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Identification of Mitogen-Activated Protein Kinase-Activated Protein Kinase-2 as a Vimentin Kinase Activated by Okadaic Acid in 9L Rat Brain Tumor Cells

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Abstract Organization of intermediate filament, a major component of cytoskeleton, is regulated by protein phosphorylation/dephosphorylation, which is a dynamic process governed by a balance between the activities of involved protein kinases and phosphatases. Blocking dephosphorylation by protein phosphatase inhibitors such as okadaic acid (OA) leads to an apparent activation of protein kinase(s) and to genuine activation of phosphatase-regulated protein kinase(s). Treatment of 9L rat brain tumor cells with OA results in a drastically increased phosphorylation of vimentin, an intermediate filament protein. In-gel renaturing assays and in vitro kinase assays using vimentin as the exogenous substrate indicate that certain protein kinase(s) is activated in OA-treated cells. With specific protein kinase inhibitors, we show the possible involvement of the cdc2 kinase- and p38 mitogen-activated protein kinase ($p38^{MAPK}$)-mediated pathways in this process. Subsequent in vitro assays demonstrate that vimentin may serve as an excellent substrate for MAPK-activated protein kinase-2 (MAPKAPK-2), the downstream effector of $p38^{MAPK}$, and that MAPKAPK-2 is activated with OA treatment. Comparative analysis of tryptic phosphopeptide maps also indicates that corresponding phosphopeptides emerged in vimentin from OA-treated cells and were phosphorylated by MAPKAPK-2. Taken together, the results clearly demonstrate that MAPKAPK-2 may function as a vimentin kinase in vitro and in vivo. These findings shed new light on the possible involvement of the $p38^{MAPK}$ signaling cascade, via MAPKAPK-2, in the maintenance of integrity and possible physiological regulation of intermediate filaments. J. Cell. Biochem. 71:169–181, 1998.

Key words: intermediate filaments; mitogen-activated protein; kinase-activated protein kinase-2; vimentin; okadaic acid; phosphorylation

In eucaryotic cells, protein phosphorylation/dephosphorylation is one of the most important regulatory processes, and almost all signaling pathways involve activation of protein kinases that are counteracted by protein phosphatases (PPases) [Cohen, 1992; Mumby and Walter, 1993]. In addition, several lines of evidence indicate that protein phosphorylation/dephosphorylation directly regulates the dynamics of intermediate filaments (IFs) and that PPases play a crucial role in these processes [Eriksson et al., 1992b; Inagaki et a., 1994]. For instance, downregulation of type 1 and type 2A PPase (PP1 and PP2A) by inhibitors such as okadaic acid (OA) [Yatsunami et al., 1991; Lee et al., 1992; Kasahara et al., 1993; Lai et al., 1993], calyculin A [Eriksson et al., 1992a; Hirano and Hartshorne, 1993], microcystin-LR [Toivola et al., 1997], and fostriecin [Roberge et al., 1994; Ho and Roberge, 1996] have been reported to disrupt the IF networks by inducing hyperphosphorylation of the respective IF proteins. These experiments have firmly established that PP1 and PP2A are major regulators of IF protein dephosphorylation; however, little is known about their kinase counterparts for IF phosphorylation in vivo.

IF proteins are excellent substrates for multiple kinases [Inagaki et al., 1996]. The first identified vimentin kinase is cAMP-regulated protein kinase (PKA), as demonstrated by treatment of cells with bromo-cAMP [Gard and Lazarides, 1982], injection of anti-PKA antibodies [Lamb et al., 1989], and direct in vitro studies in which phosphorylation-mediated assembly/disassembly of vimentin IFs has been analyzed

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[Inagaki et al., 1987; Ando et al., 1989]. Similar dynamics of IF assembly/disassembly regulated by phosphorylation induced by several other protein kinases, including Ca^{2+}/diacylglycerol-dependent protein kinase (PKC) [Inagaki et al., 1987], cdc2 kinase [Tsujimura et al., 1994], $p37^{mos}$ kinase [Chou et al., 1996], Ca^{2+}/calmodulin-depdendent protein kinase II (CaMKII) [Ando et al., 1991], and PKN [Matsuzawa et al., 1997], have been reported. In addition, cGMP-dependent protein kinase (PKG) [Pryzwansky et al., 1995] and glycogen synthase kinase-3/protein kinase F_A [Huang et al., 1994] have been demonstrated to phosphorylate purified vimentin in vitro. Nonetheless, the physiological link of these kinases to vimentin phosphorylation and organization has not been completely elucidated.

Given the central importance of the equilibrium between kinases and phosphatases in cellular regulation, inhibition of PP1 and PP2A by OA will have important functional implications [Bialojan and Takai, 1988]. That is, inhibition of dephosphorylation of proteins that are substrates for multiple kinases would lead to the "apparent activation" of protein kinases. However, there may be "genuine activation" of certain protein kinases that are downregulated by dephosphorylation. For example, OA may act as a PKC activator [Zheng et al., 1991] and mimic the actions of PKA [Walker and Watson, 1992; Giasson et al., 1996]. Moreover, activities of PKA [Liauw and Steinberg, 1996], CaMKII [Zhu et al., 1996], and cdc2 kinase [Yamashita et al., 1990] have been shown to be downregulated by PP2A, an OA-sensitive PPase. Thus, activation of these protein kinases is reasonably expected in OA-treated cells. It is worth noting that all of these kinases have been suggested to regulate IF assembly/disassembly by protein phosphorylation in vitro; whether they exerted the same function in vivo on PPases inhibition is not known.

We previously demonstrated that vimentin is hyperphosphorylated in OA-treated 9L RBT cells [Lee et al., 1992]. In the present study with the same model, we attempt to identify the kinases that function in OA-induced disturbation and thus might be a candidate for in vivo IF kinase(s). In-gel renaturing assays and in vitro kinase assays using vimentin as the exogenous substrate clearly demonstrate the activation of certain protein kinases in OA-treated cells. Further studies using specific protein kinase inhibitors and comparative two-dimensional peptide mapping suggest possible involvement of a cdc2-like kinase(s) and a protein kinase(s) downstream of the p38 mitogen-activated protein kinase ($p38^{MAPK}$)-mediated pathway in this process. As in the previous study, we demonstrate that the MAPK-activated protein kinase-2 (MAPKAPK-2) is a genuine vimentin kinase and is activated with OA treatment. These studies, linking MAPKAPK-2 to OA-induced hyperphosphorylation of vimentin, should help to elucidate the physiological roles of MAPKAPK-2 and vimentin IFs.

MATERIALS AND METHODS

Materials

OA was purchased from Gibco Laboratories (Grand Island, NY). CaMKII and protein kinase inhibitors, including H7, H8, KT5823, KN62, olomoucine, and SB203580, were purchased from Calbiochem (San Diego, CA), and staurosporine was obtained from Sigma (St. Louis, MO). Modified trypsin, PKC, PKA, PKG, and cdc2 kinase were obtained from Promega (Madison, WI). The $p38^{MAPK}$, MAPKAPK-2, and anti-MAPKAPK-2 antibody were obtained from Upstate Biotech Inc. (Lake Placid, NY). The [γ-^{32}P]ATP (5,000 Ci/mmol) was obtained from Amersham (Buckinghamshire, England). [^{32}P]orthophosphate ($^{32}P_i$; specific activity of 8,500–9,120 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma. Vimentin was purified from bovine lens by a combination of ion exchange and chromatofocusing chromatography, as previously described [Perng et al., 1994]. Recombinant mouse heat-shock protein 25 (HSP25) was obtained according to the method of Gaestel et al. [1991].

Cell Culture

The 9L RBT cells, derived from rat gliosarcoma [Weizsaecker et al., 1981], were a gift from Dr. M.L. Rosenblum (University of California at San Francisco) and were maintained in Eagle's Minimum Essential Medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin. 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/cm^2. Exponentially growing cells at 80–90% confluency were used.

Drug Treatment, Protein Fractionation, Metabolic Labeling, and SDS-PAGE

All drug stocks, including OA and protein kinase inhibitors, were diluted to appropriate concentrations with culture medium before adding to the cells. The cells were treated at 37°C according to several protocols as specified in the following sections and figure captions. To study the effect of OA on protein phosphorylation, cells were prelabeled with 0.5 mCi/ml of $^{32}P_i$ for 1 h and treated with 400 nM OA in the presence of the isotope for different durations as indicated. Alternatively, cellular proteins were separated into detergent-soluble and -insoluble fractions by extraction with buffer A (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, and 1% nonident P-40) for 10 min on ice. Solubilized proteins were then collected, centrifuged, and aliquoted into equal amounts of 2× sodium dodecylsulfate (SDS) sample buffer before polyacrylamide gel electrophoresis (PAGE). The detergent-insoluble proteins, which remained attached to the substratum after the extraction step, were briefly washed in phosphate buffered saline (PBS) and solubilized in sample buffer for electrophoresis. For the studies of protein kinase inhibitors on protein phosphorylation in OA-treated cells, the respective inhibitors at specified concentrations (Table 1) were added along with $^{32}P_i$ to the cells that were then treated with OA for 2 h. After treatment, the cells were briefly washed and directly lysed in SDS sample buffer for gel electrophoresis. SDS-PAGE was performed according to the method of Laemmli [1970] with 10% or 12.5% w/v acrylamide for resolving gels and 4.75% acrylamide for stacking gels. After electrophoresis, the gels were processed for Coomasie Blue staining, followed by autoradiography when necessary [Lee et al., 1992]. Sample preparations and other related experimental procedures were carried out as previously described [Lee et al., 1992]. The intensity of bands of interest in autoradiographs was quantified by densitometric scanning (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting Analysis

After electrophoresis, the gels were electrotransferred onto a nitrocellulose membrane (Hybond-C extra, Amersham) in 50 mM Tris-borate, pH 8.3, and 1 mM EDTA. The membrane was incubated for 1 h with 3% nonfat milk 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 1:1,000 in TTBS containing 1% nonfat milk) for 6 h and then processed for detection by ECL chemiluscence (Amersham).

In Vitro Assays of Protein Kinase Activities in OA-Treated Cells

Cells grown in 25-cm^2 flasks were treated with 400 nM OA for up to 2 h. At 0.5-h intervals, the cells were washed twice with PBS and then lysed in 300 µl of buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM benzamidine, and 0.1% [w/v] β-mercaptoethanol) for 30 min on ice. The cell lysates were collected by centrifugation, and the protein content of each sample was adjusted to 1 mg/ml. The protein concentration of the cell lysate was determined by the Bradford [1976] method. For in-gel kinase assay, cell lysates were resolved by 10% SDS-polyacrylamide gels containing 0.3 mg/ml vimentin. After electrophoresis, the gels were denatured, renatured, and processed for autoradiography as described elsewhere [Huot et al., 1995]. Alternatively, 10 µg of sample were incubated with 10 µg vimentin, 100 µM [γ-^{32}P]ATP in 25 mM Tris-HCl, pH 7.0 containing 30 mM NaCl, 3 mM $MgCl_2$, 2.5 µM PKI (a specific kinase inhibitor of PKA), and 10 µM H7 in a total volume of 20 µl. The reaction was allowed to proceed at 37°C for 30 min and then terminated by adding 5 µl 5× sample buffer. The samples were then subjected to SDS-PAGE and processed for autoradiography.

Assay for MAPKAPK-2 Activity

MAPKAPK-2 activity was measured by using vimentin or recombinant mouse HSP25 as substrates. After treatment, cells were washed twice with PBS and then lysed in 300 µl of buffer B for 30 min on ice. The cell lysates were collected by centrifugation and then used. Five hundred micrograms of cell lysate were incubated with 6 µg of anti-MAPKAPK-2 antibody for 2 h at 4°C. Immune complexes were precipitated with Protein G-Sepharose (Pharmacia Biotech Inc., Gaithersburg, MD) and then washed five times

with assay buffer (20 mM MOPS, pH 7.2, containing 2.5 mM PKI, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 15 mM $MgCl_2$). For the subsequent kinase assay, 10 μg of vimentin or HSP25 and 100 μM [γ-^{32}P]ATP in assay buffer were incubated in a total volume of 100 μl, and the reaction was allowed to proceed at 30°C for 30 min and was terminated with 25 μl 5× sample buffer. Phosphorylation of substrates was then determined after 12.5% SDS-PAGE, followed by autoradiograph and densitometric analyses.

In Vitro Phosphorylation of Vimentin by Specific Protein Kinases

For in vitro phosphorylation by PKC, PKA, PKG, CaMKII, and cdc2 kinase, 0.3 mg/ml of purified vimentin was incubated with 100 μM [γ-^{32}P]ATP in 25 mM Tris-HCl, pH 7.0, containing 30 mM NaCl, 3 mM $MgCl_2$, and other supplements as follows [Ando et al., 1989, 1991; Tsujimura et al., 1994]: PKC: 2 μg/ml kinase, 0.8 mM $CaCl_2$, and 50 μg/ml phosphatidylserine; PKA: 0.1 μg/ml kinase; PKG: 2 μg/ml kinase, 4 mM cGMP; CaMKII: 0.5 μg/ml kinase, 30 μM $CaCl_2$, and 20 μg/ml calmodulin; cdc2 kinase: 2 μg/ml kinase. For p38^{MAPK} or MAPKAPK-2, 0.2 U kinase and 0.3 mg/ml of purified vimentin were incubated with 100 μM [γ-^{32}P]ATP in assay buffer specified in the previous section. The reactions were allowed to proceed for 30 min at 30°C and was terminated by the addition of 5× sample buffer. After electrophoresis, the phosphovimentin from each sample was processed for phosphamino acid analysis and peptide mapping as described below.

Phosphoamino Acid Analysis and Two-Dimensional Thin Layer Electrophoresis (2D-TLE) Peptide Mapping of $^{32}P_i$-Labeled Vimentin

$^{32}P_i$-labeled cell lysates or phosphovimentins obtained as described in the previous section were resolved by SDS-PAGE. After being electrotransferred onto polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, MA), the protein bands containing the labeled vimentin were excised from the membranes and processed for phosphoamino acid analysis or two-dimensional tryptic peptide mapping, as described elsewhere [Boyle et al., 1991]. After hydrolysis in 5.7 N HCl at 110°C for 1 h, the hydrolysates were dried and mixed with 5 μl of 0.1 mg/ml phosphoamino acid standard mixtures containing phosphoserine, phosphothreonine, and phosphotyrosine in pyridine acetate solution (pyridine:acetic acid:water = 1:10:189, v/v) at pH 3.5. The samples were spotted on 20- × 20-cm cellulose-coated plates and subjected to thin-layer high-voltage electrophoresis in the same buffer at 1,000 V for 1 h. Phosphoamino acid standards were stained with ninhydrin, and the ^{32}P-labeled phosphoamino acids were located by autoradiography. For peptide mapping, the protein bands were incubated with modified trypsin in 1:100 (w/w) at 37°C for 24 h. The samples were then dried in vacuo, solubilized in pH 1.9 buffer (formic acid: acetic acid:water = 50:150:1,800, v/v), and then spotted onto cellulose-coated plates. The first dimension was performed in pH 1.9 buffer at 1,000 V for 50 min, and the plate was further chromatographed in buffer consisted of n-butanol:pyridine:acetic acid:water (785:607:122:486, v/v) in the second dimension at room temperature. Radioactive spots were visualized with Phosphorimage (Molecular Dynamics) and processed for presentation with Adobe Photoshop.

RESULTS

OA Induces Hyperphosphorylation of Vimentin and Activates Cytosolic Protein Kinase(s) in 9L RBT Cells

Phosphorylation of intracellular proteins in OA-treated 9L RBT cells was determined by metabolic labeling. In untreated samples, the incorporation of $^{32}P_i$ into different proteins increased gradually as the labeling time was extended. Treatment of 400 nM OA for 1 h had little effect on protein phosphorylation in general. However, the phosphorylation level of vimentin was greatly enhanced after 2 h of treatment, and there was no further increase when the treatment time was extended to 3 h (Fig. 1A). Regarding reorganization as related to phosphorylation, the distribution of vimentin was also examined by detergent extraction. The data showed that as the phosphorylation level of vimentin increased, vimentin molecules translocated toward soluble fraction as demonstrated by immunoblotting (Fig. 1B). As a first step to characterize which protein kinase is activated and is thus responsible for vimentin hyperphosphorylation under OA treatment, cellular extracts were assayed for endogenous kinase activity by using purified vimentin from bovine lens. In-gel kinase renaturation assay

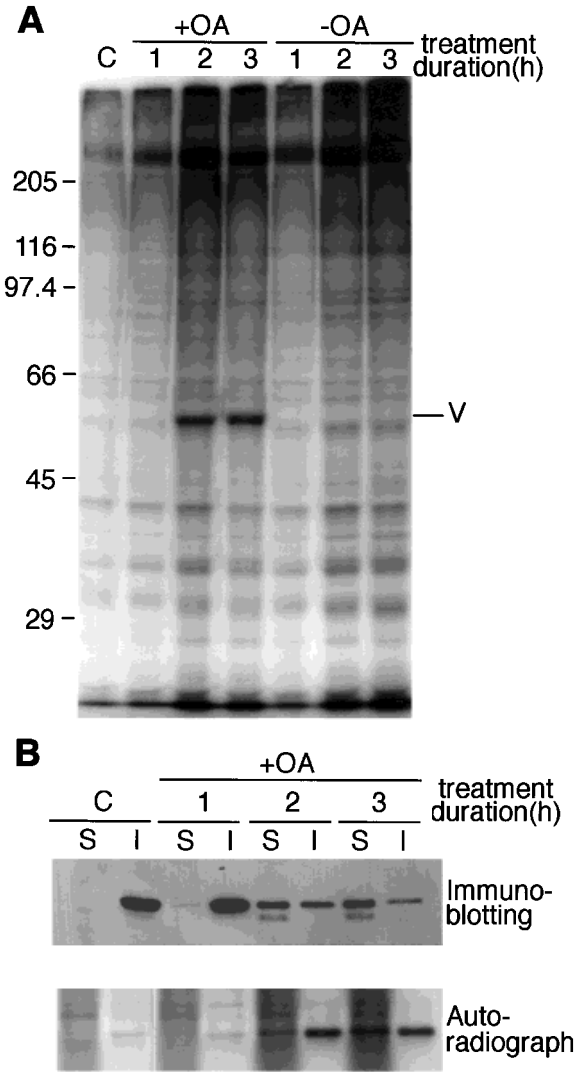

Fig. 1. Time-dependent phosphorylation (**A**) and translocation (**B**) of vimentin in okadaic acid (OA)-treated 9L RBT cells. A: Cells were prelabeled with [^{32}P]orthophosphate (0.5 mCi/ml) for 1 h, followed by treatment with 400 nM OA for up to 3 h in the presence of the isotope. At 1-h intervals, the cells were lysed with sodium dodecylsulfate (SDS) sample buffer, and the cell lysates were resolved by SDS–polyacrylamide gel electrophoresis (PAGE). After gel electrophoresis, the gels were processed for autoradiography. B: Alternatively, the cells were extracted with detergent and fractionated in detergent-soluble (S) and detergent-insoluble (I) fractions as described in Materials and Methods. The fractions were then resolved by SDS-PAGE followed by immunoblotting against vimentin (B, top) and autoradiography (B, bottom). Only the region containing vimentin was shown. **Lane C:** Untreated controls. Molecular weight standards are shown at the left. V, vimentin band.

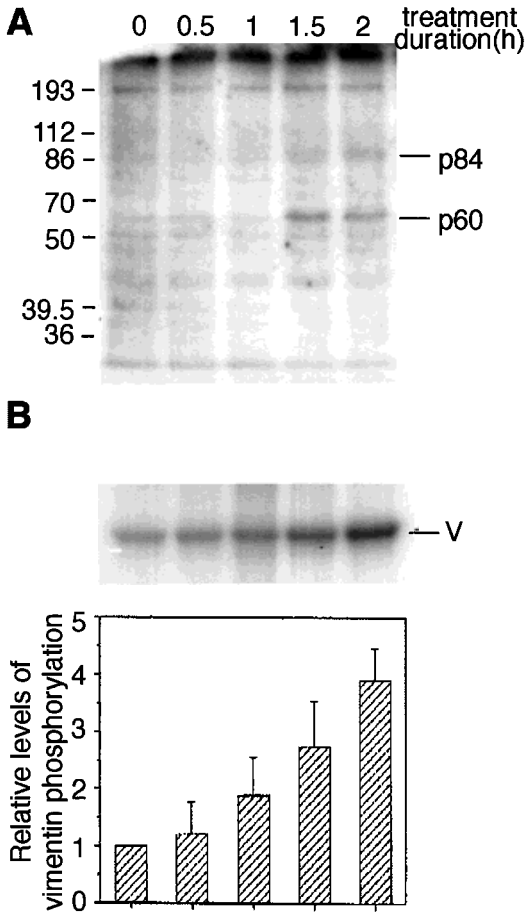

Fig. 2. Time-dependent activation of vimentin kinases in okadaic acid (OA)-treated 9L cells. **A:** Cells were treated with 400 nM OA for up to 2 h. At 0.5-h intervals, the cells were lysed and the cytosolic fractions were obtained. The samples (30 μg each) were resolved in 10% sodium dodecylsulfate (SDS)-polyacrylamide gels containing 0.3 mg/ml purified vimentin. After electrophoresis, the gels were denatured in 9 M urea, renatured, and then incubated with [γ-^{32}P]ATP to visualize protein bands that exhibit kinase activity by autoradiography. Autoradiographs with molecular weight standards indicated at the left. **B:** Alternatively, 10 μg of each sample were incubated with purified vimentin in the presence of [γ-^{32}P]ATP and $MgCl_2$ at 30°C for 30 min. The samples were resolved by SDS–polyacrylamide gel electrophoresis, and the resulting gels were processed for autoradiography. Subsequently, the relative phosphorylation levels of vimentin were quantified by densitometric scanning. Data represent the means ± SD of three independent experiments.

was performed, and the results showed two polypeptides with apparent molecular weights of 60 kDa and 84 kDa, with sharply increased phosphotransferase activity toward vimentin (Fig. 2A). Subsequently, by in vitro kinase assay using vimentin as an exogenous substrate, the kinetics of kinase activity toward vimentin showed a trend similar to that of phosphorylation of vimentin in cells: a continuous increase from 0.5 to 2 h of treatment (Fig. 2B). These results clearly demonstrate that certain protein kinases were indeed activated with treatment of cells with OA.

Kinase Inhibitors Olomoucine and SB203580 Revert OA-Induced Vimentin Hyperphosphorylation

The abilities of different kinase inhibitors (Table 1) to antagonize the OA effect in 9L RBT cells were subsequently exploited to evaluate the involvement of specific kinases. Initial studies were conducted to assure the inhibitory effects of the kinase inhibitors by in vitro kinase assay using specific peptide substrates. The kinase inhibitors could be introduced at a concentration 1–10 times higher than their K_i toward target enzymes without prominent cytotoxicity (data not shown). After pretreatment of cells with different kinase inhibitors for 1 h and subsequent treatment with OA for 2 h in the presence of kinase inhibitors, the cells were processed for detection of protein phosphorylation. As shown in Figure 3A, staurosporine at 0.1 μM could completely abolish the phosphorylation of almost all proteins and the hyperphosphorylation of vimentin in OA-treated cells; the data further substantiated that there was protein kinase activated in this process. However, H7, a PKC inhibitor, and H8, a PKA inhibitor, had much less effect on the inhibition of OA-induced hyperphosphorylation of vimentin, which decreased to 87.4% and 90.3%, respectively. Furthermore, the involvement of CaMKII was also evaluated by adding the inhibitor KN62. The results indicate that the inhibitor had no influence on the process. Interestingly, olomoucine, a specific cdc2 kinase inhibitor, and SB203580, which specifically inhibits $p38^{MAPK}$, appeared to reduce significantly the phosphorylation level of vimentin (73.3% and 54.4%, respectively) on OA treatment (Fig. 3B). Taken together, these results, wherein no specific kinase inhibitors other than staurosporine exerted complete inhibition of the OA-induced protein phosphorylation, can be ascribed to an unidentified staurosporine-sensitive kinase or to a concerted effect of a number of kinases that confer the hyperphosphorylation of vimentin with OA treatment. Therefore, the introduction of a single highly selective kinase inhibitor fails to exert prominent effects because other kinases would compensate for such inhibition. However, the results indicated that cdc2 kinase and $p38^{MAPK}$ are, at least in part, involved in OA-induced vimentin hyperphosphorylation under the experimental condition.

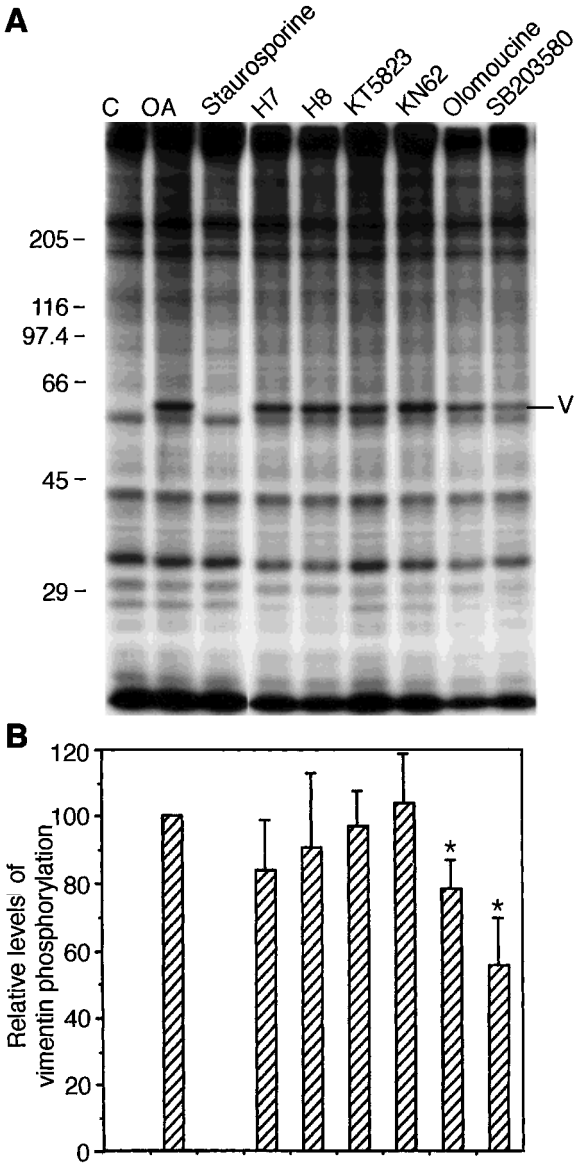

Fig. 3. Effect of specific protein kinase inhibitors on okadaic acid (OA)-induced vimentin phosphorylation. **A:** Cells were prelabeled with [^{32}P]orthophosphate (0.5 mCi/ml) in the presence of a protein kinase inhibitor, as indicated, for 1 h. The cells were then treated with 400 nM OA in the presence of the isotope and the inhibitors for another 2 h. After treatment, the cells were lysed with sodium dodecylsulfate (SDS) sample buffer and the cell lysates were resolved by SDS–polyacrylamide gel electrophoresis followed by autoradiography. **B:** Subsequently, the relative phosphorylation level of vimentin in each lane was quantified by densitometric scanning. Data represent means ± SD from three independent experiments and compared with those from OA-treated cells. *$P < 0.001$.

MAPKAPK-2 Phosphorylates Vimentin In Vitro and Is Activated by OA Treatment

To address further the issue as to whether $p38^{MAPK}$-mediated pathway contributes to

TABLE I. Concentrations Used and Specificities of the Employed Protein Kinase Inhibitors

Inhibitors	Concentration (μM)	K_i (μM) toward target protein kinases[a]						References
		PKC	PKA	PKG	CaMKII	cdc2 kinase	$p38^{MAPK}$	
Staurosporine	0.1	0.0007	0.007	0.0085	—	<0.01	—	Ingaki et al. [1986] Schachtele et al. [1988] Jarvis et al. [1994]
H7	50	6.0	3.0	5.8	—	—	—	Hagiwara et al. [1987]
H8	50	15	1.2	0.48	—	—	—	Kase et al. [1987]
KT5823	20	—	—	0.0024	—	—	—	Tokumitsu et al. [1990]
KN62	20	>100	>100	—	6.0	—	—	Ido et al. [1991]
Olomoucine	50	>2000	>2000	>1000	—	7.0[b]	—	Vesely et al. [1994]
SB203580	50	>100	>100	—	—	—	0.6[b]	Saklatvala et al. [1996]

[a]PKC, Ca^{2+}/diacyglycerol-dependent protein kinase (PK); PKA, cAMP-dependent PK; PKG, cGMP-dependent PK; CaMKII, Ca^{2+}/calmodulin-dependent PK II; $p38^{MAPK}$, mitogen-activated PK p38. Chemical names of the inhibitors are as follows: H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H8, N-[2(methylamino)ethyl]-5-isoquinoline sulfonamide; KT5823, (N-methyl-(8R,(S,11S)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H, 8H, 11H-2, 7b, 11a-triazadibenza [a, g]cycloocta [c,d,e]-trinden-1-one); KN62, 1-[N,O-bis-1,5-isoquinoline sulfonyl)-N-methyl-L-tyrosyl-4-phenylpiperazine; olomoucine, [2-(2-hydroxylethylamino)-6-benzylamino-9-methylpurine]; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5(4-pyridyl)imidazole.

[b]IC_{50} (μM) of inhibitor tested against purified protein kinases.

vimentin hyperphosphorylation under OA treatment, the activities of $p38^{MAPK}$ and MAPKAPK-2, two kinases in this signaling pathway, toward vimentin were evaluated individually by in vitro kinase reaction (Fig. 4). In sharp contrast to $p38^{MAPK}$, which cannot phosphorylate vimentin at all, MAPKAPK-2 phosphorylates vimentin extensively. The activity of MAPKAPK-2 under OA treatment was investigated further by kinase assay with immunoprecipitated MAPKAPK-2 using vimentin or HSP25 as the exogenous substrate. The phosphorylation of vimentin increased significantly by immunocomplexes obtained from OA-treated cells; moreover, SB203580 could partly inhibit the vimentin phosphorylation (Fig. 5), which is similar to the results obtained from intact cells (Fig. 3). A similar trend was demonstrated when using HSP25 as the substrate (Fig. 5). These results further confirm activation of MAPKAPK-2 with OA treatment.

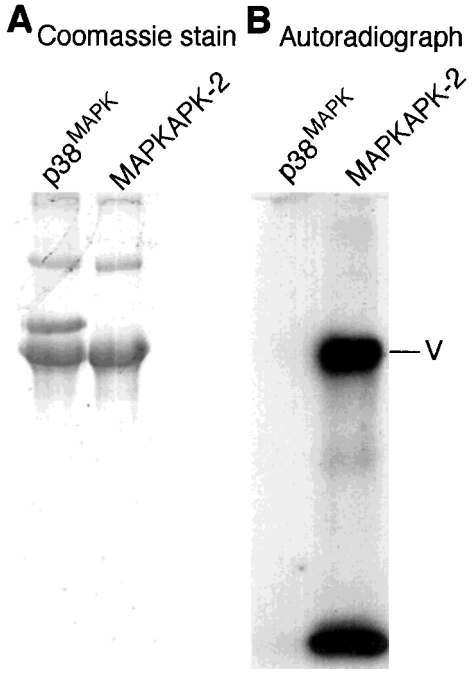

Fig. 4. In vitro phosphorylation of vimentin by mitogen-activated protein kinase (MAPK) p38 and MAPK-associated protein kinase-2. Purified vimentin (V) was incubated with 0.2 U $p38^{MAPK}$ and MAPKAPK-2 in the presence of [γ-^{32}P]ATP and $MgCl_2$, respectively, at 30°C for 30 min. At the end of the reaction, the samples were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis, and the gels were processed for Coomassie Brilliant Blue staining (**A**) and autoradiography (**B**).

Comparative 2D Peptide Mappings Indicate MAPKAPK-2 Phosphorylates Vimentin In Vitro in OA-Treated Cells

The comparative 2D tryptic peptide mapping was employed to examine the involvement of kinases in vimentin hyperphosphorylation with OA treatment. As a first step, the phosphovimentin obtained from OA-treated cells was charaterized by phosphoamino acid analysis, which showed that only serine, not threonine or tyrosine, was phosphorylated (Fig. 6A). The tryptic phosphopeptides of vimentin on 2D-TLE plates, by using electrophoresis in the first dimension and chromatography in the second, were examined with two different comparative strategies using either an equal amount of proteins or total radioactivity to probe the presence of new phosphorylation sites. When equal amounts of vimentin from untreated and treated cells were used, a general increase in five major phosphopeptides was detected (a–e in Fig. 6B, upper panel). To check whether OA induces the appearance of new phosphopeptides, equal amounts of radioactivity from control and OA-treated cells were analyzed by 2D-TLE. Analysis of phosphopeptides with almost equal radioactivity required 30 times more protein from control cells. The results show that, although no obvious additional phosphorylated sites appeared with OA treatment, the phosphate moieties on two phosphopeptides (a and d in Fig. 6B, lower panel) increased rather significantly as compared with other phosphopeptides. Therefore, these phosphopeptides probably serve as the target for OA-activated kinases, in which activity was low in interphase cells due to the counteraction by OA-sensitive PPase(s) on the substrate(s) or on the kinase itself. The tryptic maps of vimentin from OA-treated cells were compared further with those obtained from vimentin phosphorylated in vitro by the following protein kinases, including PKC, PKA, PKG, CaMKII, cdc2 kinase, and the newly identified vimentin kinase, MAPKAPK-2 (Fig. 6C). As shown in the 2D-TLE plates, the phosphopeptide e appeared in all preparations. Notably and consistent with the studies using kinase inhibitors, the peptide maps of vimentin from OA-treated cells, which contained five dispersed spots, are very different from those phosphorylated in vitro by PKC, PKA, PKG, and CaMKII in which a group of phosphopeptides clustered on the right lower part of the plates is detected (compare Fig. 6B with Fig. 6C). Moreover, the patterns from cdc2 kinase-phosphorylated vimentin showed a certain degree of simi-

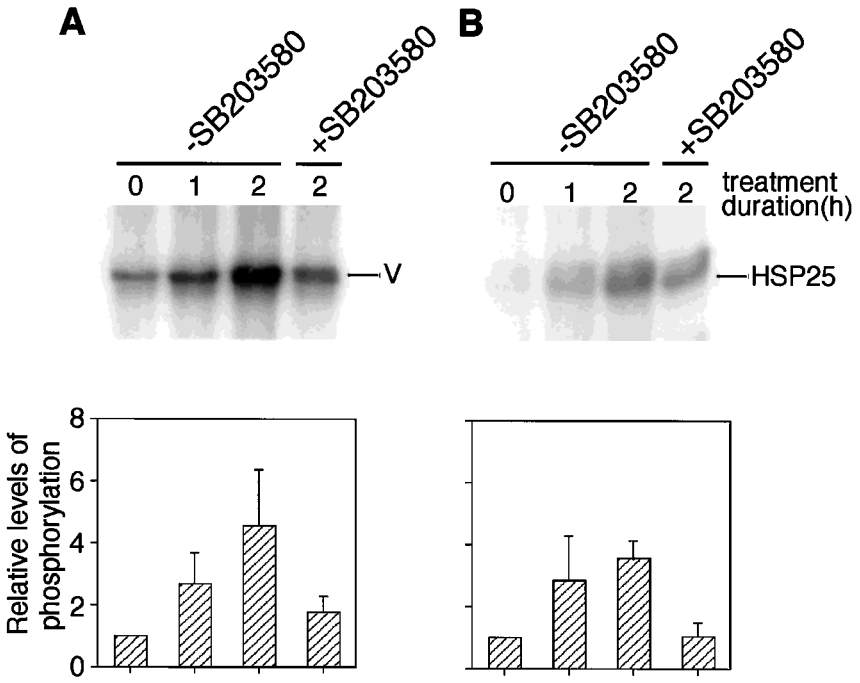

Fig. 5. Activation of mitogen-activated protein kinase (MAPK)-associated protein kinase-2 in okadaic acid (OA)-treated 9L cells. Cells were treated with 400 nM OA with or without SB203580, the specific inhibitor for $p38^{MAPK}$, for up to 2 h. After 1-h intervals, the cells were lysed and the cytosolic fractions were obtained. MAPKAPK-2 in these samples was immunoprecipitated by antibodies and then protein G-Sepharose. The immunocomplexes were processed further for kinase assay by using vimentin (**A**) and heat-shock protein 25 (HSP25; **B**) as substrates. The reactions were incubated in the presence of [γ-^{32}P]ATP and $MgCl_2$ at 30°C for 30 min. The level of phosphorylation was quantified by densitometric scanning. Data represent the means ± SD of three independent experiments. Similar results were obtained in three independent experiments.

larity to that of OA-phosphorylated vimentin as indicated by the similar positioning of several phosphopeptides (peptides c–e). Strikingly, with vimentin phosphorylated by MAPKAPK-2, some phosphopeptides, the peptides b–e in the map from OA-induced phosphorylated vimentin, emerged with other minor phosphopeptides. This result, indicating that MAPKAPK-2 could phosphorylate vimentin at sites at which phosphorylation also occurred under OA treatment, further support the notion that MAPKAPK-2 is directly involved in the OA-induced hyperphosphorylation of vimentin.

DISCUSSION

Concerning the physiological significance of vimentin phosphorylation, the functional in vivo vimentin kinases should be identified. We report that, by using OA to inhibit PPases and thus strengthen the kinase function, the $p38^{MAPK}$-mediated pathways are linked to the OA-induced hyperphosphorylation of vimentin. Moreover, vimentin is an excellent substrate for MAPKAPK-2, the downstream effector of $p38^{MAPK}$.

Based on the in-gel and in vitro assays of protein kinase activities in the cellular extracts from the OA-treated cells, it is clear that certain protein kinases are activated time dependently during OA treatment, although the inhibition of PPases still contribute to OA-induced vimentin hyperphosphorylation. A number of protein kinases, including PKC, PKA, PKG, CaMKII, and cdc2 kinase, have been suggested to regulate IF assembly/disassembly by protein phosphorylation in vitro and in vivo; whether they exerted the same function under OA treatment has been elucidated by inhibitor studies and comparative peptide mapping. By using kinase inhibitors antagonizing PKC, PKA, PKG, and CaMKII, these inhibitors seemed to exert no obvious influences on the process of OA-induced hyperphosphorylation of vimentin despite the fact that this protein may serve as an excellent substrate for these kinases. However, olomoucine, a specific cdc2 kinase inhibitor, partly but significantly reverted the vimentin hyperphosphorylation induced by OA. Two-dimensional peptide mapping also leads to similar conclusions, as indicated by the strong re-

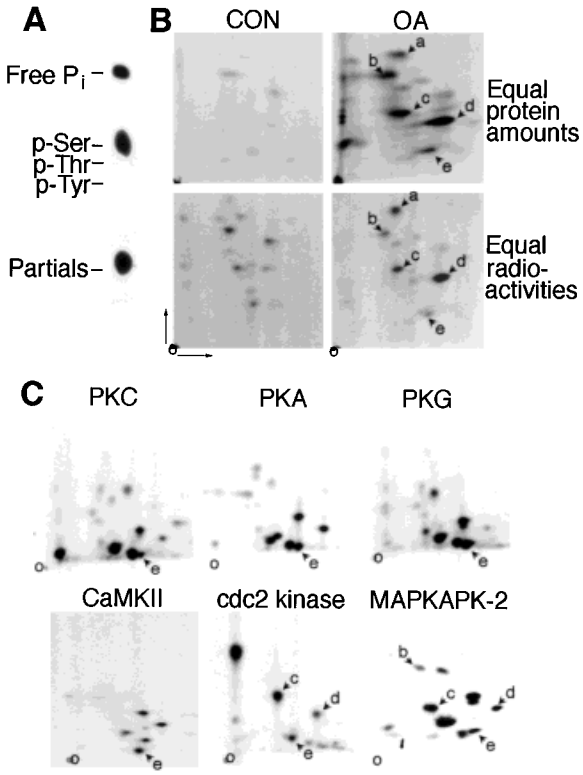

Fig. 6. Phosphoamino acid analysis (**A**) and phosphopeptide mapping of radiolabeled phosphovimentin isolated from okadaic acid (OA)-treated cells (**B**) or phosphorylated by protein kinases in vitro (**C**). A: Cells were prelabeled with [^{32}P]orthophosphate (P_i; 0.5 mCi/ml) for 1 h followed by treatment with OA for 2 h in the presence of the isotope. After treatment, the cells were lysed, and the cell lysates were resolved by 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis. The proteins in the gels were electrotransferred onto polyvinylidene difluoride membranes, and the radiolabeled phosphovimentin band was identified and excised, processed for acid hydrolysis, and then subjected to thin-layer chromatography followed by autoradiography. The positions of unlabeled phosphoamino acid standards are also indicated. B: For two-dimensional thin layer electrophoresis (2D-TLE) tryptic peptide mapping, radiolabeled phosphovimentin samples were obtained from control and OA-treated cells. The samples were then digested with trypsin and then processed for 2D-TLE peptide mapping with either equal protein amounts or equal radioactivities. C: Alternatively, purified vimentin was phosphorylated by Ca^{2+}/diacyglycerol-dependent protein kinase (PKC), cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), cdc2 kinase, and mitogen-actived protein kinase-activated protein kinase-2 (MAPKAPK-2) in the presence of [γ-^{32}P]ATP and then subjected to tryptic digestion. The radiolabeled phosphopeptides were subsequently resolved by 2D-TLE followed by autoradiography. The origins of these maps are marked with open circles. Directions of electrophoresis at pH 1.9 (horizontal arrow) and ascending chromatography (vertical arrow) are indicated. Representative graphs from three independent experiments are shown.

semblance between maps from cdc2 kinase-phosphorylated vimentin, but not PKC-, PKA-, PKG-, or CaMKII-phosphorylated vimentin, and OA-induced phosphorylated vimentin. Consistent with our results, Kasahara et al. [1993] demonstrated that only staurosporine, but not H7, H8, or W7, could abolish OA-induced hyperphosphorylation of cytokeratins in BALB/MK-2 keratinocytes, and they suggested the involvement of cdc2 kinase or another kinase in this process. However, PP2A has been show to regulate cdc2 kinase activity in a negative manner [Yamashita et al., 1990], therefore, inhibition of PP2A by the administration of OA is expected to result in the activation of cdc2 kinase.

The inclusion of SB203580, a specific $p38^{MAPK}$ inhibitor, and OA treatment results in a significant suppression of vimentin phosphorylation. Mitogen-activated protein kinase p38, one of three MAPK cascades that also include p42/$p44^{MAPK}$ and c-Jun kinase subfamilies, is also an important mediator of signal transduction from cell surface to nucleus. Mammalian $p38^{MAPK}$ was originally identified in murine pre-B cells transfected with the lipopolysaccharide (LPS) complex receptor CD14 and in murine macrophages, where it is activated in the response to LPS [Han et al., 1994]. The kinase also appears to be involved in the production of cytokines by stimulated monocytes and to mediate the aggregation of platelets in response to collagen [Saklatvala et al., 1996]. In parallel, $p38^{MAPK}$ was also identified as a reactivating kinase that activated MAPKAPK-2, which in turn phosphorylated the small HSP (i.e., HSP25 in murine cells and HSP27 in human cells); the central response occurred in inflammatory cytokines and from cellular stresses such as heat shock, osmotic stress, or ultraviolet light [Saklatvala et al., 1996].

Activation of MAPKAPK-2 has been directly shown in stress treatment including heat shock, osmotic stress, arsenite [Rouse et al., 1994], tumor necrosis factor-α (TNF-α) [Engel et al., 1995], interleukin-1 [Guy et al., 1995], anisomycin [Cano et al., 1996], ischemic preconditioning [Maulik et al., 1996], and phorbol ester [Zu et al., 1996]. However, to our knowledge, only HSP25/27 has been identified as a physiological substrate of MAPKAPK-2, although the kinase could phosphorylate glycogen synthase [Stokoe et al., 1992] and tyrosine hydroxylase [Sutherland et al., 1993] in vitro. Further identification of protein substrates should help in the under-

standing of MAPKAPK-2 cellular function. Strikingly, by in vitro kinase reaction, vimentin was proven to be an excellent substrate for MAPKAPK-2. Moreover, tryptic maps had a stronger resemblance with those from OA-phosphorylated vimentin, and phosphoamino acid analysis also indicated MAPKAPK-2 phosphorylates vimentin only at serine (data not shown), which is consistent with the results in OA-treated cells. These data and the fact that SB203580 antagonizes OA-induced hyperphosphorylation of vimentin indicate that the $p38^{MAPK}$ pathway, especially MAPKAPK-2, may have a part in maintaining the integrity of IFs in cells. Furthermore, we have shown MAPKAPK-2 may be activated with OA treatment. Because activation of MAPKAPK-2 by $p38^{MAPK}$ requires phosphorylation and the kinase can be inactivated in vitro by PP2A [Stokoe et al., 1992], it is plausible that PPase inhibitor would directly affect kinase activity. Moreover, Guy et al. [1995] suggested that the activation of MAPKAPK-2 in TNF or the interleukin-1 signaling pathway involved the phosphorylation and thus inactivation of PP2A by a tyrosine kinase, followed by the activation of $p38^{MAPK}$ and MAPKAPK-2. OA-induced activation of MAPKAPK-2 may exploit the same mechanism, another mechanism through another unknown mediator, or both.

The results obtained by comparative peptide maps and inhibitor studies clearly indicate that cdc2 kinase and MAPKAPK-2, but not PKC, PKA, PKG, CaMKII, are involved in OA-induced phosphorylation of vimentin. Moreover, MAPKAPK-2 is identified as a new vimentin kinase. Whether the phosphorylation of vimentin by MAPKAPK-2 affects the assembly/disassembly dynamics of vimentin IFs remains to be clarified. Further investigation of the link between MAPKAPK-2 and phosphorylation-coupled regulation of vimentin and of HSP25 is also warranted.

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