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Retaining of the Assembly Capability of Vimentin Phosphorylated by Mitogen-Activated Protein Kinase-Activated Protein Kinase-2

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Abstract Intermediate filament (IF) networks can be regulated by phosphorylation of unit proteins, such as vimentin, by specific kinases leading to reorganization of the IF filamentous structure. Recently, we identified mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP kinase-2) as a vimentin kinase (Cheng and Lai [1998] J. Cell. Biochem. 71:169–181). Herein we describe the results of further in vitro studies investigating the effects of MAPKAP kinase-2 phosphorylation on vimentin and the effects of the phosphorylation on the filamentous structure. We show that MAPKAP kinase-2 mainly phosphorylates vimentin at Ser-38, Ser-50, Ser-55, and Ser-82, residues all located in the head domain of the protein. Surprisingly, and in stark contrast to phosphorylation by most other kinases, phosphorylation of vimentin by MAPKAP kinase-2 has no discernable effect on its assembly. It suggested that structure disassembly is not the only obligated consequence of phosphorylated vimentin as regulated by other kinases. Finally, a mutational analysis of each of the phosphorylated serine residues in vimentin suggested that no single serine site was primarily responsible for structure maintenance, implying that the retention of filamentous structure may be the result of the coordinated action of several phosphorylated serine sites. This also shed new lights on the functional task(s) of vimentin that is intermediate filament proteins might provide a phosphate reservoir to accommodate the phosphate surge without any structural changes. J. Cell. Biochem. 89: 589–602, 2003.

Key words: vimentin; mitogen-activated protein kinase-activated protein kinase-2; intermediate filament; assembly; phosphorylation

Vimentin constitutes the main intermediate filament (IF) cytoskeletal organization in mesenchymal cells and many tumor cells [Bershadsky and Vasiliev, 1988]. The tripartite structure of IF proteins consists of a central protease-resistant rod domain with high α-helical content (80%) and two flanking head/tail domains [Stewart, 1993]. The rod domain is involved in coiled–coil interactions for antiparallel stacking of tetramers to form protofilaments [Herrmann et al., 1996]. Further assembly of IF proteins involve complex interactions of the rod and head domains [Herrmann et al., 1992; Hofmann and Herrmann, 1992].

Based on structural and biochemical studies in vitro, IFs are believed to be primarily responsible for the structural integrity of the cytoplasm and appear to be static relative to other cytoskeletal elements. However, IFs do have dynamic properties [Herrmann and Aebi, 1998]. IF molecules are rapidly transported along microtubule tracks as part of certain physiological activities, such as locomotion, spreading, and axon outgrowth [Chou et al., 2001]. Also, the structural organization of the IF network is regulated spatially and temporally by phosphorylation [Inagaki et al., 1996]. For example, IF structure changes dramatically during mitosis, when the entire IF network is converted into protofilament aggregates. This phenomenon is associated with increased phosphorylation of

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IFs [Chou et al., 1989]. Cdc2 kinase was the first kinase identified in IF phosphorylation in mitotic cells [Chou et al., 1990, 1991]. Tsujimura et al. [1994] found a strong correlation between the degree of disassembly of vimentin filaments and the amount of mitotically activated Cdc2 kinase during mitosis. As a second example, Rho kinase phosphorylates IFs specifically at the cleavage furrow during cytokinesis [Goto et al., 2000]. This cleavage-specific phosphorylation event appears to be essential for proper segregation of daughter cells [Yasui et al., 1998].

In addition to its involvement during development, phosphorylation and thus reorganization of vimentin also occurs in the cells undergoing stimulation by cAMP increasing agents [Lamb et al., 1989], the phorbol ester PMA [Huang et al., 1988], chemotactic factors [Wyatt et al., 1991], hormones [Spruill et al., 1983], anti-cancer drugs [Chu et al., 1998], the phosphatase inhibitor pervanadate [Feng et al., 1999], and other phosphatase inhibitors [Yatsunami et al., 1991; Eriksson et al., 1992; Lee et al., 1992; Hirano and Hartshorne, 1993; Ho and Roberge, 1996]. In vitro biochemical studies also show that IF proteins can be substrates for a wide range of kinases, including cAMP-dependent protein kinase A (PKA), protein kinase C (PKC) [Inagaki et al., 1987], Ca^{++}/calmodulin-dependent protein kinase II (CaMKII) [Tokui et al., 1990], Cdc2 kinase [Chou et al., 1990], autophosphorylation-dependent protein kinase [Huang et al., 1994], PKN [Matsuzawa et al., 1997], Rho-kinase [Goto et al., 1998], mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP kinase-2) [Cheng and Lai, 1998], and p21-activated kinase (PAK) [Ohtakara et al., 2000]. Although phosphorylation of IF is linked to structural changes in most instances, there are exceptions, such as $p37^{mos}$-phosphorylated vimentin where no changes in structure were observed [Chou et al., 1996]. Thus, the physiological function of IF phosphorylation requires further investigations.

MAPKAP kinase-2 is the downstream effector of the p38 mitogen-activated protein kinase (MAPK) pathway transmitting signals from the cell surface to the nucleus [Cano and Mahadevan, 1995]. MAPKAP kinase-2 was originally identified in skeletal muscle as an enzyme activated in vitro by the p42/p44 isoforms of MAPK [Stokoe et al., 1992]. Rouse et al. [1994] further identified a p42/44 homologue (RK also known as p38MAPK or RK/p38MAPK), as a genuine kinase and activator of MAPKAP kinase-2 in mammalian cells. This p38MAPK kinase cascade transmits signals for cytokines, growth factors, stress, and even apoptosis in cells. Upon phosphorylation by p38MAPK, MAPKAP kinase-2 translocated from the nucleus to the cytoplasm and was activated to phosphorylate its substrates [Ben-levy et al., 1998]. MAPKAP kinase-2 has a nuclear substrate, serum response factor (SRF) [Heidenreich et al., 1999], and cytosolic substrates, such as small heat shock proteins (HSP) [Landry et al., 1992]. However, the physiological significance of MAPKAP kinase-2 function is not clear as yet. Further characterization of substrates for MAPKAP kinase-2 would facilitate our understanding of this kinase and the entire p38MAPK signaling cascade.

Recently, we reported that MAPKAP kinase-2 is activated in 9L rat brain tumor (RBT) cells during protein phosphatase inhibitor treatment and that vimentin is a substrate for MAPKAP kinase-2 in vitro [Cheng and Lai, 1998]. In the present study, we investigated the characteristics of MAPKAP kinase-2-phosphorylated vimentin. We determined that MAPKAP kinase-2 phosphorylated vimentin at ser-38, ser-50, ser-55, ser-82 sites to a level of approximately 1.5 mol phosphate/mol protein. Electron microscopic studies further showed that vimentin, phosphorylated by MAPKAP kinase-2, in contrast to kinases, does not lose its capacity to organize into filaments.

MATERIALS AND METHODS

Materials

MAPKAP kinase-2 was purchased from Upstate Biotech, Inc. (Lake Placid, NY). The [γ-^{32}P]-ATP (5,000 Ci/mmol) was obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Lysyl endopeptidase was from Wako (Nagoya, Japan) and modified trypsin endopeptidase was from Promega (Madison, WI). The human vimentin cDNA construct, pET-vim, was kindly provided by Dr. Roy A. Quinlan (Dundee University, Dundee, Scotland). Oligonucleotides were synthesized at Quality, Inc. (Taipei, Taiwan). DNA endonucleases, ligases, and other DNA modifying enzymes were from New England Biolabs (Beverly, MA). Chemicals

for electrophoresis were from Bio-Rad Laboratories, Inc. (Richmond, CA). Site-specific antibodies recognizing respective phosphoserine-6, -33, -38, -50, -55, and -82 of vimentin were kindly provided by Dr. Roy A. Quinlan and Dr. Masaki Inagaki (Aichi Cancer Center Research Institute, Aichi, Japan). Other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Purification of Native Vimentin

Native vimentin was purified from bovine lenses through a procedure of urea extraction, ion exchange, and chromatofocusing chromatography as described [Perng et al., 1994]. The protein concentration was adjusted to 2 mg/ml and stored at −70°C in a buffer containing 8 M urea, 5 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10 mM β-mercaptoethanol, and 0.4 mM phenylmethylsulfonatefluoride until use.

Construction of Expression Vectors Encoding Wild-Type Vimentin and Point-Mutated Vimentin Variants

The full-length human vimentin cDNA was amplified from pET-Vim by polymerase chain reaction (PCR) with oligonucleotides: VmF (5′-CGGGAT*CCATGG*CCACCAGGTCC-3′) containing a *NcoI* site and VmR (5′-CCC*AAGCTT*-TTATTCAAGGTCATCGTGATGC-3′) containing a *HindIII* site. After digested with *NcoI* and *HindIII*, the wild-type vimentin cDNA fragment was subcloned into the *NcoI*/*HindIII* sites of pET23d (Novagen, Madison, WI) to generate the resultant plasmid pET23d-Vm for expression. Vimentin variants with the respective serine residue at 6, 33, 38, 50, 55, or 82 being point-mutated into alanine were also constructed. Construction of the VmS6A mutant and the VmS33A mutant were carried out with the Transformer™ mutagenesis system (Clontech, Palo Alto, CA) according to manufacturer's instruction. The respective oligonucleotides for construction of VmS6A and VmS33A mutants were VmS6A-F (5′-GCCACCAGGTCCGTG**GCC**TC-GTCCTCCTACCGC-3′), and VmS33A-F (5′-AGCTACGTGACTACG**GCC**ACCCGCACCTA-CAGC-3′), in which the changed nucleotides were bold and the alanines were underlined. Alternatively, the VmS38A, S50A, S55A, and S82A mutants were obtained by PCR mutagenesis [Higuchi et al., 1988] using pET-Vim as the template followed with subcloning into pET23d expression vector. The PCR mutagenesis includes three PCR reactions. The first PCR reaction was to generate the upstream fragments of the mutation sites using VmF as the forward primer and respective reverse primers as the followings to introduce the mutation: VmS38A-R (5′-GCGCGCTGCCCAG**GGC**GT-AGGTGCGGGTG-3′), VmS50A-R (5′-GACGA-GGCGTAGAG**GGC**GCGGCTGGTGCTGG-3′), VmS55A-R (5′-CATACACGCCGCCCGG**GGC**-CGAGGCGTAGAGG-3′), or VmS82A-R (5′-CA-GCGAGAAGTCCAC**CGC**GTCCTGCAGGAGC-CG-3′), where the codons corresponding to alanine amino acid positioned at 38, 50, 55, 82 were underlined and the changed nucleotides were bold. The second step of PCR was to generate the downstream fragments of the mutation sites with VmR as the reverse primer and respective forward primer: VmS38A-F (5′-CAC-CCGCACCTAC**GCC**CTGGGCAGCGCGC-3′), VmS50A-F (5′-CCAGCACCAGCCGC**GCC**CT-CTACGCCTCGTC-3′), VmS55A-F (5′-CCTC-TACGCCTCG**GCC**CCGGGCGGCGTGTATG-3′), or VmS82A-F (5′-CGGCTCCTGCAGGAC-**GCG**GTGGACTTCTCGCTG-3′). The upstream fragments from the first PCR and the corresponding downstream fragments from the second PCR reactions were then mixed, denatured, and then annealed through the terminal overlapping regions. The mixture was then served as templates for the final combinational PCR with the outer primers, VmF and VmR. After digested with *NcoI*/*HindIII*, the individual vimentin variant was cloned into pET23d expression vector. The sequences of all constructs were confirmed by automatic sequencing (Mission Biotech, Taipei, Taiwan).

Enrichment of Recombinant Wild-Type Vimentin and its Variants

Recombinant wild-type vimentin and its variants were expressed and purified as following. After transformation of respective vimentin constructs into *E. coli* strain BL21(DE3), a single colony bearing respective plasmids was inoculated and grown in 10 ml of LB medium with 100 μg/ml ampicillin at 37°C for overnight. The culture was then transferred into 1 L LB broth with 100 μg/ml ampicillin in a 37°C shaker until the O.D. reached around 0.6. Recombinant protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h, the cells were harvested by centrifugation at 3,800 *g* for 30 min at 4°C and then lysed in buffer A (50 mM Tris-HCl, pH 7.8,

200 mM NaCl, 10 mM EDTA, 5% glycerol, 10 μg/ml lysozyme, 1% Triton X-100, and 0.2% v/v protease inhibitor cocktail (Calbiochem, San Diego, CA) for 1 h on ice followed by sonication. The samples were then centrifuged at 3,800 *g* for 15 min and the supernatants were discarded. The insoluble fractions were washed once and further dissolved in buffer B (8 M urea, 5 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10 mM β-mercaptoethanol, and 0.4 mM phenylmethylsulfonatefluoride) and then the solubilized fractions were obtained by centrifugation at 12,000 *g* for 30 min to remove any insolubles. The protein concentration was adjusted to 2 mg/ml and stored at −70°C until use.

Phosphorylation of Vimentin by MAPKAP Kinase-2 or by PKA

Purified vimentin (0.3 mg/ml), either obtained from bovine lenses as native proteins or obtained from bacterial expression, was phosphorylated by 0.2 U of MAPKAP kinase-2 to a stoichiometry of 1.5 mol phosphate/mol vimentin in a final volume of 50 μl solutions containing 20 mM MOPS, pH 7.2, 2.5 μM PKI (inhibitor of protein kinase A), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 100 μM ATP at 37°C for 4 h. Phosphorylation of vimentin by PKA was performed as described by Inagaki et al. [1987]. Purified vimentin (0.3 mg/ml), either native or recombinant, was incubated with 90 U of PKA, 0.1 mM ATP, 0.3 mM $MgCl_2$, 30 mM NaCl, and 25 mM Tris-HCl, pH 7.0, in a final volume of 50 μl at 30°C for 2 h.

Lysyl Endopeptidase Digestion of Purified Bovine Vimentin

Lysyl endopeptidase was used to investigate if phosphorylation of vimentin occurred in the head domain of vimentin since the first accessible site for lysyl endopeptidase in vimentin located in amino acid 97, which is downstream of the head domain. Purified bovine vimentin was phosphorylated by 0.2 U of MAPKAP kinase-2 to a stoichiometry of 1.5 mol phosphate/mol vimentin in a final volume of 50 μl solutions containing 20 mM MOPS, pH 7.2, 2.5 μM PKI (inhibitor of protein kinase A), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP at 37°C for 4 h. MAPKAP kinase-2-phosphorylated vimentin were precipitated with trichloroacetic acid and then incubated with or without 25 μg/ml lysyl endopeptidase (Wako) in 50 mM NH_4HCO_3, pH 7.8 for 3 h at 30°C and the samples were then separated by 17% SDS–PAGE. Radiolabeled polypeptide bands were visualized by autoradiography.

SDS–PAGE and Immunoblotting Analysis

SDS–PAGE was performed according to the method of Laemmli [1970] with 10 or 17% w/v acrylamide for resolving gels and 4.75% acrylamide for stacking gels. After electrophoresis, the gels were processed for Coomassie Brilliant Blue staining, followed by autoradiography when necessary [Cheng and Lai, 1998]. For immunoblotting, the gels were electrotransferred onto a nitrocellulose membrane (Hybond-C extra, Amersham Pharmacia Biotech, Inc.) in 50 mM Tris-borate, pH 8.3, and 1 mM EDTA. The membrane was incubated for 2 h with 3% nonfat milk in TTBS (10 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 anti-vimentin antibodies or certain site-specific antibodies against phosphovimentin in 1:1000 for 18 h and then processed for detection by alkaline phosphatase colorimetric developing techniques.

Identification of the Phosphorylation Sites of MAPKAP Kinase-2-Phosphorylated Vimentin by Manual Edman Degradation

Native vimentin was phosphorylated by MAPKAP kinase-2 in the presence of [γ-^{32}P] ATP as described. After precipitated with trichloroacetic acid, vimentin was oxidized with formic acid, and proteolytically digested with lysyl endopeptidase followed by a reverse-phase Pro column (Amersham Pharmacia Biotech, Inc.) with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid to obtain the ^{32}P-labeled amino-terminal head domain of vimentin. This phosphopeptides were then incubated with modified trypsin (1:100, v/v) in 100 μl of 50 mM NH_4HCO_3, pH 7.8 at 37°C for 5 h. The digested peptides were then fractionated on a reverse-phase Pep column (Amersham Pharmacia Biotech, Inc.) with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid for 120 min. The fractions containing radioactive peptides were determined by scintillation counting and collected for manual Edman degradation [Boyle et al., 1991; Ku and Omary, 1994] and protein sequencing. After

dried in vacuo, the purified peptide was dissolved in 20 μl water and an aliquot was taken as starting material. The volume was restored to 20 μl, and an equal volume of 5% phenyl isothiocyanate in pyridine was added. The sample was incubated at 45°C for 30 min, and then extracted with 200 μl of heptane:ethyl acetate (10:1) and with 200 μl heptane:ethyl acetate (2:1). The final aqueous phase was dried in vacuo and then taken up in 50 μl trifluoroacetic acid and incubated at 45°C for 10 min. The trifluoroacetic acid was then removed in vacuo, and the residue was taken up in a volume of water equal to 20 μl minus the volume taken out as a sample of the starting material. An aliquot equal to that taken for the starting material was removed (cycle 1), the volume was then restored to 20 μl, and the whole procedure is repeated. Afterwards, the samples were analyzed by electrophoresis on thin-layer chromatography (TLC) plates in a buffer of pH 1.9 at 1,000 V for 30 min in Hunter thin-layer electrophoresis (TLE) apparatus (HTLE-7000; C.B.S Scientific Co., Del Mar, CA) and then processed for radiography [Boyle et al., 1991]. Alternatively, the samples were kept until radioactivity decayed and then the amino acid sequences of the peptides were deduced by automatic sequencing (Milligen/Biosearch Pro-Sequencer 6600, Burlington, MA).

Assembly of Vimentin and Analysis of Vimentin Filaments by Electron Microscopy

In vitro assembly of soluble tetrameric recombinant vimentin into a filamentous form was accomplished by altering the buffer strength to physiological ionic strength at neutral pH. Soluble tetrameric vimentin in buffer B was dialyzed stepwise against buffer C (5 mM Tris-HCl, pH 7.5, containing 160 mM NaCl and 5 mM β-mercaptoethanol) at room temperature for 4 h. For centrifugation analysis, the samples were layered upon 0.85 M sucrose and centrifuged at 32,000 *g* for 30 min at 4°C in a Beckman TL100 ultracentrifuge (Beckman Coulter, Fullerton, CA). The pellet and the supernatant fractions were then analyzed by SDS–PAGE as described by Nicholl and Quinaln [1994]. Alternatively, the samples were directly applied onto 300-mesh formvar/carbon-coated copper grids (Electron Microscopic Science, Fort Washington, PA) and allowed to set for 1 min. The grids were then negatively stained with 2% (w/v) uranyl acetate at room temperature and examined with a Hitachi H-600 electron microscope at 75 kV.

RESULTS

Stoichiometric Phosphorylation of Vimentin by MAPKAP Kinase-2

In a previous study, we demonstrated that MAPKAP kinase-2 is a genuine vimentin kinase [Cheng and Lai, 1998]. As a first step for investigating the characteristics of vimentin phosphorylated by MAPKAP kinase-2, the stoichiometry of phosphorylation was determined by means of monitoring transfer of radioactive phosphate. Vimentin was phosphorylated by MAPKAP kinase-2 in the presence of [γ-^{32}P] ATP and radioactive vimentin was recovered. The data showed that the radioactive phosphate transferred by MAPKAP kinase-2 onto vimentin occurred in a time-dependent manner and plateaued at 1.5 mol phosphate/mol protein (Fig. 1A). The Mg^{2+}-dependence of the reaction, which is a major characteristic of kinase reactions, was also examined. Results showed the reaction in buffer containing 10 mM Mg^{2+} resulted in the highest phosphate level transferred onto vimentin (Fig. 1B). To reveal which domain of vimentin was phosphorylated by

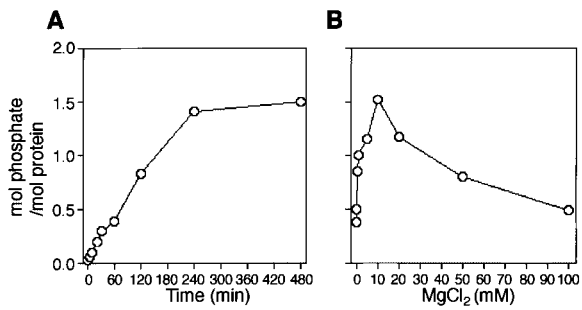

Fig. 1. Stoichiometry of phosphorylation of vimentin by MAPKAP kinase-2. **A**: Time course of phosphorylation of bovine vimentin by MAPKAP kinase-2. Purified bovine vimentin (0.3 mg/ml) were incubated with MAPKAP kinase-2 in the buffer described in ''Materials and Methods.'' At indicated intervals, the reaction mixtures were stopped by precipitated with trichloroacetic acid and then processed for scintillation counting. The resulted radioactivity was transformed into mol phosphate/mol protein. **B**: Magnesium dependence of phosphorylation of vimentin by MAPKAP kinase-2. Purified bovine vimentin (0.3 mg/ml) were incubated with MAPKAP kinase-2 in the buffer as described in ''Materials and Methods,'' however, with different concentrations of $MgCl_2$. The reaction mixtures were processed for scintillation counting and the amount of phosphate were graphed. Shown are the mean values from three individual experiments.

MAPKAP kinase-2, we utilized lysyl endoproteinase to digest in vitro radiolabeled vimentin. Since the first accessible lysine residue is located between the head domain and central domain of vimentin, the protease digestion of vimentin by lysyl endopeptidase leaves the 12-kDa amino-terminal head domain intact, which was analyzed by SDS–PAGE. The radiograph showed that after digestion of radiolabeled of vimentin by lysyl endoproteinase, most of the radioactivity co-migrated with a 12-kDa protein band, the size predicted for the amino-terminal head domain of vimentin (Fig. 2). Combined with the results from previous studies, which indicated most of phosphoamino acids in MAPKAP kinase-2-phosphorylated vimentin were phosphoserine [Cheng and Lai, 1998], we concluded the MAPKAP kinase-2, like most of the vimentin kinases, phosphorylated vimentin mainly at serine residues in the head domain.

Identification of MAPKAP Kinase-2 Phosphorylation Sites on Vimentin

We further defined MAPKAP kinase-2 phosphorylation sites on vimentin either by manual Edman degradation and/or by immunoblotting with phospho-specific antibodies. For manual Edman degradation, native vimentin was phosphorylated by MAPKAP kinase-2 in the presence of [γ-^{32}P]ATP to 1.5 mol phosphate/mol protein and subjected to lysyl endoproteinase treatment. The 12-kDa radioactive head domain of vimentin was purified by chromatography and subjected to trypsin digestion. The resulting phospho-peptides were fractionated on acetonitrile gradients on a Pep reverse-phase column (Amersham Pharmacia Biotech, Inc.). As shown in Figure 3A, three radioactive

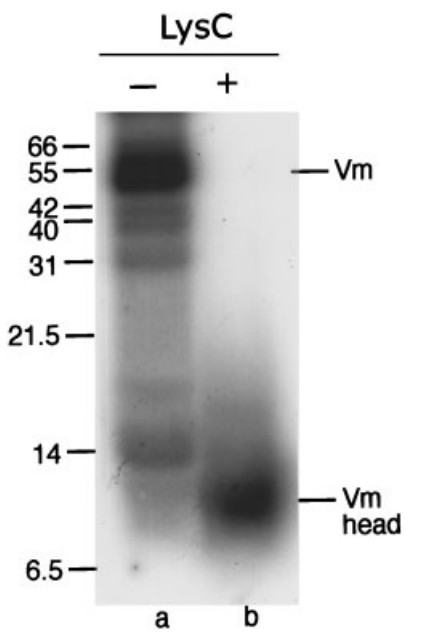

Fig. 2. The assignment of phosphorylated domain in MAPKAP kinase-2-phosphorylated vimentin. Vimentin (Vm) (15 μg) phosphorylated by MAPKAP kinase-2 was incubated in the presence (**b**) or absence (**a**) of lysyl endopeptidase (Lys-C) and subjected to SDS–PAGE analysis. Radiolabeled bands were visualized by autoradiography. Molecular weights are marked at the left and the positions of vimentin and the head domain of vimentin are marked at the right.

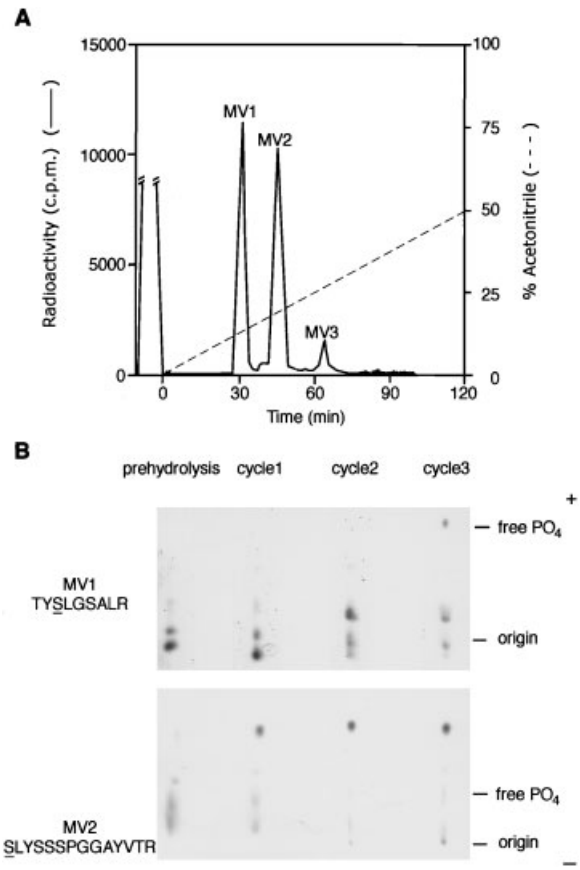

Fig. 3. Identification of phosphorylation sites on MAPKAP kinase-2-phosphorylated native vimentin by trypsin digestion and manual Edman degradation. **A**: After phosphorylated by MAPKAP kinase-2 in the presence of [γ-^{32}P]-ATP, native vimentin were digested with lysyl endopeptidase at 37°C. The radioactive amino-terminal head domain of vimentin was then digested with trypsin and fractionated by a Pep column with 0–50% acetonitrile gradients (– – –). The radioactivity of each fraction (1 ml) was measured in ^{32}P liquid scintillation counter (——). **B**: Phosphopeptides indicated above (MV1 and MV2) was processed for three cycles of manual Edman degradation. A fraction (1/5) of the starting material and of each cycle product was subjected to thin-layer electrophoresis (TLE). The number of cycles is indicated at the bottom and the position of labeled free phosphate and the origin of the maps are indicated at the right. Shown left are the sequences of trypsinized phosphopeptides.

peptides, designated as MV1, MV2, and MV3 were obtained. The phosphopeptides were subsequently subjected to manual Edman degradation and the products were analyzed by TLC followed by autoradiography (Fig. 3B). The generation of radioactive phosphates at a particular Edman degradation cycle where serine is located provides a way to locate the phosphoserine residue(s) on each peptide. For MV1, radioactivity was present in degradation cycle 3, i.e., there was phosphoserine in the third residue of this particular phosphopeptide. Confirmed by protein sequencing, we deduced the MV1 as TYS^{38}LGSALR. The same procedure was performed on peptide MV2 with radioactivity present in the first round. The deduced sequence of MV2 was S^{50}LYSSSPGGGAYVTR. Unfortunately, we were unable to deduce the sequence of peptide MV3.

Subsequently, we verified the MAPKAP kinase-2 phosphorylated sites on vimentin by employing the phospho-specific antibodies developed by the laboratory of Dr. Inagaki [Goto et al., 1998]. Meanwhile, in order to see if recombinant proteins have the same phosphorylating sites as native proteins do, both native vimentin and recombinant vimentin were phosphorylated. MAPKAP kinase-2 phosphorylated native vimentin showed reactivity toward phospho-ser-55 and -ser-82 antibodies although no phospho-ser-38 and -ser-50 were observed with native protein using this method of detection. For the phosphorylated recombinant proteins, phospho-ser-38, -ser-50, -ser-55, and -ser-82 showed up (Fig. 4). Altogether with the results of manual sequencing, both native vimentin and recombinant vimentin revealed the occurrences of same phosphorylation sites. Therefore, we were confident to use recombinant vimentin for the following studies. Furthermore, it was noticed that phosphorylated native protein was less reactive toward phosphorylation site-specific antibodies than phosphorylated recombinant vimentin was. It might be due to lower degree of phosphorylation of native proteins or the native proteins are not fully accessible to antibodies.

Fig. 4. Immunoblotting of MAPKAP kinase-2 phosphorylated vimentin with individual phospho-specific antibodies. Vimentin was incubated without or with MAPKAP kinase-2 to get phosphorylated at 1.5 mol phosphate/mol protein in vitro. Both native vimentin (Nat.) and bacterially expressed recombinant (Re.) vimentin were analyzed. After SDS–PAGE, samples were transferred onto a nitrocellulose membrane. The membrane was immunoblotted with the antibodies specific to phosphoserine-6, -33, -38, -50, -55, and -82, respectively, and then was processed for colorimetric assay.

Effect of MAPKAP Kinase-2 Phosphorylation on Vimentin IF Structure

Recombinant vimentin proteins were phosphorylated either by MAPKAP kinase-2 or by PKA prior to further assembly into filaments. The organization of vimentin was subsequently examined by centrifugation to fractionate species by size. Fractions were then analyzed by SDS–PAGE (Fig. 5A). Results revealed that almost all MAPKAP kinase-2 phosphorylated vimentins resided in the pellet fraction from the gradient, suggesting that the phosphorylated form of vimentin was either in a filamentous organization or in irregular aggregates. Subsequent electron microscopic studies showed that MAPKAP kinase-2 phosphorylated vimentin was organized in a filamentous structure (Fig. 5B). In addition, compared with the filaments formed by vimentin without MAPKAP kinase-2 phosphorylation, the MAPKAP kinase-2 phosphorylated filaments were rough on their surface (see arrows in Fig. 5B). The parallel experiments with vimentin phosphorylated with PKA were consistent with previously reported studies [Inagaki et al., 1987]. After

centrifugation, most of the PKA-phosphorylated vimentins distributed into the supernatant fractions and showed little filamentous structure under the electron microscope. Both of the studies indicated loss of filament forming ability of vimentin after phosphorylated by PKA (Fig. 5). Thus, in stark contrast to vimentin phosphorylated by PKA, MAPKAP kinase-2-phosphorylated vimentin retains its filament-forming ability.

Effect of MAPKAP Kinase-2 Phosphorylation on Mutant Vimentins

The retaining of filament-forming capability of MAPKAP kinase-2-phosphorylated vimentin might have resulted from the efforts of a single site or the concerted actions of multiple phosphorylation sites. To probe the possibility of the contribution of a unique serine to the filament-forming capability of vimentin phosphorylation by MAPKAP kinase-2, we constructed a set of six vimentin variants, in which respective serine in the head domain was point-mutated into alanine residue. These vimentin variants, designated as VmS6A, VmS33A, VmS38A, VmS50A, VmS55A, and VmS82A, were expressed in bacteria and purified. The size and purity of proteins were then confirmed by SDS–PAGE analysis followed by Coomassie Brilliant Blue staining (Fig. 6). The vimentin variants were then dialyzed into assembly buffer and the assembly statuses in physiological condition were examined by electron microscopic analysis with negative staining (Fig. 7). Interestingly, these mutants, bearing one mutant residue at a time, remain capable of forming filaments (Fig. 7) that were indistinguishable from the filaments made of recombinant wild-type vimentin (Fig. 5B). To examine the effects of phosphorylation by MAPKAP kinase-2 and PKA on these mutants, vimentin proteins were phosphorylated with MAPKAP kinase-2 or PKA followed by assembly step. MAPKAP kinase-2 phosphorylation apparently introduced no appreciable structural alterations on vimentin variants. It implied that the capability

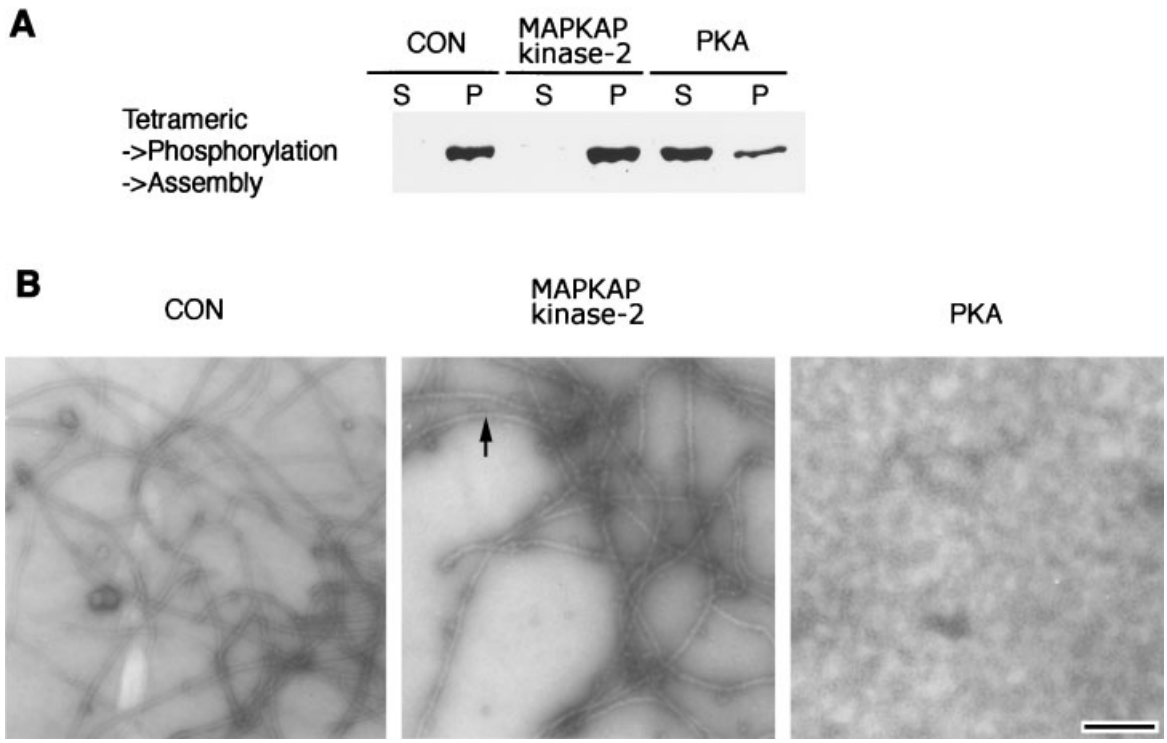

Fig. 5. Assembly competence of MAPKAP kinase-2-phosphorylated and PKA-phosphorylated vimentin. **A**: Recombinant vimentin was phosphorylated as described in ''Materials and Methods.'' The phosphorylation reaction was proceeded in the absence or presence of MAPKAP kinase-2 or PKA prior to changing into the assembly buffer in a total of 4 h. After the samples were subjected to high-speed centrifugation (32,000 *g*, 30 min, 4°C), the supernatant (S) and the precipitate (P) were analyzed by SDS–PAGE followed with Coomassie Brilliant Blue staining. **B**: Alternatively, after assembly step, the samples were placed directly on carbon film-coated specimen grids, stained with 2% uranyl acetate, and then subjected to the electron microscopy studies. The arrow in the filaments constituting MAPKAP kinase-2-phosphroylated vimentin indicated the appearance of roughness. Bar, 194 nm.

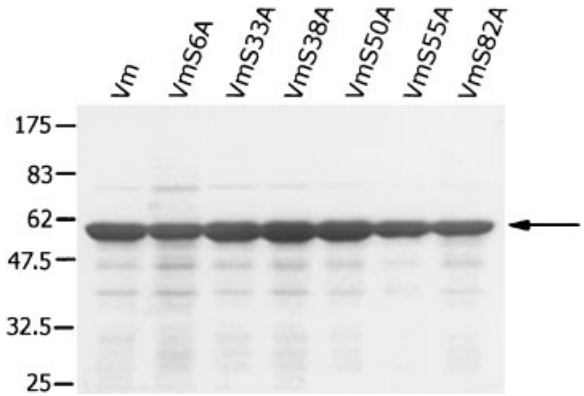

Fig. 6. The SDS–PAGE analysis of the expression of the point-mutated vimentin proteins. A set of point-mutated vimentin, designated as VmS6A, VmS33A, VmS38A, VmS50A, VmS55A, and VmS82A in which the specified serine were altered into alanine residues, were constructed and then cloned into pET23d expression vector. After individual vector transformed into *E. coli* BL21(DE3) strain, a single colony was inoculated in 10 ml for growing. The culture was then transferred into 1 L LB with 100 μg/ml ampicillin until the O.D. reached around 0.6. After addition of 1 mM IPTG for 3 h to induce the expression of recombinant proteins, the cells were collected by centrifugation and the expressed vimentin from the same amount of cells were enriched by two cycles of centrifugation. The samples thus obtained were then processed for SDS–PAGE analysis followed with Coomassie Blue staining. The arrow on the right showed the position of vimentin and molecular weight markers were at the left.

of organizing into filaments for MAPKAP kinase-2-phosphorylated vimentin might be due to coordinated actions of multiple phosphorylation sites instead of one unique site. It is also interesting that the filaments composed of VmS38A phosphorylated by MAPKAP kinase-2 seemed more readily to trap the dye particles in electron microscopic analysis, indicating the possible existence of some kinks in the intersection of filaments. Whether it implies interference in parallel packing of intermediate filaments needs further investigations. Notably, the studies with PKA showed that VmS6A mutation surprisingly retains filament-forming ability of vimentin after PKA phosphorylation, which is opposite to the behavior of wild-type vimentin toward PKA, while the rest of mutations underwent full disassembly after PKA phosphorylation as expected. It indicated that Ser-6 plays an important role in phosphorylation-mediated disassembly of vimentin by PKA. In addition, individual mutations at other phosphorylable serines still display wild-type-like disassembly, indicating that loss of function on single serine residue, except Ser-6, could be compensated by other functioning residues to result in reorganization of vimentin filaments after phosphorylation. It will be very interesting to investigate the effects of phosphorylation on vimentin carrying multiple mutations at different phosphorylation sites.

DISCUSSION

IF proteins are continually reported being excellent substrates for kinases. Most of the time, the phosphorylation event was correlated with structural changes [Inagaki et al., 1996]. In the present study, we demonstrated that MAPKAP kinase-2 would phosphorylate vimentin on Ser-38, Ser-50, Ser-55, and Ser-82 at 1.5 mol phosphate/mol protein. It showed no appearances of unique phosphorylation site(s) in vimentin phosphorylated by MAPKAP kinase-2, compared with phosphorylation by other kinases. It is also noteworthy that some phosphorylation sites were coincident with supposed unique sites for other kinases, such as Ser-55 for cdc2 kinase [Chou et al., 1991; Tsujimura et al., 1994] and Ser-82 for CaMKII [Ando et al., 1991]. On the other hand, in stark contrast to the disassembly of vimentin filaments resulted from phosphorylation by most kinases, MAPKAP kinase-2-phosphorylated vimentin is independent of IF structural changes.

Herein we provided evidences that reorganization of IFs is not the only obligated result of in vitro phosphorylation of IFs although IF phosphorylation is mostly accompanied with reorganization and/or disassembly of IF filaments. Phosphorylation of vimentin by PKA or PKC was among the first phosphorylation events investigated [Inagaki et al., 1987; Ando et al., 1989]. Not only were the PKA and PKC phosphorylation sites in vimentin identified, but also the phosphorylation-mediated reorganization of IFs was evidenced. Vimentin filaments underwent complete disassembly after phosphorylation by PKA or PKC at the N-terminal head domain of vimentin [Inagaki et al., 1987; Ando et al., 1989]. Similar phenomena were observed when IF was phosphorylated by cdc2 kinase [Chou et al., 1990], CaMKII [Tokui et al., 1990], or Rho kinase [Goto et al., 1998]. As far as we know, phosphorylation of IF proteins by individual protein kinase was still mostly associated with the loss of filamentous structure. One exception was regarding $p37^{mos}$ protein kinase. Vimentin filaments showed no obvious structural changes after vimentin filaments phosphorylated by $p37^{mos}$ protein

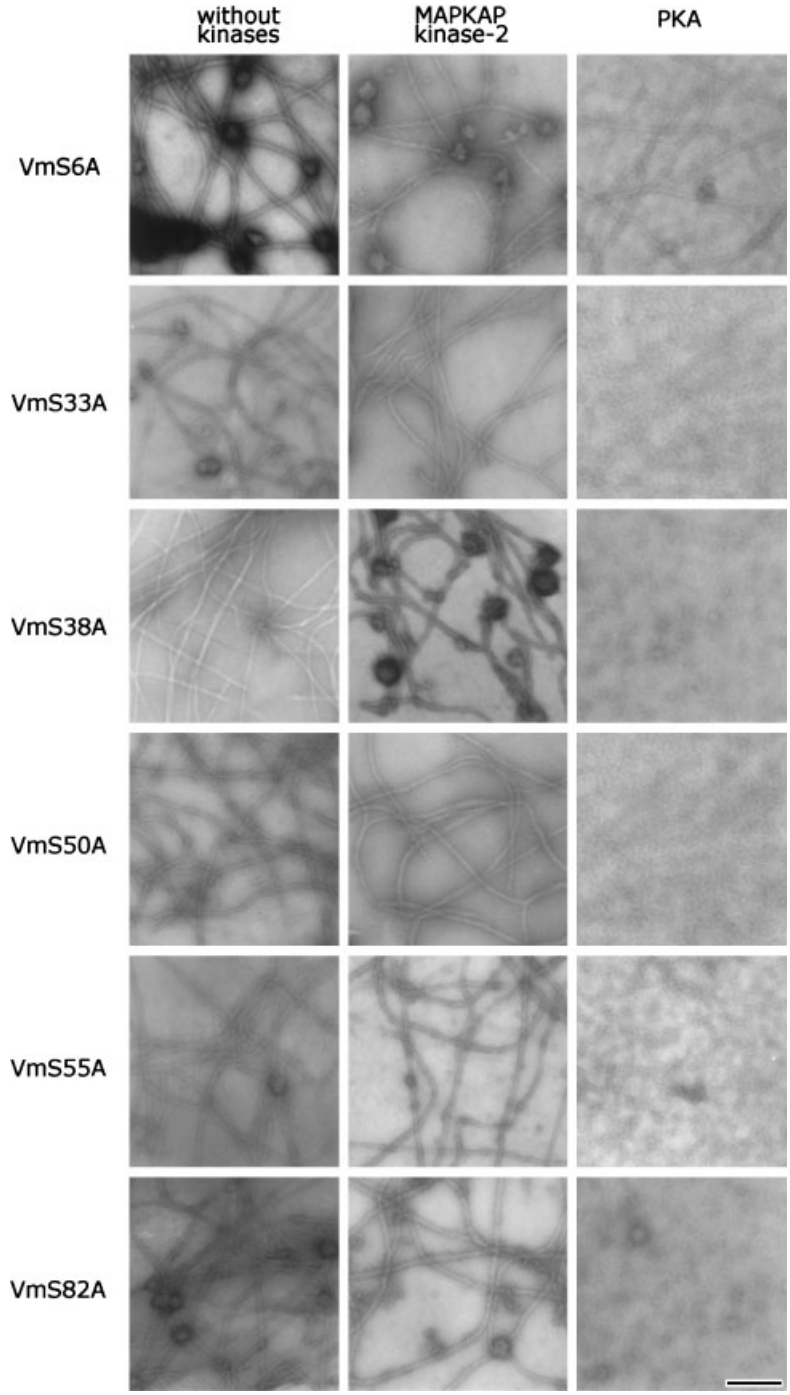

Fig. 7. The effects of MAPKAP kinase-2 phosphorylation on the assembly capabilities of vimentin variants. The point-mutated vimentin was expressed and purified as described in Figure 6. The samples thus obtained were processed for assembly step with or without prior phosphorylation by MAPKAP kinase-2 or PKA. Subsequently, the samples were placed directly on carbon film-coated specimen grids, stained with 2% uranyl acetate, and then subjected to electron microscopy. Shown are representatives from three independent experiments. Bar, 194 nm.

kinase. The proposed reason for this was that the phosphorylation sites by $p37^{mos}$ kinase were on the C-terminal region of vimentin [Chou et al., 1996]. We demonstrated here that vimentin phosphorylated by MAPKAP kinase-2, mainly on its N-terminal domain, has little effects on its structure in vitro. Alterations of IF structure would be dependent on both stoichiometry and sites of phosphorylation [Carpenter et al., 1992; Chou et al., 1996].

Therefore, the fact that phosphorylation of vimentin by MAPKAP kinase-2 resulted in little structural changes of vimentin IFs might be due to weak phosphate level, either in terms of the level for the whole protein or in terms of the level of specific phosphorylation sites, or due to no critical site(s) being phosphorylated by MAPKAP kinase-2. Compared with that PKA phosphorylated vimentin at 4 mol phosphate/mol protein on 11 sites and PKC did at 1.7 mol phosphate/mol protein on six sites [Inagaki et al., 1987; Geisler et al., 1989], MAPKAP kinase-2 phosphorylated vimentin at a weak level of 1.5 mol phosphate/mol protein on four sites. This implied a threshold of vimentin phosphorylation for reorganization or disassembly might be needed. Moreover, it is not clear whether the stoichiometry of each phosphorylation site is the same or there is certain site(s) with higher phosphate level, which could critically decide the organization of phosphorylated vimentin. It is also possible that the sites phosphorylated by MAPKAP kinase-2 are not essential for IF reorganization.

Alternatively, based on the fact that $p37^{mos}$ kinase-phosphorylated vimentin has little effects on its structure might be due to that the phosphorylation sites were located in the carboxyl terminal of vimentin protein and the possibility of the carboxyl-terminal phosphorylation of vimentin by MAPKAP kinase-2 is not completely excluded, it might be interesting to determine if the phosphorylation on the carboxyl-terminal domain of IF proteins has crucial effects on vimentin filaments organization.

The in vitro studies here showed MAPKAP kinase-2 caused no dramatic changes on vimentin structure upon phosphorylation. However, in the OA-treated 9L RBT cells, activation of MAPKAP kinase-2 was associated with reorganization and increased phosphorylation of vimentin filaments [Cheng and Lai, 1998]. There should be other effectors leading to reorganization of vimentin networks in the OA-treated cells. Reorganization of vimentin filaments could rely solely on dose-dependent phosphorylation. For this, the phosphate equilibrium in the cells needs to be balanced by protein kinases and protein phosphatases, and the inhibition of protein phosphatases by protein phosphatase inhibitor would result in a net increase of cellular kinase activities. In fact, some kinases, such as Cdc2 kinase [Yamashita et al., 1990] and Rho kinase [Inada et al., 1999], are activated in the cells treated with protein phosphatase inhibitors [Cheng and Lai, 1998 and references therein]. In this regard, the disassembly of IF in OA-treated cells would be a result of concerted activations of several kinases. Upon OA treatment, MAPKAP kinase-2 could sequentially or synergistically work with other kinases to induce severe changes on vimentin structure. Provided that MAPKAP kinase-2 activation is one of the prerequisite phosphorylation events on vimentin, other phosphorylable residues on vimentin proteins would become accessible to other kinases after MAPKAP kinase-2 phosphorylation and then the following phosphorylations by other kinases exceed the threshold for structural changes. On the other hand, phosphorylation by MAPKAP kinase-2 might work synergistically with other proteins, besides kinases, to ultimately disrupt IF structure. Several protein factors, such as microtubules [Vilalta et al., 1998], actin filaments [Coulombe et al., 2000], and α-crystallin [Nicholl and Quinaln, 1994], have been shown to affect IF structures. Vilalta et al. [1998] demonstrated that prior stabilization of microtubules by taxol would block the effect of OA on vimentin reorganization in cells. This observation indicated that, at least, disruption of microtubule network in OA treatment is linked to the reorganization of IF networks. Some evidences also showed the interactions between the actin network and intermediate filament network [Shah et al., 1998]. It is also possible that the activation of MAPKAP kinase-2 result in phosphorylation of HSP25, which subsequently lead to inductive polymerization of actin and then possibly induced the reorganization of IF networks in OA-treated cells. MAPKAP kinase-2 activation was well correlated with HSP25 phosphorylation in the cells upon heat shock [Gaestel et al., 1991; Landry et al., 1992] and in the cells treated with interleukin-1, growth factors [Saklatvala et al., 1991], and tumor necrosis factor [Rogalla et al., 1999]. That implied MAPKAP kinase-2 might play an important role in the cytoskeleton interactions upon stress through the phosphorylation of HSP25, which subsequently affects the status of actin polymerization. The physiological roles of p38 MAPK pathways on IF phosphorylation or cytoskeleton organization is worthy of further investigations.

It is plausible that vimentin might provide a sequestering reservoir to accommodate the

urgent elevated activity of MAPKAP kinase-2 upon stress and that a stress relieving mechanism could result from this. That could also be applied onto the situation with other kinases. From our previous studies with different types of cultured cells, we observed that vimentin usually experiences hyper-phosphorylated in the cells treated okadaic acid, a phosphatase inhibitor [Lai et al., 1993a]. We also showed that phosphorylation of vimentin is a prominent phenomena in the cells upon stress [Lai et al., 1993b]. Both implied that vimentin could function as a phosphate reservoir in the stressed cells. Altogether, new functional task(s) of vimentin, independent of structural changes, may be achieved by regulation of activity of MAPKAP kinase-2 or other kinase(s) with the necessity of phosphorylation level or multiple phosphorylation sites. In this regard, it would be interesting to compare the phosphorylation sites on vimentin filaments phosphorylated in vitro by individual kinase and the sites phosphorylated in stressed cells.

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