclonal antibody to vimentin (diluted 1:200 in TTBS containing 1% gelatin) at room temperature for 2 h. After three washes with TTBS, immunocomplexes on the membranes were reacted with goat anti-mouse antibody conjugated with alkaline phosphatase (diluted 1:2,000 in TTBS containing 1% gelatin) at room temperature for 30 min. The membrane was then rinsed three times with TTBS, dried, and developed into a color immunoblot at room temperature in developing buffer (15 mg of nitro blue tetrazolium, 0.7% N,N-dimethylformamide, 30 mg of 5-bromo-4-chloro-3-indolyl phosphate per 100 ml, 1 mM $MgCl_2$, and 100 mM $NaHCO_3$, pH 9.8).

Indirect Immunofluorescence and Microscopy

For indirect immunofluorescence studies, cells were grown on chamber slides (Nunc) and drug treatment conditions were identical to those described above. After treatments, the slides were briefly washed with PBS and the cells were then fixed and permeabilized with methanol at −20°C for 10 min. After a brief rinse in PBS, the fixed cells were incubated for 1 h at room temperature with monoclonal antibody against vimentin diluted in PBS containing 3% BSA (1:20). The cells were then washed with 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 for

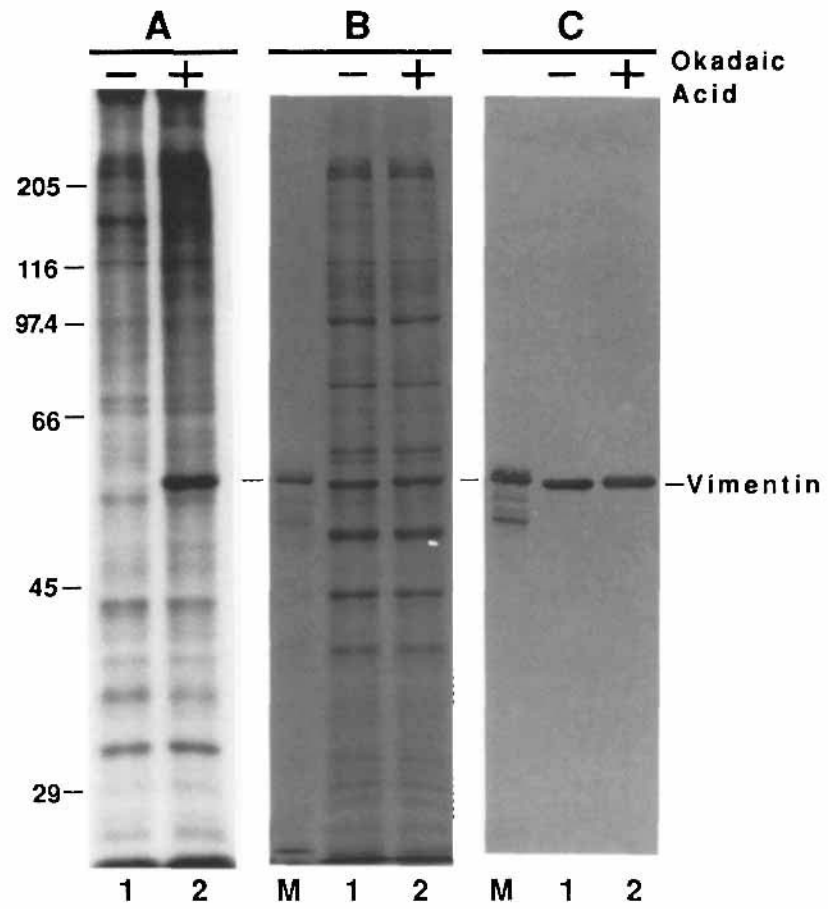

Fig. 1. Hyperphosphorylation of vimentin in OA-treated 9L cells. Cells were pre-labeled with [^{32}P]orthophosphate for 1 h and then treated with 600 nM OA for 1 h. The cells were then lysed with SDS sample buffer and the proteins were resolved by 10% SDS-PAGE. After electrophoresis, the gels were processed for autoradiography (**A**) or stained with Coomassie blue (**B**). Alternatively, the proteins were electrotransferred onto a nitrocellulose membrane and then processed for immunoblotting by using monoclonal antibody to vimentin as a probe. The immunocomplexes on the membrane were reacted with goat anti-mouse antibody conjugated with alkaline phosphatase and then developed into a color immunoblot (**C**). **Lane M:** Vimentin from bovine lens as a positive control. **Lane 1:** Untreated cells. **Lane 2:** OA-treated cells.

examination. A Nikon microscope equipped with epifluorescence optics (Nikon Optiphot, Tokyo, Japan) was used for obtaining photographs of the fluorescent images.

RESULTS

Dose-Dependent Effects of OA on the Phosphorylation of Vimentin

The patterns of total cellular proteins and phosphoproteins were analyzed after the cells were pre-labeled with [^{32}P]orthophosphate and then treated with 600 nM OA for 1 h. Figure 1A shows that the levels of protein phosphorylation were augmented in cells incubated with OA. Specifically, a protein band with a molecular weight of 57 kDa was hyperphosphorylated (Fig. 1A) but the protein patterns remained unchanged (Fig. 1B). The 57 kDa protein was subsequently identified as vimentin as demonstrated by immunoblotting analysis (Fig. 1C). The enhanced phosphorylation of vimentin in OA-treated cells occurred in a concentration-dependent manner. As shown in Figure 2, phosphorylation of vimentin was initially increased about eightfold in cells that were treated with 400 nM OA for 1 h but could be further enhanced up to twentyfold when OA concentration was increased to 800 nM. The OA-induced vimentin hyperphosphorylation was also time dependent. The levels of vimentin phosphorylation were initially augmented in cells incubated with 400 nM OA for 30 min (Fig. 3A) but could be increased up to 10- and 26-fold when cells were treated with 400 nM OA for 60 and 90 min,

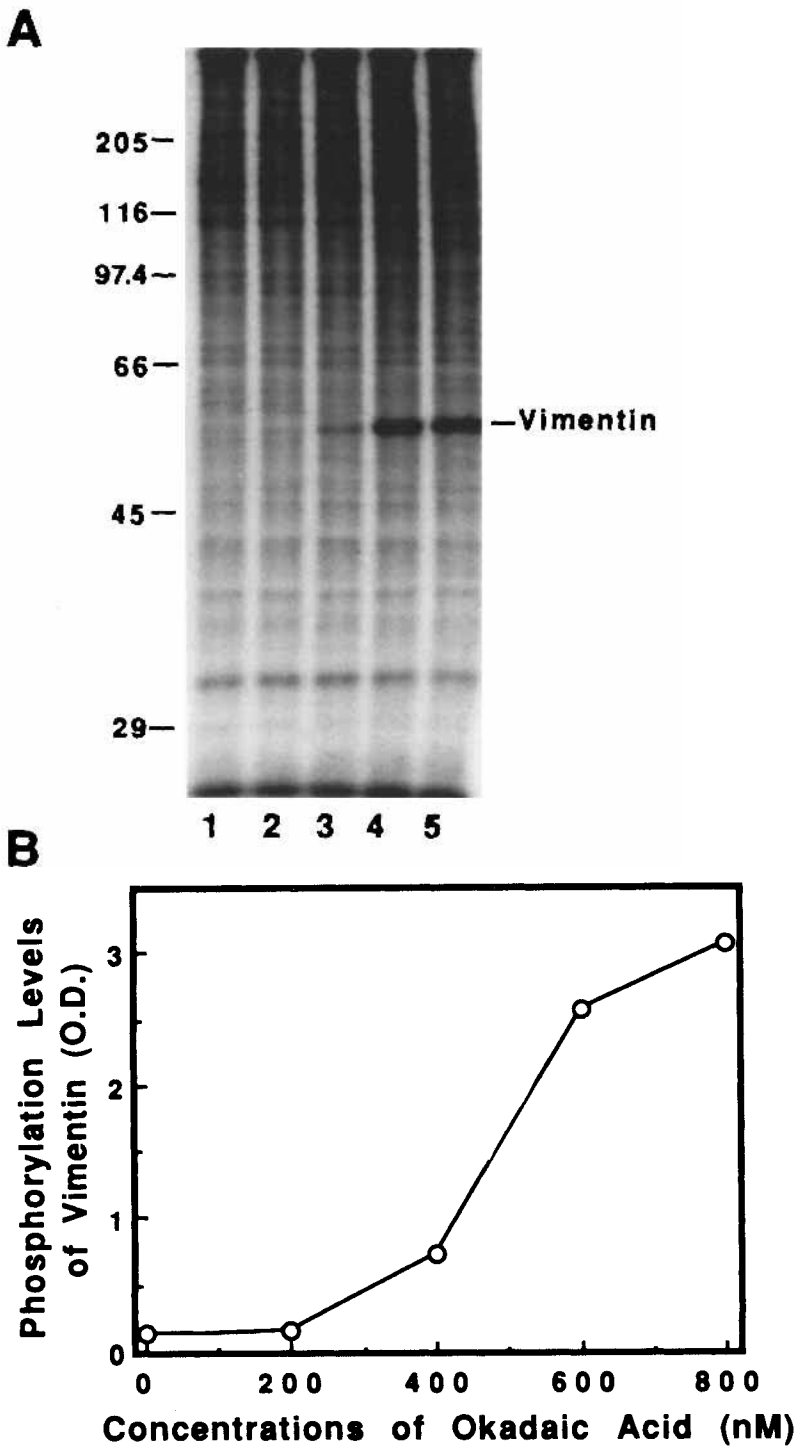

Fig. 2. Concentration-dependent effect of OA on vimentin phosphorylation in 9L cells. Cells were pre-labeled with [^{32}P]orthophosphate for 1 h and then treated with OA at the concentrations as indicated for 1 h. The cells were then lysed with SDS sample buffer and the proteins were resolved by 10% SDS-PAGE. After electrophoresis, the gels were processed for autoradiography (**A**) in which the relative phosphorylation levels of vimentin were determined by densitometry (**B**). Background levels of optical density were subtracted and the amounts of phosphorylation were presented as O.D. units calculated from the peak areas. **Lane 1:** controls. **Lanes 2–5:** Cells were treated with 200, 400, 600, and 800 nM OA, respectively.

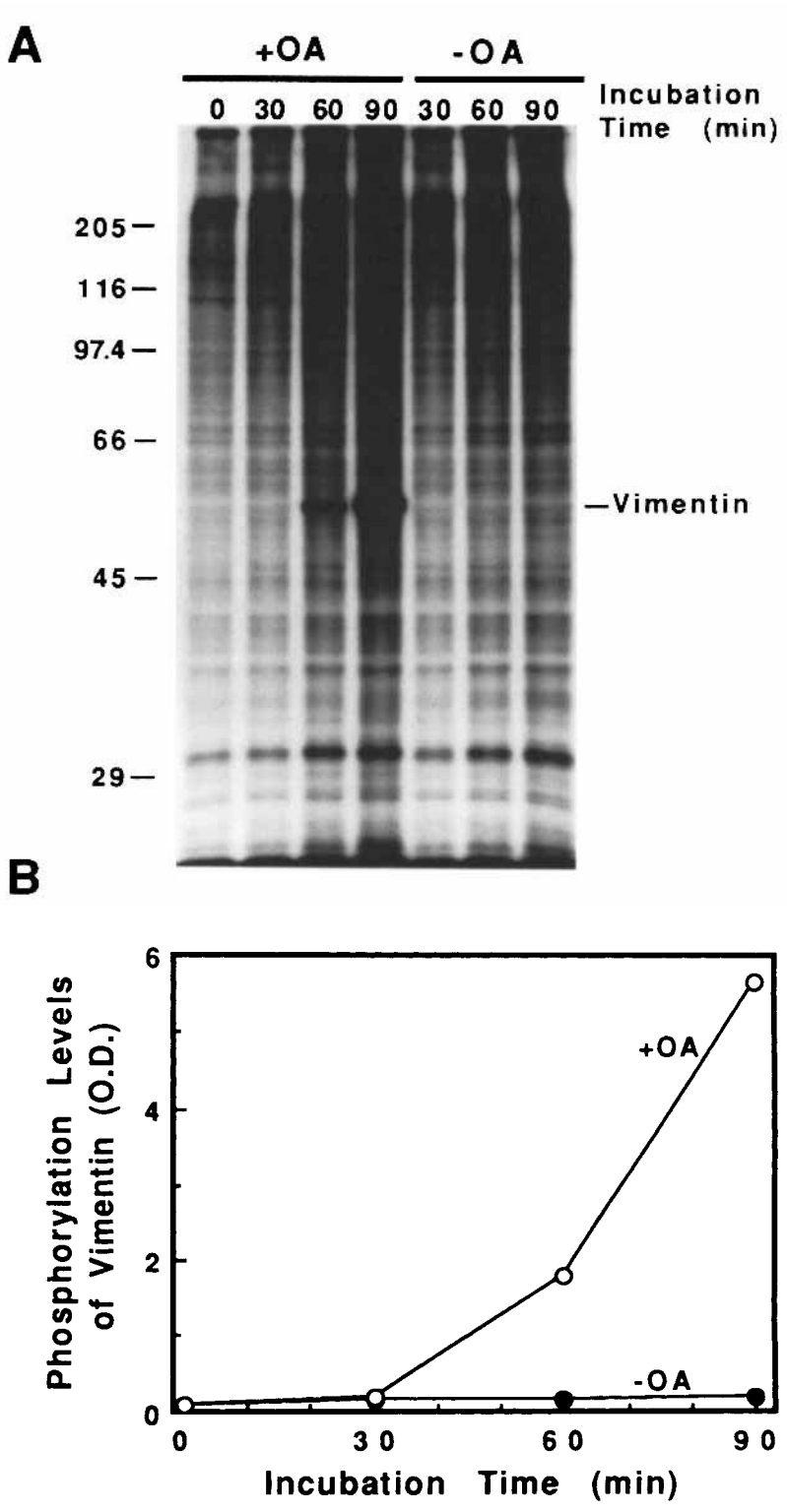

Fig. 3. Time-dependent effect of OA on vimentin phosphorylation in 9L cells. **A:** Autoradiograph of the phosphoproteins after treatments with OA. **B:** Quantitation of the phosphorylation of vimentin by densitometry. Experimental conditions were as described in the legend to Fig. 2 except that 400 nM OA was used for different incubation time points as indicated.

respectively (Fig. 3B). It was also found that the phosphate moieties on vimentin were not stable under alkaline conditions (not shown), further indicating that OA may enhance vimentin phosphorylation specifically on serine/threonine residues.

Reversible Effect of OA on Vimentin Phosphorylation and Cell Morphology

The enhanced phosphorylation of vimentin induced by OA was found to be reversible. After OA removal and incubation of the cells in normal growing conditions, dephosphorylation of the hyperphosphorylated vimentin was monitored at different time intervals. Figure 4 shows that the dephosphorylation occurred in a time-dependent manner after drug removal and was essentially completed after 11 h of recovery. This observation suggested that normal turnover of the phosphate moieties on the vimentin molecules was immediately in place upon removal of OA.

Figure 5 shows the morphological changes of the cells during the hyperphosphorylation and dephosphorylation of vimentin induced by OA. In close correlation with the phosphorylation levels of vimentin, cell rounding up was initially observed when the cells were treated with 400 nM OA for 60 min (Figs. 3, 5C) and reached its maximal level after 90 min (Figs. 3, 5D). Prolonged incubation may result in the detachment of the cells (not shown). After removal of OA, cells gradually returned to their normal appearance and the reversible morphological changes was also in close correlation with the dephosphorylation states of vimentin (Figs. 4, 5E–I). Completed recovery of the normal cell morphology was also observed after 11 h incubation of cells under normal growing condition (Fig. 5I). Immuno-staining experiments further revealed that the vimentin IFs were disassembled and clustered around the nucleus in the OA-treated cells but could be reorganized to the original filamentous form after OA was removed and the cells were recovered in normal growing conditions (Fig. 6). All the results taken together demonstrate that OA induces hyperphosphorylation-reorganization of vimentin and the subsequent cell morphological changes of 9L rat brain tumor cells in a reversible manner.

Characterization of Phospho-Vimentin in OA-Treated Cells

OA-induced hyperphosphorylation of vimentin was further analysed by two-dimensional PAGE. Figure 7 demonstrated that the pIs of authentic vimentin (Vm) and its degradation products (VDPs) were affected by OA in contrast to actin and the constitutive heat-shock protein72 (HSP72). From Coomassie-stained gels, 3–4 isoforms of Vm and VDPs having different pIs were generated in OA-treated cells. Autoradiograms further showed that the phosphorylation levels of Vm and VDPs were drastically increased and that at least 7 isoforms of Vm and 3–4 isoforms of VDPs were clearly detected (Fig. 7C,D). These isoforms of Vm and VDPs were probably generated by phosphorylation induced by OA.

As a result of hyperphosphorylation induced by OA, the solubility of vimentin was found to be concomitantly increased in OA-treated cells when lysed with buffer containing 1% NP-40 and 0.15 M NaCl (Fig. 8). In untreated cells, the detergent-soluble and -insoluble vimentin were of equal amounts. However, when cells were treated with 400 nM OA for 1 h, about 80% of vimentin became soluble (Fig. 8C). Since the detergent-solubility of vimentin could be due to an intracellular partitioning between cytosolic and nuclear-cytoskeletal fractions [Collier and Schlesinger, 1986], the results indicated that there was 4 times as much of vimentin in the cytosol as in the nuclear-cytoskeletal in the cells treated with OA (Fig. 8C). Since the total amount of intracellular vimentin remained the same, the results further indicated that vimentin molecules were redistributed from the nucleus-cytoskeleton fraction to the cytosol after OA treatment. The levels of phosphorylation of vimentin were simultaneously monitored in these studies. In untreated cells, it was found that soluble vimentin was in a non-phosphorylated state, whereas the phosphorylated vimentin was associated with the nuclear-cytoskeletal fraction (Fig. 8B). By contrast, a higher level of phosphorylation of vimentin was observed in the cytosolic fraction of the OA-treated cells and approximately 70% of the phosphate moieties was detected in cytosolic phospho-vimentin (Fig. 8C). The results suggest that a proportion of vimentin may be translocated into the cytosol and becomes detergent-soluble after hyperphosphorylation by OA. Taken together, the results further support the notion that OA induces hyperphosphorylation of vimentins and thereby promotes reorganization and redistribution of vimentin IFs in 9L rat brain tumor cells.

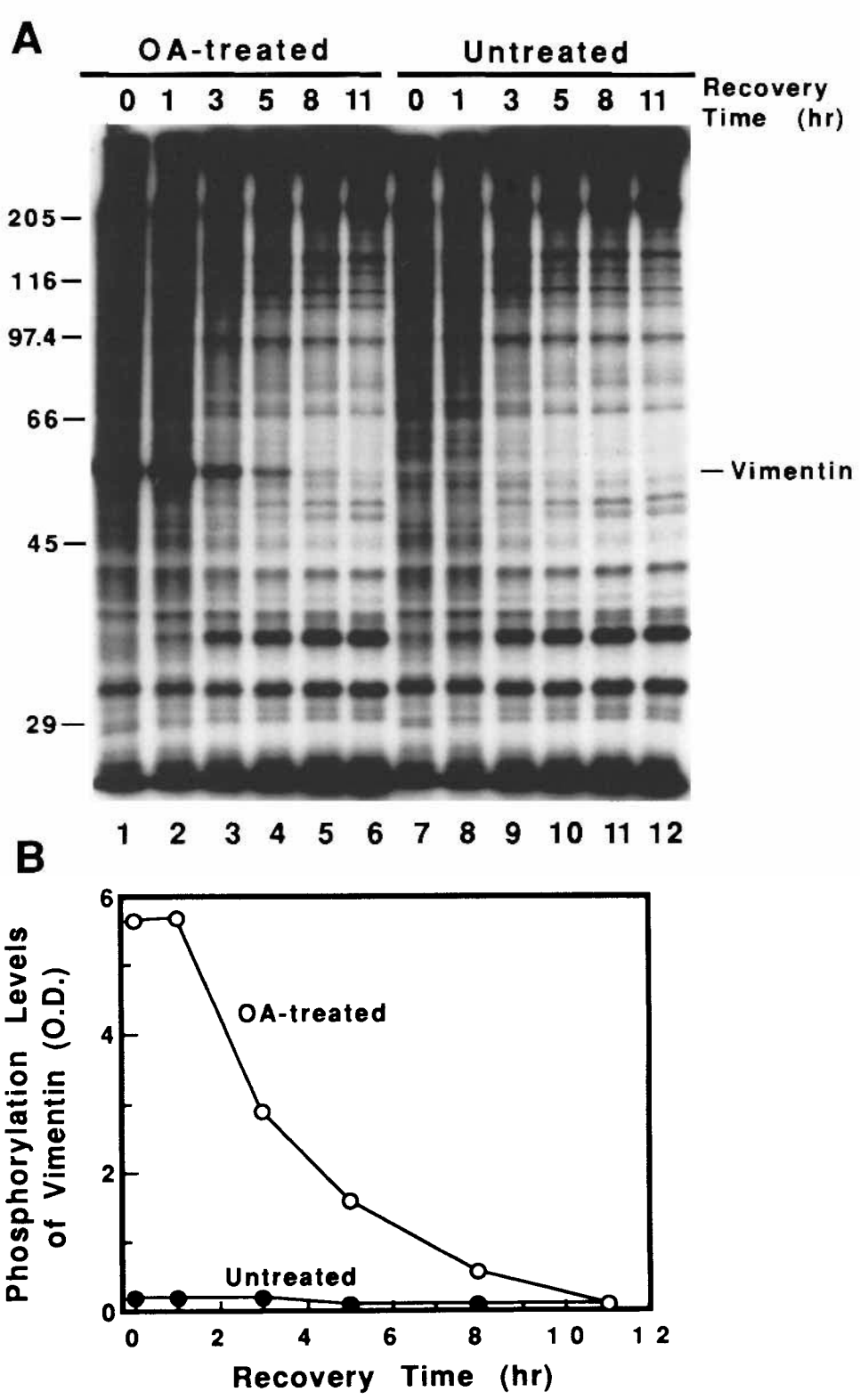

Fig. 4. Time-dependent dephosphorylation of phosphovimentin after removal of OA from treated 9L cells. **A:** Autoradiograph of the phosphoproteins after treatment with OA. **B:** Quantitation of the phosphorylation of vimentin by densitometry. Cells were pre-labeled with [^{32}P]orthophosphate for 1 h and treated with 400 nM OA for another 90 min. The cells were then washed and allowed to recover under normal growing conditions for various durations as indicated (**lanes 1–6**) and then processed as described in the legend to Fig. 2. Untreated cells were also labeled and processed simultaneously (**lanes 7–12**).

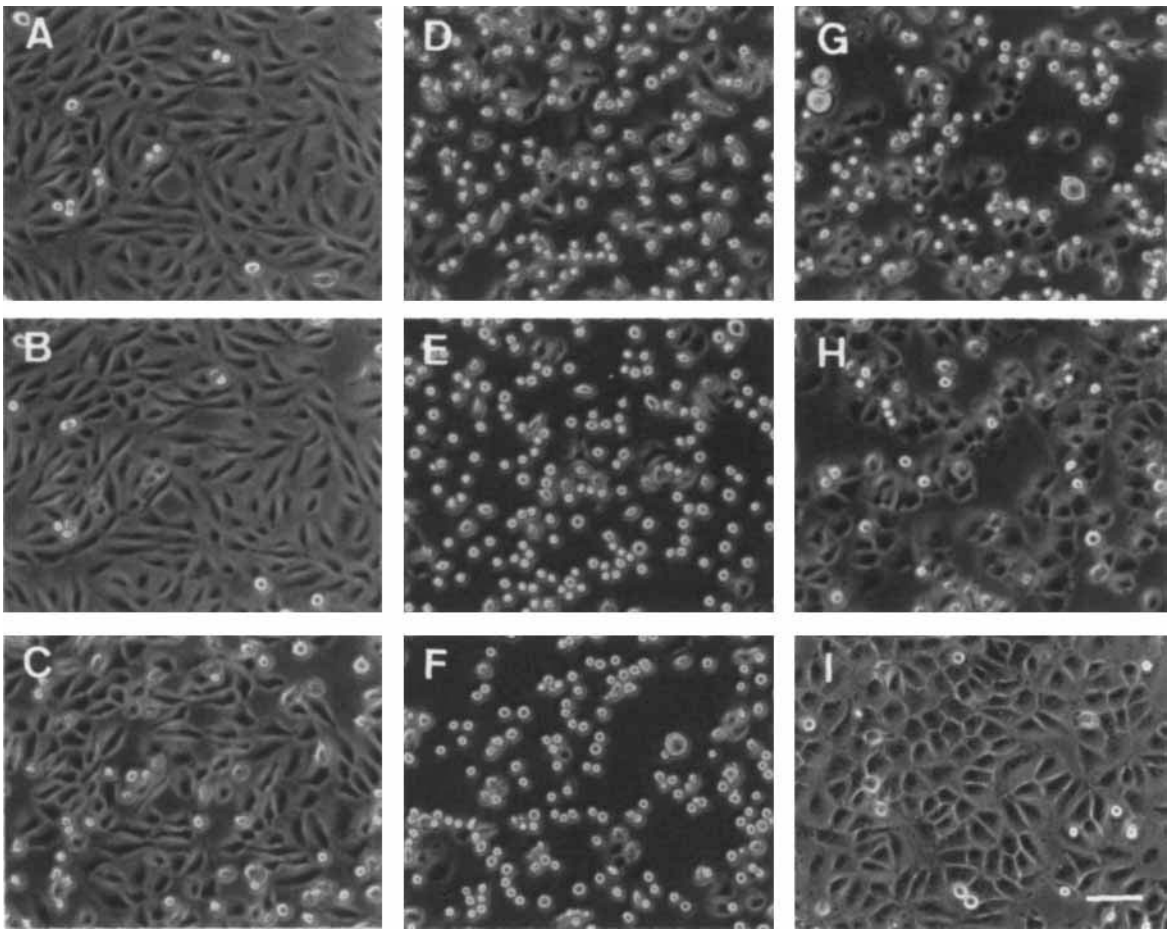

Fig. 5. Morphological changes of 9L cells after exposure to and removal of OA. **A–D:** Cells were incubated with 400 nM OA for various durations before being photographed. A: Untreated cells. B–D: Cells treated for 30, 60 and 90 min, respectively. **E–I:** Cells were treated with 400 nM OA for 90 min. OA was then removed and cells were allowed to recover under normal growing conditions for 1, 3, 5, 8, and 11 h, respectively. Bar represents 50 μm.

DISCUSSION

We have demonstrated that vimentin is the major cellular protein hyperphosphorylated in 9L rat brain tumor cells treated with OA. A 26-fold increase in phosphorylation and at least 7 isoforms of phosphovimentin generated by OA characterized the hyperphosphorylated nature of vimentin. Hyperphosphorylation of vimentin by dinophysistoxin-1 in human fibroblasts [Yatsunami et al., 1991] and by calyculin A in mouse embryo fibroblasts [Chartier et al., 1991] have recently been reported. However, the isoforms of hyperphosphorylated vimentin having different pIs as presented in this report does not agree with the observation made by Yatsunami et al. [1991] in which the pI of authentic vimentin remained unchanged. Moreover, the cellular and biochemical effects generated by this toxin were investigated in detail. In the present studies, we further identified that hyperphosphorylation of vimentin induced by OA was in a dose-dependent manner and was virtually reversible, that the process could affect vimentin-containing IFs organization and subsequently resulted in the round morphology of cells and the morphological changes were also reversible, and that the process also changed the solubility of vimentin which resulted in the subcellular redistribution of vimentin in the cell.

As summarized in Table I, several protein kinases have been identified as vimentin kinases from a number of in vitro or in vivo studies. Vimentin becomes higher phosphorylated in cells undergoing mitosis and the cdc2 kinase is responsible for this process [Chou et al., 1989, 1990, 1991]. Increase in phosphorylation of vimentin has also been observed in cells subjected to a variety of treatments such as cAMP increasing

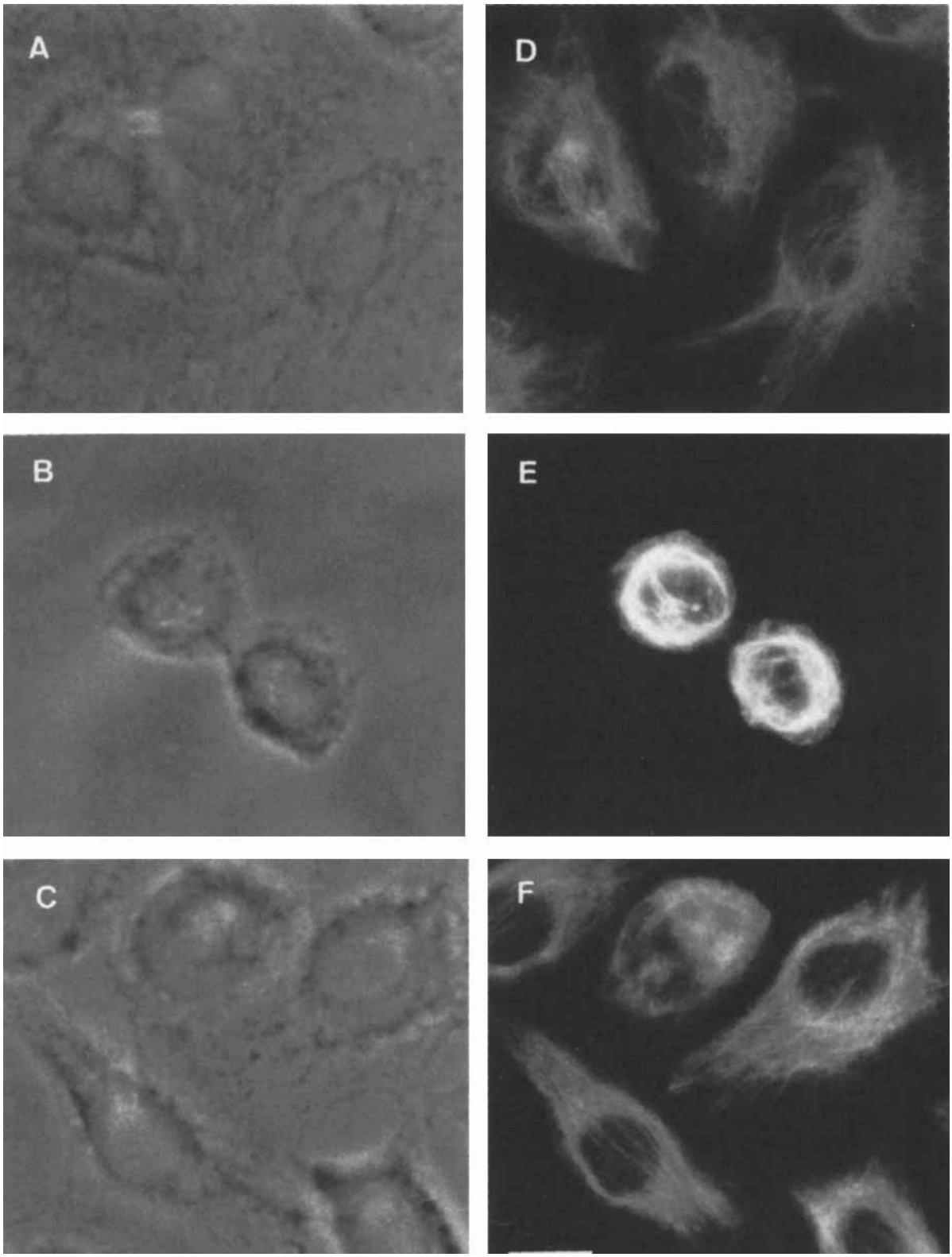

Fig. 6. Morphology and organization of vimentin IFs in control, OA-treated, and recovered cells. Cells were treated with 400 nM OA for 90 min. OA was then removed and the cells were allowed to recover under normal growing conditions for 11 h. The control, treated, and recovered cells were processed and analyzed by indirect immunofluoresence using mouse monoclonal antibodies against vimentin. **A–C:** Phase contrast microscopies. **D–F:** Immunofluoresent microscopies. A,D: Untreated controls. B,E: Cells treated with 400 nM OA for 90 min. C,F: OA-treated cells recovered for 11 h. Bar represents 15 μm.

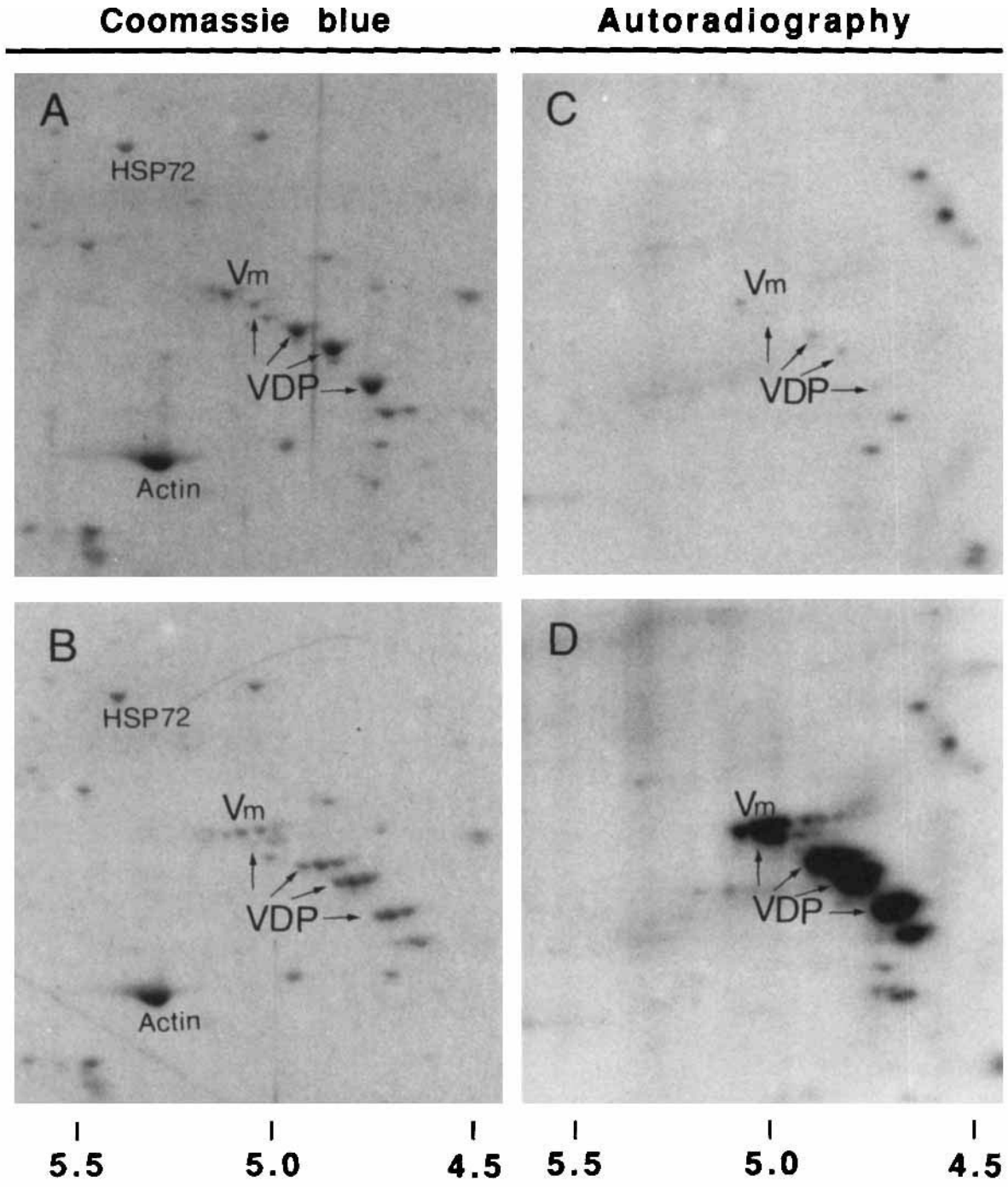

Fig. 7. Two-dimensional gel analysis of OA-induced changes in the electrophoretic mobilities of vimentin and its degradation products. Cells were pre-labeled with [^{32}P]orthophosphate and treated with OA as described in Fig. 5D. Untreated cells were also labeled and processed simultaneously. After treatments, the cells were lysed and the lysates were resolved by two-dimensional PAGE. The gels were then stained with Coomassie blue (A,B) and processed for autoradiography (C,D). Only the regions nearby vimentin and its degradation products are shown. **A,C:** Control cells. **B,D:** OA-treated cells. Vm, vimentin; VDP, degradation products of vimentin; HSP72, constitutive heat-shock protein with molecular mass of 72,000. The pH range is shown at the bottom of the panels.

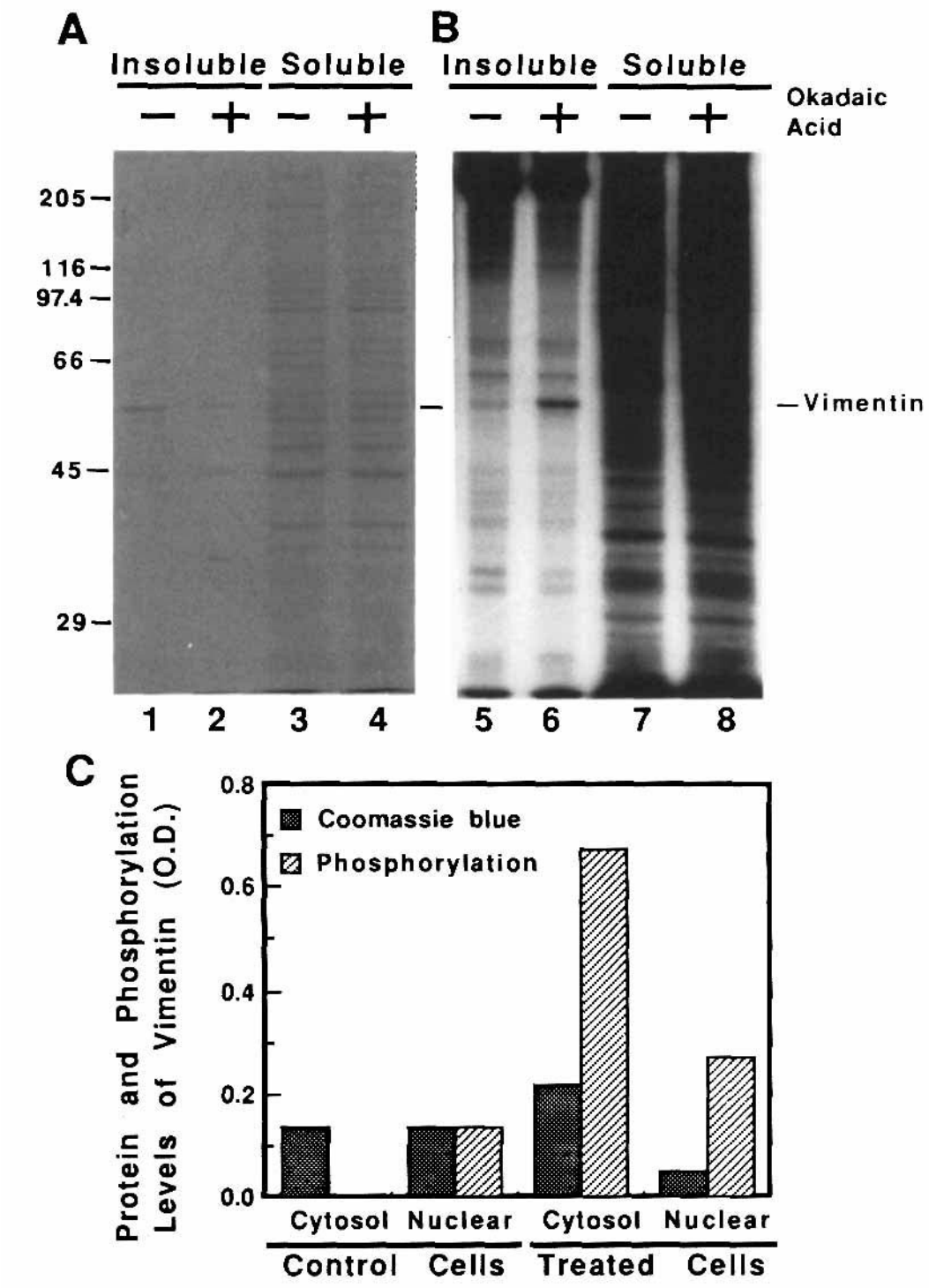

Fig. 8. Distribution of vimentin and phosphoproteins between the detergent-soluble (cytosolic) and -insoluble (nuclear-cytoskeletal) fractions in cells after treatment with OA. Cells were pre-labeled with [^{32}P]orthophosphate for 1 h and then treated with 400 nM OA for 1 h. After treatments, the cells were lysed with extraction buffer containing 1% NP-40 and 0.15 M NaCl. After centrifugation, the cellular proteins were collected as soluble and insoluble fractions which were subsequently resolved by 10% SDS-PAGE. The gels were stained by Coomassie blue (**A**) and processed for autoradiography (**B**) in which the relative amount of vimentin proteins and the phosphorylation levels of vimentin were determined by densitometry (**C**).

TABLE I. In Vivo and In Vitro Phosphorylation of Vimentin

Cell origins	Treatments (conditions)[a]	Putative kinase(s)[b]	Sites of phosphorylation	Maximal level[c]	Effect on vimentin organization	References
A. In vivo studies						
Myogenic	8-Br-cAMP	PKA	?	2–3	?	Gard and Lazarides [1982]
Sertoli	FSH	PKA	?	?	aggregate	Spruill et al. [1983]
Neutrophils	fMLP	?	?	1.5	?	Huang et al. [1984]
Epithelial	hormones[d]	PKA	?	3–5	aggregate	Coca-Prados [1985]
Endothelial	thrombin	?	?	3.8	?	Bormann et al. [1986]
CHO	PKC	PMA	N-terminal region	?	?	Huang et al. [1988]
BHK-21	mitotic	cdc2	?	6.0	disassembly	Chou et al. [1989]
Fibroblasts	microinject	PKA	?	8–12	aggregate	Lamb et al. [1989]
Fibroblasts	DTX-1	?	?	10	?	Yatsunami et al. [1991]
Neutrophils	fMLP	PKG	?	?	disassembly	Wyatt et al. [1991]
Gliosarcoma	WA	?	?	2.5	aggregate	Lee and Lai [unpublished]
Gliosarcoma	OA	?	?	26	disassembly	present studies
B. In vitro studies						
Myogenic		PKA	?		?	O'Connor et al. [1991]
Mastocytoma	M-V	PKA	?	?	unaffected	Inagaki et al. [1987]
	F-V	PKA	?		depolymerized	
	M-V	PKC	?		unaffected	
	F-V	PKC	?		unaffected	
CHO		PKC	?	?	?	Huang et al. [1988]
Myogenic	angiotensin II	?	?	2–3	?	Tsuda et al. [1988]
L-929	mitotic	PKA	N-terminal region	5.7	disassembly	Evans [1988, 1989]
Mesenchymal	P-V	PKA	N-terminal region	?	depolymerized	Geisler et al. [1989]
	P-V	PKC	N-terminal region	?	unaffected	
	F-V	PKA	N-terminal region	?	unaffected	
Mastocytoma		PKA	Ser-46,6,24,38,50,65		?	Ando et al. [1989]
		PKC	Ser-8,9,20,25,33,41,6,24,38,50,65		?	
BHK-21	F-V	cdc2	?	?	disassembly	Chou et al. [1989]
BHK-21	mitotic	cdc2	Ser-55,41,65	?	aggregate	Chou et al. [1990, 1991]

[a]Abbreviations used in this column: 8-Br-cAMP, 8-bromo-cyclic AMP; FSH, follicle-stimulating hormone; fMLP, formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; DTX-01, dinophysistoxin-1; WA, withangulatin A [Chen et al., 1990; Lee et al., 1991]; OA, okadaic acid; M-V, monomeric vimentin; F-V, filamentous vimentin; P-V, protofilament vimentin.

[b]Classified according to Hunter [1991]. PKA, cAMP-dependent protein kinase; PKC, diacylglycerol regulated protein kinase (protein kinase, C); PKG, cGMP-dependent protein kinase; cdc2, cdc2-related protein kinase.

[c]Folds of increased phosphorylation compared to basal or control level.

[d]Included one of the followings: β-adrenergic agonists (isoproteronol or epinephrine), activators of adenylate cyclase (cholera toxin or forskolin), 8-Br-cAMP, and prostaglandin E_1.

agents [Gard and Lazarides, 1982; Coca-Prados, 1985], phorbol ester PMA [Huang et al., 1988], and chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine [Huang et al., 1984; Wyatt et al., 1991]. These cellular responses are not entirely cell cycle dependent, suggesting that vimentin may be a substrate for a number of protein kinases. It is likely that cdc2 kinase is directly involved in the phosphorylation of vimentin in mitotic cells while other protein kinases, such as PKA, PKC, and PKG, participate in the phosphorylation and reorganization of vimentin in other cellular activities. Since OA seems to have no direct effect on a variety of protein kinases including PKC [Suganuma et al., 1989], PKA, protein kinase F_A/glycogen synthase kinase-3, and casein kinase-2 [Haystead et al., 1989], it is conceivable that hyperphosphorylation of vimentin by OA in cells is also a consequence of phosphatase inhibition and the following "activation" of a wide variety of protein kinases as noted by Sassa et al. [1989].

Dephosphorylation of vimentin has been suggested to be regulated by Ca^{2+}-stimulated [Evans, 1989] and OA sensitive protein phosphatases [Yatsunami et al., 1991]. OA, being hydrophobic, readily enters cells and presumably inhibits protein phosphatases by specific binding [Suganuma et al., 1990; Nishiwaki et al., 1990]. It is particularly effective against PP2A (ID_{50} 1–2 nM), PP1 (ID_{50} 100–500 nM), and less so against PP2B (ID_{50} 4–5 μM) [Bialojan and Takai, 1988]. In the present studies, the most effective dose for the induction of vimentin hyperphosphorylation was 400–800 nM OA for 1 h and this effect was dose dependent. It is probable that in in vivo experiments unequal distribution and partially restricted permeability through the plasma membrane of OA may occur; however, our results indicate that PP1 and PP2A may function as the major vimentin phosphatase in the cells. We do not exclude the possibility that PP2B and other protein phosphatases such as polycation-modulated phosphatase [Bialojan and Takai, 1988] may also be involved in this process. The identity of the vimentin phosphatases obviously presents an intriguing problem that remains to be unraveled.

Organization and reorganization of vimentin-containing IFs has been reported to be regulated by phosphorylated states of vimentin (Table I). The process is crucial for a variety of cellular activities [Bershadsky and Vasiliev, 1988]. In normal growing cells, only a small fraction of the vimentin molecules are phosphorylated [Cabral and Gottesman, 1979; Nelson and Traub, 1983]. We have demonstrated that all of the phosphovimentin molecules were associated with the nuclear-cytoskeletal fraction and that all of the cytosolic vimentin was unphosphorylated. Augmented phosphorylation of vimentin would result in the disassembly of the IFs which are reorganized into higher levels of aggregates. For instance, increased phosphorylation of vimentin coincides temporally with changes in IF organization, such as the formation of a "cage" of IFs surrounding the spindle during mitosis [Zieve et al., 1980]. Recently, it has been shown that increased phosphorylation of vimentin is performed by a microinjection of the purified catalytic subunits of the PKA into rat embryo fibroblasts and this leads to the bundling together of the 10-nm filaments. Moreover, this increase in vimentin phosphorylation is not accompanied by any significant increase in soluble vimentin [Lamb et al., 1989]. The use of OA makes further investigation to the mechanism of organization and reorganization of vimentin IFs possible. It seems apparent that this process is controlled by concerted actions of protein kinases-phosphatases. Treatment of 9L cells with OA induced a complete disassembly in vimentin IFs and a dramatic shape change. It is reasonable to assume that these transformations are due to vimentin hyperphosphorylation. The most unique effect induced by OA was that all of the hyperphosphorylated vimentin became soluble when cells were treated with 400 nM OA for 90 min (unpublished data). However, at the same concentration and shorter incubation time (i.e., 60 min), the hyperphosphorylated vimentin was partitioned to both of the cytosolic and the nuclear-cytoskeletal fractions. Thus, it is apparent that the disintegrated phosphovimentin is released from the aggregated form of vimentin IFs. However, we did not rule out the possibility that cytosolic vimentin pre-existing in control cells may be the preferred substrate for the protein kinases which are "activated" during the inhibition of protein phosphatases. The factor(s) influencing equilibria of vimentin filaments in cells remain to be further dissected.

The OA-induced hyperphosphoryation of vimentin and the subsequent changes in IFs reorganization, cell morphology, and detergent-solubility of vimentin were found to be reversible. The action mechanism of OA in this process

may be as follows. OA directly inhibited protein phosphatases which resulted in the "activation" of protein kinases. Concomitant with this kinases activation, the level of hyperphosphorylation of vimentin was increased as the time durations were prolonged. Subsequently, the filamentous type of vimentins totally became the disassembled type and the cell rounded up. Eventually, further hyperphosphorylation of vimentin by OA solubilized the disassembled vimentin IFs. After removal of OA, the inhibition of protein phosphatases was relieved and organization of vimentin IFs and cell shape recovered to normal appearance due to the dephosphorylation of vimentin. The most intriguing questions that emerged are why vimentin is the prominent protein hyperphosphorylated by OA and what the physiological significance of this process in the cell is. Because of the large amount of vimentin molecules (up to 3–5% of total proteins in 9L cells) and its possession of multiple phosphorylation sites (serine and threonine) in 9 kDa N-terminal head domain [Inagaki et al., 1987; Geisler et al., 1989; Ando et al., 1989], it is probable that vimentin IFs may play an important role for reserving the phosphate moieties which are transferred by reactions dictated by protein kinases that are apparently "activated" by OA. This implication may also be extended to other IF components such as cytokeratin or neurofilament proteins. In fact, Yatsunami et al. [1991] and Chartier et al. [1991] have suggested that hyperphosphorylated proteins generated by the OA class of tumor promoters are mainly the components comprising IFs. Therefore, OA may be used as a valuable tool to study the mechanism of phosphorylation-dephosphorylation of IF proteins as well as assembly-disassembly of IFs.

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