Journal of Cellular Biochemistry 79:126–138 (2000)

Association of Protein Phosphatase 2A With Its Substrate Vimentin Intermediate Filaments in 9L Rat Brain Tumor Cells

Ting-Jen Cheng, Yi-Liang Lin, Ann-Shyn Chiang, and Yiu-Kay Lai*

Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan 30013, Republic of China

Abstract The importance of protein phosphatases in maintaining the integrity of intermediate filaments is supported by the fact that intermediate filaments would undergo a massive reorganization in cells treated with inhibitors of protein phosphatases 1 and 2A. Herein we used okadaic acid to investigate the differential roles of protein phosphatases 1 and 2A in the maintenance of intermediate filament integrity in 9L rat brain tumor cells. Protein phosphatase 2A activity was substantially inhibited after treatment with 400 nM okadaic acid for 2 h, whereas the activity of protein phosphatase 1 was only slightly affected. Furthermore, protein phosphatase 2A shows selective specificity toward phosphovimentin, which was immunologically precipitated from isotopically labeled and okadaic acid-treated cells. Further biochemical fractionation and microscopic studies revealed that vimentin intermediate filaments were colocalized with protein phosphatase 2A, but not protein phosphatase 1, in control cells. On okadaic acid treatment, vimentin filament disassembled and protein phosphatase 2A redistributed throughout the cytoplasm, suggesting that these two proteins separate from each other, whereas protein phosphatase 2A was inhibited. This working hypothesis was further supported by treatment with a low concentration (40 nM) of okadaic acid, which causes the same phenomenon. Taken together, our results showed that protein phosphatase 2A could be assigned to the intermediate filaments to serve the physiological role in maintaining the proper phosphorylation level of intermediate filaments in normal cells. This finding should pave the way for the elucidation of the regulatory mechanism of intermediate filament organization governed by protein phosphorylation. J. Cell. Biochem. 79:126–138, 2000.

Key words: type 1 protein phosphatase; type 2A protein phosphatase; okadaic acid; intermediate filaments; vimentin

Dephosphorylation of proteins is equally as important as phosphorylation of proteins for the regulation of cellular functions [Hunter, 1995]. The Ser/Thr protein phosphatases (PPases) are usually classified into four groups including types 1, 2A, 2B, and 2C (designated as PP1, PP2A, PP2B, and PP2C, respectively) according to their preference of substrate, mechanism of activation, as well as sensitivity to inhibitor proteins or naturally occurring toxins [Cohen, 1989]. PP1 and PP2A are structurally related enzymes, showing approximately 50% amino acid sequence identity in the catalytic domain [Cohen, 1989; Mumby and Walter, 1993]. To exert their physiological functions efficiently, individual members of the PPase holoenzymes consist of catalytic as well as regulatory subunits that can target the holoenzyme to distinct cellular compartments [Inagaki et al., 1994]. For example, PP1 could be targeted to glycogen particles [Hubbard et al., 1990] or sarcoplasmic reticulum [Alessi et al., 1992] by a 124-kDa glycogen targeting subunit (G-subunit), to nucleus by $PP1_N$ [Ohkura and Yanagida, 1991], or to myofibrils by $PP1_M$ [Okubo et al., 1994]. Similarly, PP2A holoenzyme is subcellularly localized through association with its own set of targeting subunits (the B subunits) to the centrosomes [Sontag et al., 1996], endoplasmic reticulum, Golgi, as well as nucleus [McCright et al., 1996]. PP2A is also found to be associated with the translation termination factor eRF1; therefore, the enzyme may also exert its function on the translational apparatus [Andjelkovic et al., 1996]. Additionally, PP2A can be targeted onto the microtubule (MT) network [Meisinger et al., 1997] and

Grant sponsor: Republic of China National Science Council; Grant number: NSC89-2311-B-007-012-B12.

*Correspondence to: Yiu-Kay Lai, Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan 30013, R.O.C. E-mail: yklai@life.nthu.edu.tw

Received 29 December 1999; Accepted 4 February 2000

directly regulate the phosphorylation state and microtubule-binding activity of Tau [Sontag et al., 1996]. On the other hand, neurofilament preparation from spinal cord is also found to be associated with PP2A [Veeranna et al., 1995; Strack et al., 1997]. However, whether PP2A and intermediate filaments (IFs) are indeed associated in cells still awaits further investigations.

Protein phosphorylation/dephosphorylation is an essential regulatory mechanism in maintaining the functionality of the cytoskeleton. The organization of IFs are repeatedly demonstrated to be governed by concerted actions of relevant protein kinase and PPases; thus the phosphorylation state of IFs is highly regulated, both temporally and spatially [Eriksson et al., 1992; Inagaki et al., 1996]. A pivotal role for PPases in maintaining the integrity of the IFs was suggested by studies in which treatment with PPase inhibitors would invariably lead to reorganization of the IF network in the treated cells. For instance, it has been shown that okadaic acid (OA) can induce hyperphosphorylation and reorganization of vimentin in primary human fibroblasts [Yatsunami et al., 1991] and 9L rat brain tumor (RBT) cells [Lee et al., 1992], and that the compound could also induce hyperphosphorylation and reorganization of cytokeratin, another type of IF proteins, in keratinocytes [Kasahara et al., 1993]. Additionally, other PPase inhibitors, i.e., calyculin A [Hirano and Hartshorne, 1993], microcystin-LR [Toivola et al., 1997], and fostriecin [Ho and Roberge, 1996], also elicit severe and rapid alterations of IF organization in a number of experimental systems. On treatment with these inhibitors, IFs were hyperphosphorylated and, consequently, disassembled into the soluble form [Lee et al., 1992; Inagaki et al., 1994]. It is worth noting that both PP1 and PP2A can be inhibited by the aforementioned inhibitors, albeit with different degrees of sensitivity. Therefore, which PPase is essential in maintaining the integrity of IFs has yet to be clarified. Moreover, although it has been shown that PP2A is involved in the regulation of MT dynamics and thus in the MT-related cellular processes, such as mitosis [Sontag et al., 1995] and apoptosis [Mills et al., 1998], little work has been conducted to further the understanding of molecular actions of the enzymes toward the IF proteins and of the physiological role in the IF-related processes.

We have previously reported that treatment with 400 nM OA induces hyperphosphorylation and reorganization of vimentin IFs in 9L rat brain tumor cells and that the process is basically reversible [Bialojan and Takai, 1988; Cohen et al., 1990; Lee et al., 1992; Lai et al., 1993]. In the present study, we demonstrate that a higher level of PP2A activity was located in the IF-enriched fraction than that in the cytosol. Moreover, the majority of PP2A activity in both fractions was inhibited in cells treated with 400 nM OA. By immunoprecipitating [^{32}P]orthophosphate-labeled vimentin from OA-treated cells followed by incubating with purified PP1 or PP2A, we found that PP2A is more active toward the phosphovimentin than PP1. Furthermore, biochemical fractionation and indirect immunofluorescence microscopic studies independently support the hypothesis that vimentin and PP2A are associated with each other in 9L RBT cells. The elucidation that PP2A, compared with PP1, is a better vimentin PPase should provide insights into the functional roles of PP2A and the regulation of vimentin IFs in living cells.

MATERIALS AND METHODS

Materials

All cultureware was purchased from Falcon (Franklin Lakes, NJ) and culture medium components were from Gibco Laboratories (Grand Island, NY). [γ-^{32}P]ATP (5,000 Ci/mmol) was obtained from Amersham (Buckinghamshire, England), and [^{32}P]orthophosphate ($^{32}P_i$) (specific activity 8,500–9,120 Ci/mmol) was from New England Nuclear (Boston, MA). OA, with a concentration of 0.5 mM, was purchased from Gibco Laboratories, and diluted to appropriate concentrations with culture medium before use. Purified PP1 and PP2A were from Upstate Biotechnology (La Jolla, CA), and polyclonal antibodies against PP1 and PP2A were from Calbiochem (San Diego, CA). Rhodamine-conjugated antibodies were from Boehringer Mannheim (Postfach, Germany), and fluorescein isothiocyanate (FITC)-conjugated antibodies were from Cappel (Turnhot, Belgium). Allophycocyanin-conjugated streptavidin was from Molecular Probes (Leiden, Netherlands). Chemicals for electrophoresis were purchased from BioRad (Richmond, CA). Other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Cell Culture

The 9L rat brain tumor cells [Weizsaecker et al., 1981] were maintained in Eagle's minimum essential medium plus 10% fetal calf serum supplement with 100 U/ml penicillin G and 100 μg/ml streptomycin in a 37°C incubator under 5% CO_2/95% air. Before 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. For microscopic studies, the cells were seeded in Petri dishes containing sterilized glass coverslips. Exponentially growing cells at 80%–90% confluency were used.

Assessment of Enzymatic Activity of PP1 and PP2A in Cell Extracts Fractionated Into Cytosol and Intermediate Filament–Enriched Fractions

PPase activity was determined by the method of Cohen et al. [1990] with slight modifications. For cellular fractionation, cells were washed in PBS and then lysed with lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 25 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 mM benzamidine, 1 μg/ml leupeptin, and 1 mM phenyl methyl sulphonyl fluoride (PMSF)] for 10 min on ice. After the samples were centrifuged at 12,000 *g* for 20 min, the supernatants were collected as the cytosol fractions and the pellets were resuspended in the lysis buffer and designated as the IF-enriched fractions [Lee et al., 1993]. The protein contents in the fractions were quantitated [Bradford, 1976] and adjusted to a concentration of 2 mg/ml. For the measurement of PPase activity, ^{32}P-phosphorylase a, which was derived from phosphorylating phosphorylase b by using [γ-^{32}P]ATP and phosphorylase kinase up to 1 mol phosphate/mol protein, was used as a substrate. In the following assays, exogenous OA and inhibitor 2 were used to distinguish the activity of PP1 from that of PP2A. For each reaction, 40 ng of protein from the fractions was incubated with 30 μg of ^{32}P-phosphorylase a, with or without exogenous PPase inhibitor, in a total volume of 30 μl in assay buffer (50 mM Tris-HCl, pH 7.5, 0.1% β-mercaptoethanol, 0.1 mM EGTA, 0.3 mg/ml bovine serum albumin, 50 mM NaF, 1 mM Na_3VO_4, 1 mM benzamidine, 1 μg/ml leupeptin, and 1 mM PMSF). The reactions were allowed to proceed at 30°C for 10 min and then were terminated by adding 100 μl of 20% trichloroacetic acid (TCA) to precipitate the proteins. After centrifugation at 12,000 *g* for 5 min, the supernatants were taken for scintillation counting, and the phosphatase activity was calculated. One unit of activity, U, in the cell extracts was defined as that amount that catalyzed the dephosphorylation of 1.0 mmol of substrate in 1 min. In this assay system, exogenous 2 nM OA was used to selectively inhibit PP2A, whereas 200 nM inhibitor 2 plus 2 nM OA was used to inhibit PP1. Therefore, PP1 activity was taken as the PPase activity measured in the presence of 2 nM OA plus 200 nM inhibitor 2 (both PP1 and PP2A were inhibited) subtracted from that measured in the presence of 2 nM OA (only PP2A was inhibited), whereas PP2A activity was taken as the amount of activity that was inhibited by 2 nM OA.

Immunoprecipitation of $^{32}P_i$-Labeled Vimentin

Cells were prelabeled with 0.5 mCi/ml of $^{32}P_i$ for 1 h and were treated with OA in the presence of $^{32}P_i$ for 2 h. After treatment, cells were lysed with lysis buffer for 10 min on ice. The lysates were then further incubated with 2 μg anti-vimentin monoclonal antibodies (Amersham) in immuno-precipitation (IPP) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 25 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 mM benzamidine, 1 μg/ml leupeptin, and 1 mM PMSF) at 4°C with gentle agitation for 1 h. Precipitation was achieved by adding 10 μg of rabbit anti-mouse immunoglobulin G (IgG) (Cappel) for 1 h followed by 100 μl of a 50% slurry suspension of protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C. Immunoprecipitates were washed five times with IPP buffer, and the pellets were solubilized in sodium dodecyl sulfate (SDS) sample buffer for SDS–polyacrylamide gel electrophoresis (PAGE) analysis [Laemmli, 1970] followed by autoradiography and Western blotting [Cheng and Lai, 1998]. Alternatively, the washed immunoprecipitates were resuspended in phosphatase buffer (50 mM Tris-HCl, pH 8.0, 1 mM EGTA, 0.1 mM β-mercaptoethanol) for reaction with 0.1 U of purified PP1 or PP2A at 30°C for 10 min.

Preparation of Cytosol, Nuclear, and Cytoskeleton Fractions

Approximately 10^7 cells were trypsinized, collected by centrifugation at 100 *g* for 8 min at

4°C, washed once with phosphate-buffered saline (PBS) and centrifuged as already described herein. The pellet was resuspended in 0.5 ml of nuclear extraction buffer (10 mM HEPES-KOH pH 7.9, 0.5% Triton X-100, 0.5 M sucrose, 0.1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM PMSF), and then homogenized by three strokes with a Dounce homogenizer. The samples were briefly centrifuged at 10,000 *g,* and the supernatant was collected as the cytosol fraction. The resulting pellet was further lysed in 0.5 ml nuclear extraction buffer supplemented with 0.5 M NaCl and 5% glycerol at 4°C for 30 min. The samples were then centrifuged at 14,000 *g* for 20 min, and the supernatant fractions were collected as the nuclear fraction while the pellets were taken as the cytoskeleton fraction. Equal amounts of proteins in each fraction were resolved by SDS-PAGE and further analyzed by Western blotting. Binding of primary antibody to the proteins was detected by using respective horseradish peroxidase-labeled secondary antibodies followed by the ECL Western blotting detection system (Amersham). Quantitation was performed by using laser densitometry (Molecular Dynamics, Sunnyvale, CA). Relative levels of proteins are defined as the pixels of respective proteins in each fraction divided from the sum of the pixels of proteins in three fractions under the same treatment.

Immunofluorescence Microscopic Analysis

Cells on coverslips were washed in PBS at room temperature and fixed in 1:1 cold methanol-acetone at −20°C for 20 min. After fixation, cells were rinsed in PBS twice and permeabilized in 0.1% Triton X-100 in PBS containing 1% fetal bovine serum for 1 h. Subsequently, the cells were processed for double staining of PP1 or PP2A and vimentin at room temperature, and all antibodies were diluted in dilution solution (0.1% Triton X-100, 0.2% bovine serum albumin, 0.5 mM PMSF, and 1 mM DTT in PBS). First, the cells were incubated with rabbit polyclonal antibody against PP1 or PP2A (diluted 1:20) for at least 4 h and then with FITC-conjugated goat anti-rabbit IgG (diluted 1:50) for 1 h. After extensive washing with PBS, cells were further stained for vimentin filaments with mouse anti-vimentin antibodies (diluted 1:50) for 1 h followed by 1 h of incubation with rhodamine-conjugated goat anti-mouse IgG (diluted 1:50). To investigate whether PP2A and the IF network are indeed colocalized in 9L cells, a modified biotin-streptavidin fluorescence staining method was used. The cells were first extracted with Triton X-100 [Wisniewski and Goldman, 1998] before the immunostaining procedure. PP2A was stained with rabbit anti-PP2A antibodies followed by biotin-conjugated goat anti-rabbit IgG (1:50) and streptavidin-allophycocyanin (1:50). The sample was then washed extensively with PBS, and the vimentin IF network was stained with mouse anti-vimentin antibodies and FITC-conjugated goat anti-mouse IgG as already described. The samples were finally washed with PBS before being mounted in an anti-bleaching mountant (5% propylgallate/80% glycerol/PBS) and stored in the dark at 4°C until observation. Images of immunocytochemically labeled samples were viewed using a Zeiss confocal microscope (LSM510) with an Argon laser and a HeNe laser, appropriate filters (excitation wavelengths are 488, 543, and 613 nm, respectively), and a Plan-neofluor 100× (N.A. 1.3) objective. Images were recorded with 512 × 512 pixel resolution and were processed using Adobe Photoshop. The resulted micrographs were printed using a Fuji digital color printer (Pictrography 3000).

RESULTS

Only PP2A Is Inhibited in 9L RBT Cells Treated With 400 nM OA

In a previous study, we reported that treatment with 400 nM OA would result in hyperphosphorylation and reorganization of vimentin IFs [Lee et al., 1992]. To further explore the biochemical mechanism(s) underlying the hyperphosphorylation of vimentin, we attempted to distinguish the inhibitory effect of OA on PP1 and PP2A in cells treated with 400 nM OA for 2 h. PPase activity in the cellular fractions was assayed in the presence of inhibitor 2 and OA, which were included to differentiate the activity of PP1 and PP2A [Cohen, 1991]. As shown in Figure 1, the enzymatic activity of PP1 (PPase activity measured <2 nM OA minus that measured <200 nM inhibitor 2 plus 2 nM OA) in the cytosol decreased 7.4% in cells after treatment with 400 nM OA for 2 h. However, PP2A activity (PPase activity measured in the absence of inhibitors minus that measured <2 nM OA) decreased 94.6% after the

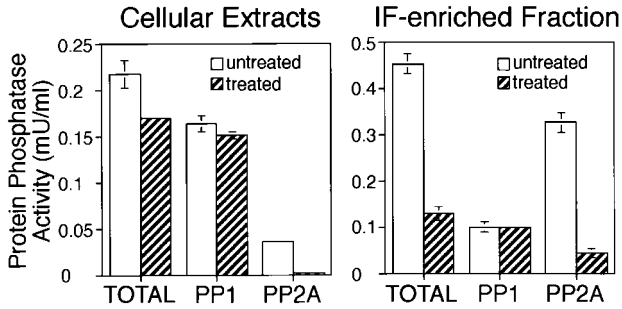

Fig. 1. Protein phosphatase activity in the cytosol and IF-enriched fractions in cells treated with 400 nM OA for 2 h. Untreated and OA-treated (400 nM, 2 h) cells were lysed and fractionated into cytosol and IF-enriched fractions. The PPase activity in the fractions were measured in the absence or presence of inhibitors where 200 nM inhibitor 2 plus 2 nM OA was used to inhibit PP1 and 2 nM OA was used to inhibit PP2A. The cellular PPase activity detected in the absence of inhibitors is denoted as "Total," the left group of bars in the graphs. The enzymatic activity of PP1 in each sample was calculated by subtracting the PPase activity measured in the presence of 2 nM OA plus 200 nM inhibitor 2 (both PP1 and PP2A were inhibited) from that measured in the presence of 2 nM OA (only PP2A was inhibited) and was denoted as "PP1," the middle group of bars in the graphs. PP2A activity was calculated by subtracting the PPase activity measured in the presence of 2 nM OA (only PP2A was inhibited) from that measured in the absence of inhibitor (no PPase was inhibited) denoted as "PP2A," the right group of bars in the graphs. Data are mean ± SD and n = 4 independent experiments. $P < 0.01$.

OA treatment. It is found that PP2A activity in the IF-enriched fraction was much higher than that in the cytosol and that PP2A activity accounted for 72.11% of the total PPase activity in the IF-enriched fraction. In OA-treated cells, the activity of PP2A decreased 86.2%, whereas that of PP1 remained unchanged (Fig. 1). These assays indicated that only PP2A is substantially inhibited in cells treated with 400 nM OA for 2 h and that a large portion of PP2A activity is associated with the IF-enriched fraction. Therefore, inhibition of PP2A is likely to be responsible for vimentin hyperphosphorylation in the OA-treated cells.

PP2A Dephosphorylates Phosphovimentin With a Higher Efficiency

To investigate whether PP1 and PP2A are indeed vimentin phosphatase, in vitro dephosphorylation experiments were performed. To prepare the substrate of this experiment, we purified hyperphosphorylated vimentin from OA-treated and $^{32}P_i$-labeled 9L RBT cells by immunoprecipitation. The immunoprecipitated $^{32}P_i$-labeled phosphovimentin, while still on the protein A-Sepharose beads, was subjected to dephosphorylation by equal units of purified PP1 and PP2A. The substrate was then eluted from the beads and analyzed by SDS-PAGE followed by autoradiography as well as Western blotting. Within 10 min, the radioactivity of the substrate decreased 20% after treatment with PP1 but decreased 80% after treatment with PP2A (Fig. 2, left panel). Equal amounts of substrate included in the reactions were ascertained by immunoblots (Fig. 2, right panel). The data indicate that PP2A is more active toward $^{32}P_i$-labeled vimentin.

Biochemical Colocalization of Vimentin and PP2A in Fractionated 9L RBT Cells During and Recovering From the OA Treatment

To investigate whether PP1 and PP2A function as vimentin phosphatases by associating with intracellular vimentin IFs, we used a biochemical fractionation technique by which cellular components were divided into cytosol (soluble), nuclear, and cytoskeleton fractions to probe the localization of vimentin and the PPases. In control cells, vimentin localized exclusively in the cytoskeleton fraction, but the PPases were found in all three fractions. On OA treatment, a significant portion of vimentin became solubilized and migrated to the cytosol fraction, and this process was basically reversible as the solubilized vimentin returned to the cytoskeleton fractions in cells recovering from the treatment (Figs. 3 and 4). Interestingly, PP2A exhibited a similar redistribution process; it seemed that a fraction of PP2A originally in the cytoskeleton fraction migrated to the soluble fraction during the OA treatment and returned during the recovery period (Figs. 3 and 4). In contrast, distribution of PP1 in the

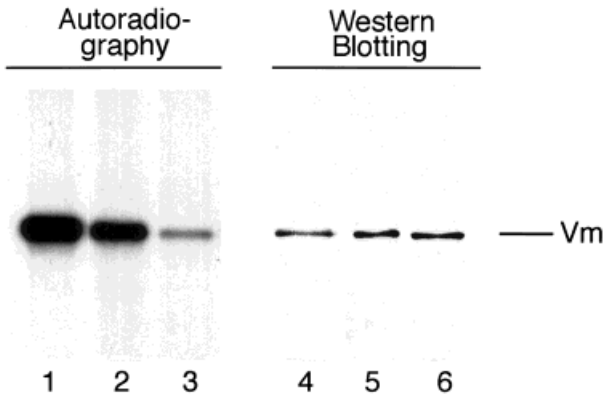

Fig. 2. Selective dephosphorylation of phosphovimentin for OA-treated 9L RBT cells by PP2A. Cells were prelabeled with $^{32}P_i$ for 1 h and then were treated with 400 nM OA for 2 h in the presence of the isotope. After treatment, the cells were lysed and phosphovimentin was precipitated by anti-vimentin antibodies and protein A-Sepharose. The immunoprecipitates were then resuspended in assay buffer and respectively incubated with PP1 (**lanes 2** and **5**) and PP2A (**lanes 3** and **6**). Equal volumes of 2× sample buffer were then added, and the samples were loaded onto SDS gels. After electrophoresis, the gels were processed for autoradiography (left panel) or Western blotting (right panel). Vm, vimentin. Noted that PP2A dephosphorylates phosphovimentin essentially with a much higher efficiency compared to that of PP1.

soluble, nuclear, and cytoskeleton fractions remained relatively constant during the treatment and recovery processes (Figs. 3 and 4). To further assure whether vimentin and PP2A are indeed associated, the cellular lysates of untreated or treated cells were fractionated and were further processed for immunoprecipitation, using antibodies against vimentin. Afterwards, the immunocomplexes were processed for Western blotting to detect the presence of PP1 and PP2A. As expected, while analyzing the cellular lysate of untreated cells, we detected no vimentin in cytosol as well as nuclear fractions. Moreover, in the cytoskeleton fraction, a portion of PP2A was coprecipitated with vimentin and no PP1 was detected (Fig. 5, left panel). In OA-treated cells, most of vimentin was present in the cytosol fraction, and neither PP2A nor PP1 has been detected in the precipitated immunocomplexes (Fig. 5, right panel). This further substantiates the possible association of PP2A and vimentin in normal cells and that this association would be disturbed while the filamentous structure of IF was reorganized.

Association of PP2A and Vimentin as Revealed by Immunofluorescence Microscopy

The possible association of vimentin and PP2A as indicated by the biochemical fractionation studies was further certified by using immunofluorescence microscopy. Cells were treated as described (400 nM OA, 2 h), and the intracellular localization of vimentin, PP1, and PP2A was revealed by double immunostaining by using anti-vimentin together with either anti-PP1 or anti-PP2A as the primary antibodies. In control cells, vimentin exhibited its normal filamentous structure (Fig. 6A,E), but PP1 was found to be abundant in the nuclear region and dispersed throughout the cytoplasm (Fig. 6C). After the OA treatment, vimentin appeared to disassemble and retract from the cytoplasm to the perinuclear region (Fig. 6B,F). Concurrently, the proportion of PP1 in nuclei increased and dispersed homogeneously in the nuclear compartment (Fig. 6D). Interestingly, the staining pattern of PP2A closely resembled that of vimentin in the untreated cells; in fact, PP2A appeared to disperse along the tracks coinciding with the vimentin filaments (Fig. 6E,G). On OA treatment, unlike vimentin filaments, which underwent an extensive rearrangement resembling a retraction of the IF network toward the nuclear periphery (Fig. 6B,F), PP2A appeared to disperse throughout the cell and no longer colocalized with vimentin (Fig. 6H). Taken together, the immunofluorescence microscopic analyses demonstrated that PP2A localized along vimentin filaments in control cells but departed from vimentin and dispersed throughout the cytoplasm after it

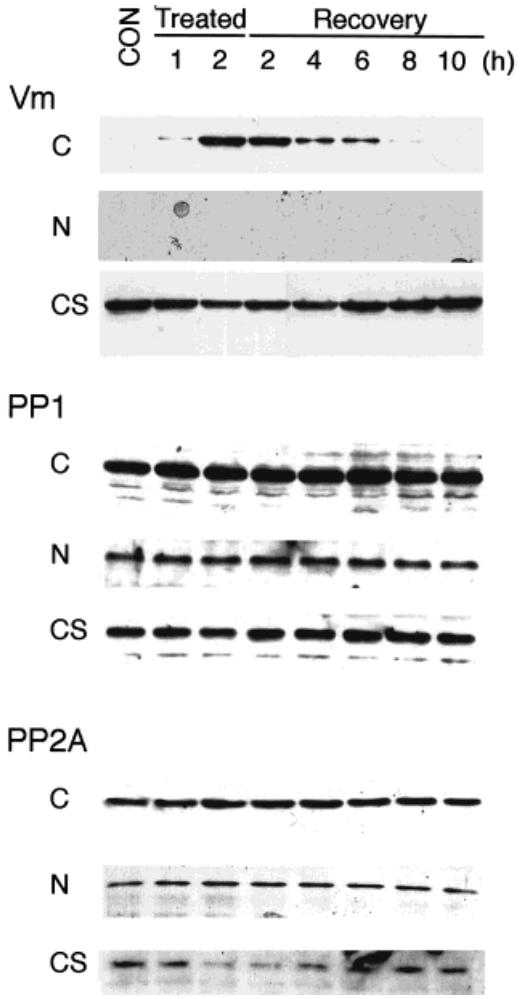

Fig. 3. Redistribution of vimentin and PP2A from cytoskeleton fraction to cytosol fraction in OA-treated 9L RBT cells. Cells were treated with 400 nM of OA for 1 or 2 h and were allowed to recover under normal growing conditions for up to 10 h. The cells were harvested at the end of the treatment or after recovery at time intervals as indicated. Subsequently, the cells were fractionated into cytosol (C), nuclear (N), and cytoskeleton (CS) fractions as described in the Materials and Methods. Equal amounts of cellular proteins in each fractions were fractionated by SDS/PAGE, and the relevant proteins, vimentin (Vm), PP1, and PP2A, were respectively recognized by immunoblotting as indicated. Representative data from three independent experiments are shown.

was inhibited by OA. To ascertain whether PP2A is directly associated with vimentin filaments in cells, we used a biotin-streptavidin system to strengthen the resolution in the immunostaining procedure. Shown in Figure 6 is the combination of six slices of cell images. A portion of the staining pattern of PP2A appeared merged with that of vimentin around

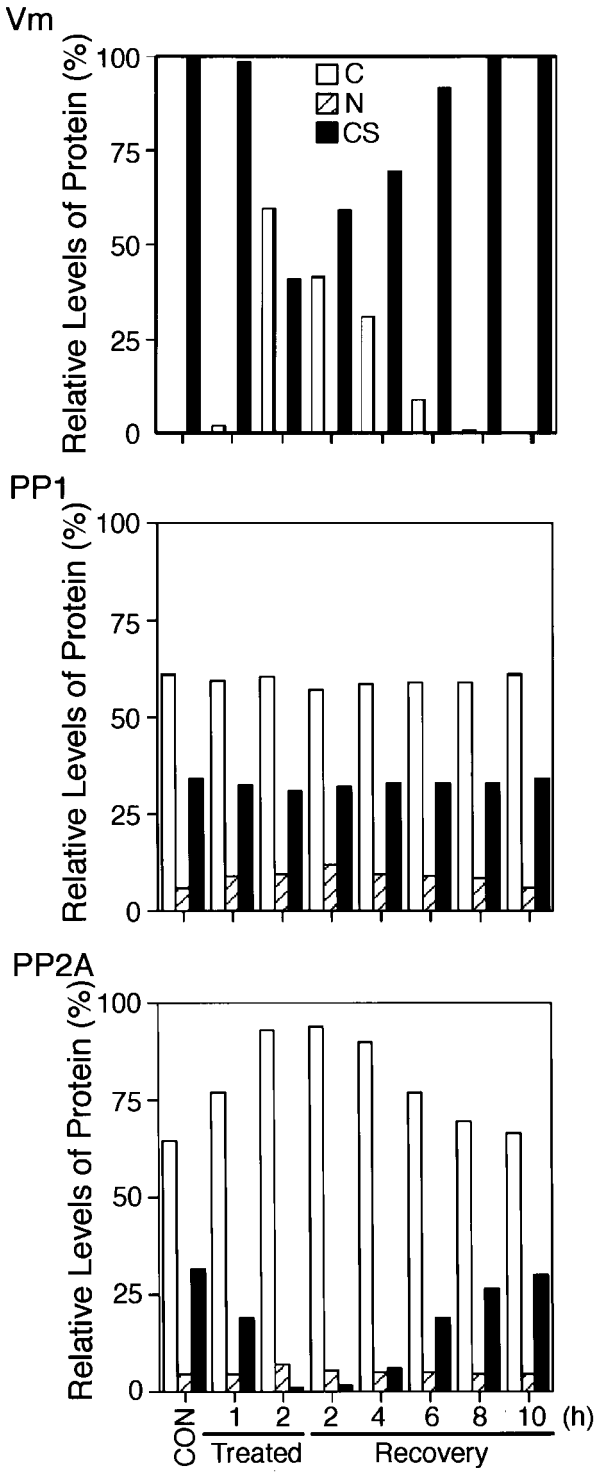

Fig. 4. Quantitative analysis of redistribution of vimentin and PP2A from cytoskeleton fraction to cytosol fraction in OA-treated 9L RBT cells. The bands of interest in the immunoblots of Figure 3 were subsequently quantified by densitometric scanning. Representative data from three independent experiments are shown.

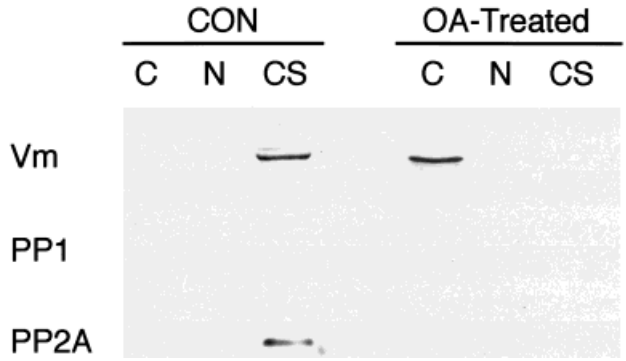

Fig. 5. Association of PP2A and vimentin revealed by immunoprecipitation. Cells were untreated (CON, left panel) or treated with 400 nM of OA for 2 h (OA-treated, right panel). The cells were harvested at the end of the treatment. Subsequently, the cells were fractionated into the cytosol (C), nuclear (N), and cytoskeleton (CS) fractions as described in the Materials and Methods section. After preclearing by incubation with 1 μg of protein G-Sepharose (Amersham Pharmacia Biotech) for 2 h at 4°C followed by centrifugation to remove pellets, the cell lysates were incubated with anti-vimentin antibodies for 2 h. Immunocomplexes were further precipitated with 1 μg of protein G-sepharose, washed five times with IPP buffer, and finally mixed with an equal amount of 2× sample buffer for SDS-PAGE followed by Western blotting using antibodies against vimentin (Vm), PP1, and PP2A.

the nucleus (Fig. 7A,C,E). Moreover, in the area that is farther from the nucleus where the vimentin IFs are wider spread, a significant portion of the spotty staining of PP2A appeared to line up along with the vimentin filaments (horizontal arrows in Fig. 7). Noticeably, there was no staining of PP2A in the region where there was no vimentin filament (vertical arrows in Fig. 7). This result clearly showed that a portion of PP2A and the vimentin network are indeed colocalized.

Low Concentration of OA (40 nM) Also Induces Reorganization of Vimentin Filaments and PP2A

To further assure that inhibition of PP2A is a major inducer of IF reorganization on OA treatment, we used 40 nM OA (the concentration that was known to inhibit PP2A only) to examine whether the alterations of IFs and PPases are the same as that under 400 nM OA. As shown in Figure 8, vimentin filaments reorganized into perinuclear region after 12-h treatment with 40 nM OA (Fig. 8B,H). Concurrently, PP1 became more concentrated in the nuclear region (Fig. 8E) and PP2A began to disperse in the cytoplasm (Fig. 8K) as in cells treated with 400 nM for 2 h (compared to Fig. 6). After 24-h treatment, vimentin exhibited a strong nuclear rim staining (Fig. 8C,I) and, interestingly, PP2A also showed a faint nuclear rim staining with dispersed spots in the cells (Fig. 8L). Therefore, low concentrations of OA would also cause the reorganization of vimentin filaments as well as concurrent redistribution of PP2A. These observation substantiate our notion that PP2A is an authentic vimentin phosphatase that associates with vimentin IFs and may be responsible for maintaining the integrity of vimentin IFs in living cells.

DISCUSSION

We have demonstrated that phosphovimentin can be efficiently dephosphorylated by PP2A. By using biochemical fractionation and immunofluorescence staining, we have also shown that PP2A colocalizes with the vimentin filaments in the control cells, but both of them become solubilized and separate from each other in cells treated with OA. Our results showed that both purified PP1 and PP2A can act on phosphovimentin obtained from OA-treated and $^{32}P_i$-labeled cells and that PP2A dephosphorylates the phosphovimentin much more efficiently. Conversely, there is no difference in dephosphorylation efficiency if the substrate ($^{32}P_i$-labeled phosphovimentin) was prepared from in vitro phosphorylation of purified vimentin by protein kinase A, protein kinase C (PKA, PKC), or cdc2 kinase in the presence of [γ-^{32}P]ATP (data not shown). Therefore, vimentin may be phosphorylated by a concerted action of multiple kinases and this, instead of that phosphorylated by an individual kinase, serves as an authentic selective substrate toward PP1 and PP2A in vivo. However, another possibility is that, instead of authentic selectiv-

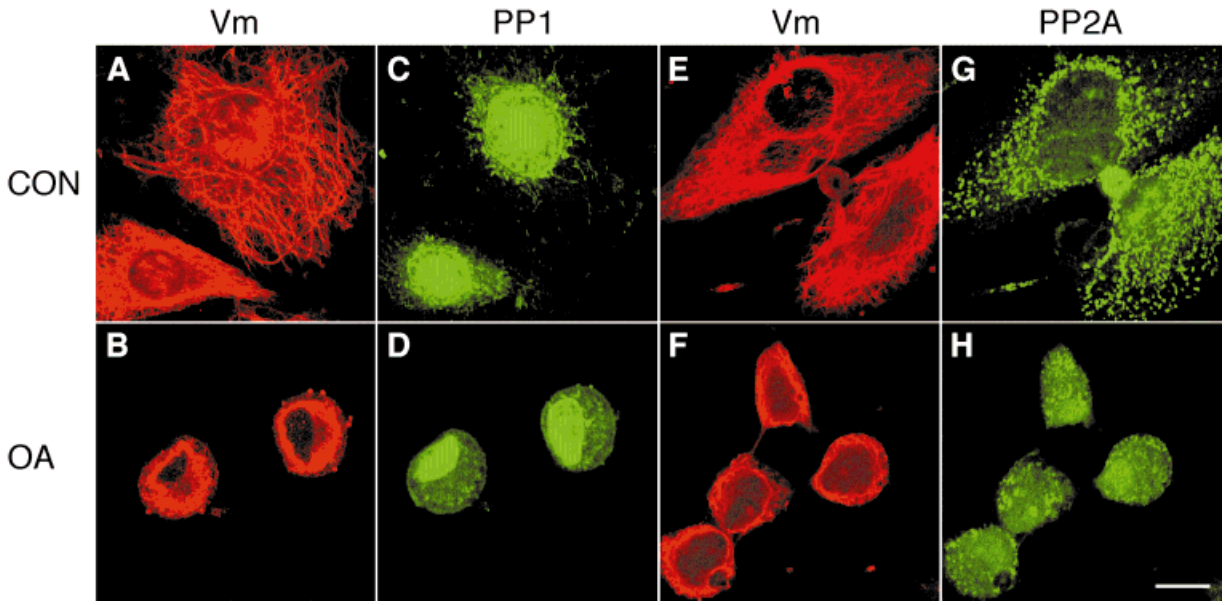

Figure 6.

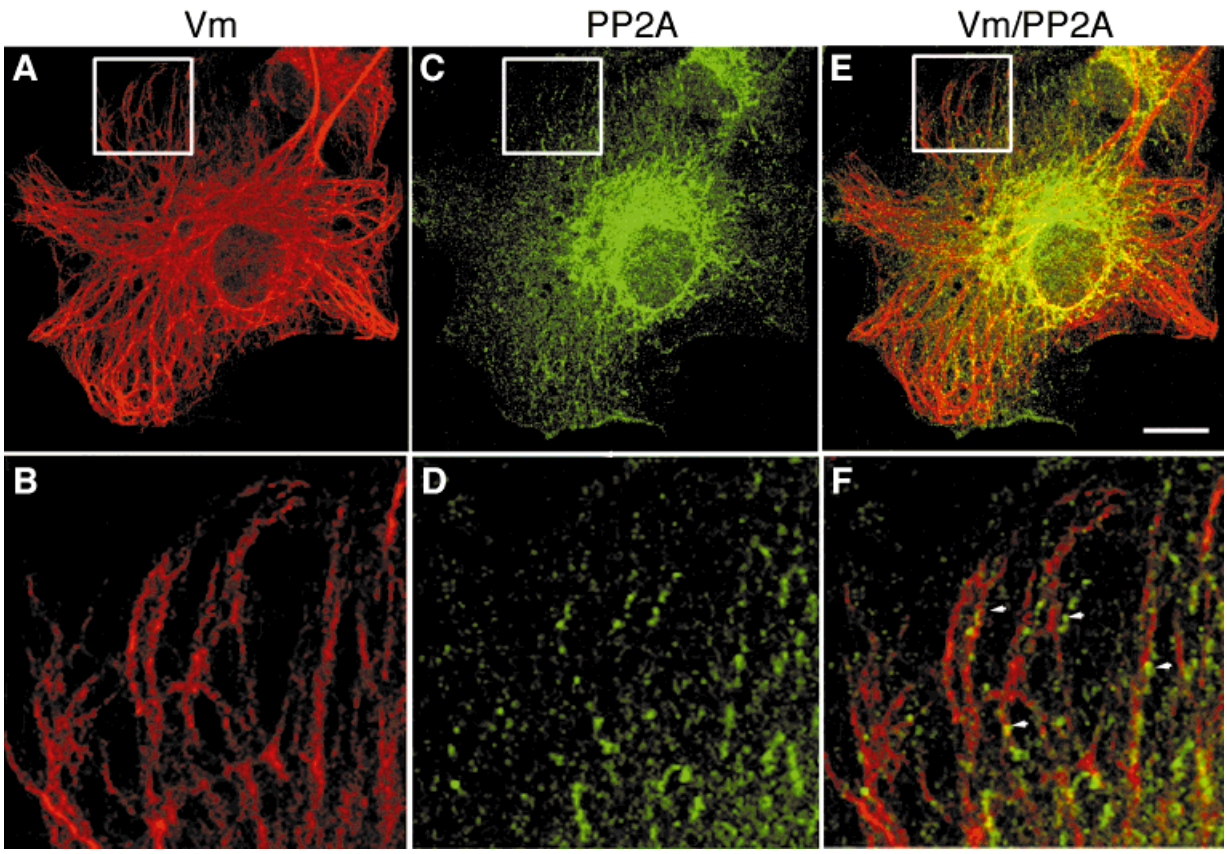

Figure 7.

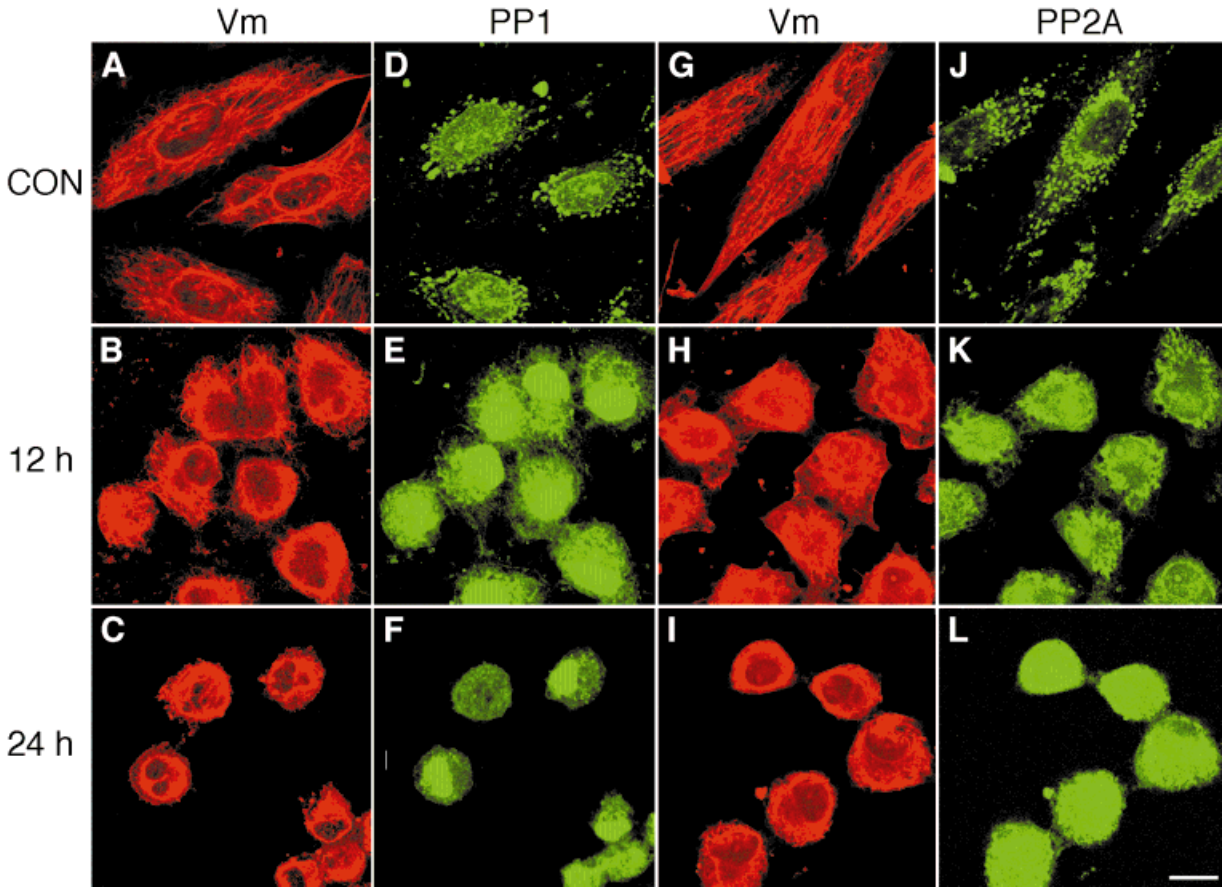

Fig. 8. Treatment of 9L RBT cells with 40 nM OA for prolonged periods yields similar effects on cellular distribution of vimentin and PPases compared to those with 400 nM OA for 2 h. Cells were treated with 40 nM for 12 or 24 h and then processed for double immunostaining as previously described. Again, the micrographs show that vimentin, PP1, and PP2A redistribute in cells on the OA treatment and that PP2A, but not PP1, is colocalized with vimentin in both the untreated and treated cells.

ity for phosphovimentin, PP2A, not PP1, is selectively targeted to dephosphorylate vimentin by some specific regulatory component(s) to maintain the integrity of vimentin filaments.

In contrast to protein kinases, whose substrate specificity is mainly determined by the presence of sequence motifs flanking the targeted amino acid residue, PPases appear to

←

Fig. 6. PP2A, but not PP1, apparently is associated with vimentin in control and OA-treated 9L RBT cells. Before (CON) and after treatment of 400 nM OA for 2 h (OA), the cells were fixed, permeabilized, and processed for double staining using mouse anti-vimentin (**A, B, E,** and **F**) together with either rabbit anti-PP1 (**C** and **D**) or anti-PP2A (**G** and **H**) as the primary antibodies. Rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG were used as the secondary antibodies. The micrographs in (A–D) clearly show that PP1 localizes around the nucleus in the control cells, and that there may be a certain degree of nuclear localization after the OA treatment; also, its distribution is distinctly different from that of vimentin. By contrast, the distributions of PP2A and vimentin are almost superimposed in both the untreated and treated cells (E–H). Vm, vimentin. Scale bar = 10 μm.

Fig. 7. Colocalization of vimentin and PP2A in 9L cells revealed by double-staining confocal micrograph. Untreated 9L cells were fixed, permeabilized, extracted with Triton X-100, and processed for double staining using rabbit anti-PP2A and mouse anti-vimentin as the primary antibodies. Subsequently, PP2A was visualized by using biotin-conjugated goat anti-rabbit IgG (1:50) and streptavidin-allophycocyanin, whereas vimentin was visualized by using FITC-conjugated goat anti-mouse IgG. Micrographs are the projection of six optical sections (collected at 2-μm intervals) showing the vimentin network (**A** and **B,** green channel in **E**) and the distribution of PP2A [**C** and **D,** red channel in (E)] were shown (upper panels). Lower panels [(B), (D), and **F**) are the magnification of the box marked in (E). Regions with staining for both vimentin and PP2A appeared yellow in the merged images [(E) and (F)]. The micrographs clearly show that a significant portion of PP2A docks on the vimentin filaments. Vm, vimentin. Scale bar = 10 μm.

lack any obvious sequence specificity. Instead, the substrate specificity is primarily determined by spatiotemporal distribution. Consistent with our current findings, there are two recent reports that suggest the role of PP2A in the dephosphorylation of IF proteins, more specifically, neurofilament (NF) proteins. It has been shown that NF-H phosphorylated in vitro by cdk5 is efficiently dephosphorylated by skeletal muscle PP2A and by a partially purified PP2A-like PPase from spinal cord [Veeranna et al., 1995] and that NF-L is dephosphorylated by NF-associated PP2A [Saito et al., 1995]. With regard to cellular proteins that are involved in maintaining cell shape and mobility, PP2A is found to be associated with epidermal filaggrin [Kam et al., 1993], which promotes the aggregation of keratin filaments in vitro and functions as an IF-associated protein [Dale et al., 1990]. Additionally, PP2A containing the Bα subunit is reported to be associated with MTs in a variety of cells, and binds and regulates the phosphorylation state of Tau, which promotes assembly and stability of MTs [Sontag et al., 1995]. Conversely, PP1 has been shown to be associated with myofibrils [Chisholm and Cohen, 1988] as well as to be able to dephosphorylate myosin light chain, and thus functions in the modulation of actin MF organization [Fernandez et al., 1990]. Furthermore, it has been shown that a significant portion of PP1 is localized in the cytoplasm, where the bulk of the actin is also located [Fernandez et al., 1990]. Therefore, PP1 may be responsible for maintaining the proper dynamic states of actin MFs, directly or indirectly.

We have shown that a significant portion of PP2A colocalizes with vimentin filaments and is involved in its dynamics properties. Moreover, we also observed that a slight portion of PP2A was also associated with MT in 9L RBT cells (data not shown). Therefore, besides PP2A directly associated with IF to regulate, another possibility is that the regulatory role of PP2A to vimentin IF organization may also be exerted via other mediator(s) such as tubulin. This finding would be consistent with Sontag et al. [1995], who demonstrated that PP2A enzymes containing the Bα subunit associate with MTs in a variety of cell types. PP2A also has been reported to bind and regulate the phosphorylation state of Tau protein, which promotes assembly and stability of MTs [Sontag et al., 1995]. In addition, several lines of evidence indicated that MTs interacts with IF based on the same associated protein between these two systems. For instance, a ubiquitous 70-kDa protein termed β-internexin was initially identified as an MT-associated protein [Green and Liem, 1989] and shown indirectly to associate with IF [Napolitano et al., 1985]. Therefore, PP2A might regulate the cytoskeleton integrity via association with both microtubules and vimentin to regulate their assembly state.

In addition to association with vimentin IF network to regulate its integrity, PP2A may also regulate the phosphorylation state of vimentin via several protein kinase(s) such as cdc2 kinase [Yamashita et al., 1990], p42 mitogen-activated protein kinase [Chajry et al., 1996], PKA [Liauw and Steinberg, 1996], PKC [Sontag et al., 1997], and CaMKII [Zhu et al., 1996], all of which are vimentin kinases, and have been shown to be downregulated by PP2A. Recently, we have shown evidence of activation of MAPKAPK-2 on OA treatment, and inhibitor for the upstream activator of MAPKAPK-2 could partially inhibit vimentin hyperphosphorylation under OA treatment [Cheng and Lai, 1998]. Based on the fact PP2A would inactivate MAPKAPK-2, whether PP2A would act in concert with MAPKAPK-2 or other kinase(s) for maintaining cytoskeletal integrity warrants further investigation.

The present data indicate that, in mammalian cells, PP2A plays an important role in regulation of IF organization. In control cells, PP2A associates with IF to maintain the integrity by counteracting the action of kinase(s). On OA treatment, the inhibited PP2A could no longer operate, and thus the phosphate moieties on the cytoskeletal proteins are bound to increase. Concomitantly, several kinases that had activity regulated by phosphorylation could be activated and phosphorylate its substrates. Because the MT and IF systems are also highly regulated by phosphorylation, the whole cytoskeletal network would collapse because of abruptly increased phosphorylation. This would be consistent with the studies in which several reports show that PPase inhibitors induce disassembly of IFs [Inagaki et al., 1996] and microtubule disintegration [Howell et al., 1997] under this condition. In this state, nonfunctional PP2A would leave the substrate and disperse throughout the cytoplasm. During recovery, PP2A resumes its function and

gradually reassociates with IFs to remove excess phosphate and maintain low phosphate moiety and thus the integrity of IFs.

Altogether, these results demonstrate that PP2A, better than PP1, serves as a phosphatase to dephosphorylate vimentin, and show that PP2A operates in vivo to dephosphorylate vimentin by associating with vimentin filaments. Although further work is still required to confirm the collaboration of kinase(s) and PP2A in regulation of the IF structure as well as the cytoskeleton, the present work illustrates for the first time that PP2A plays a major role in modulating the IF integrity in living cells.

REFERENCES

Alessi D, MacDougall LK, Sola MM, Ikebe M, Cohen P. 1992. The control of protein phosphatase-1 by targeting subunits. The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. Biochemistry 210:1023–1035.

Andjelkovic N, Zolnierowicz C, Van Hoof C, Goris J, Hemmings BA. 1996. The catalytic subunit of protein phosphatase 2A associated with the translation termination factor eRF1. EMBO J 15:7156–7167.

Bialojan C, Takai A. 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem J 256:283–290.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Chajry N, Martin PM, Cochet C, Berthois Y. 1996. Regulation of p42 mitogen-activated protein kinase activity by protein phosphatase 2A under conditions of growth inhibition by epidermal growth factor in A431 cells. Eur J Biochem 235:97–102.

Cheng T-J, Lai Y-K. 1998. Identification of mitogen-activated protein kinase activated protein kinase-2 as a vimentin kinase activated by okadaic acid in 9L RBT rat brain tumor cells. J Cell Biochem 71:169–181.

Chisholm AA, Cohen P. 1988. The myosin-bound form of protein phosphatase 1 (PP-1M) is the enzyme that dephosphorylates native myosin in skeletal and cardiac muscle. Biochim Biophys Acta 971:163–169.

Cohen P. 1989. The structure and regulation of protein phosphatases. Annu Rev Biochem 58:453–508.

Cohen P. 1991. Classification of protein-serine/threonine phosphatases: identification and quantitation in cell extracts. Methods Enzymol 201:389–398.

Cohen P, Holmes CF, Tsukitani Y. 1990. Okadaic acid: a new probe for the study of cellular regulations. Trends Biochem Sci 15:98–102.

Dale BA, Resing KA, Haydock PV. 1990. Filaggrins. In: Goldman RD, Steinert PM, editors. Cellular and molecular biology of intermediate filaments. New York: Plenum Publishing. p 393–412.

Eriksson JE, Opal P, Goldman RD. 1992. Intermediate filament dynamics. Curr Opin Cell Biol 4:99–104.

Fernandez A, Brautigan DL, Mumby M, Lamb NJC. 1990. Protein phosphatase type-1, not type-2A, modulates actin microfilament integrity and myosin light chain phosphorylation in living nonmuscle cells. J Cell Biol 111: 103–112.

Green LAD, Liem RKH. 1989. β-Internexin is a microtubule-associated protein identical to the 70-kDa heat shock cognate protein and the clathrin uncoating ATPase. J Biol Chem 264:15210–15215.

Hirano K, Hartshorne DJ. 1993. Phosphorylation of vimentin in the C-terminal domain after exposure to calyculin A. Eur J Cell Biol 62:59–65.

Ho DT, Roberge M. 1996. The antitumor drug fostriecin induces vimentin hyperphosphorylation and intermediate filament reorganization. Carcinogen 17:967–972.

Howell B, Odde DJ, Cassimeris L. 1997. Kinase and phosphatase inhibitors cause rapid alterations in microtubule dynamic instability in living cells. Cell Motil Cytoskeleton 38:201–214.

Hubbard MJ, Dent P, Smythe C, Cohen P. 1990. Targeting of protein phosphatase-1 to the sarcoplasmic reticulum of rabbit skeletal muscle by a protein that is very similar or identical to the G-subunit that directs the enzyme to glycogen. Eur J Biochem 189:243–249.

Hunter T. 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80:225–236.

Inagaki M, Matsuoka Y, Isujimura K, Ando S, Tokui T, Takakhashi T, Inagaki N. 1996. Dynamic property of intermediate filaments: regulation by phosphorylation. BioEssays 18:481–487.

Inagaki N, Ito M, Nakano T, Inagaki M. 1994. Spatiotemporal distribution of protein kinase and phosphatase activities. Trends Biochem Sci 199:448–452.

Kam E, Resing KA, Lim SK, Dale BA. 1993. Identification of rat epidermal profilaggrin phosphatase as a member of the protein phosphatase 2A family. J Cell Sci 106: 219–226.

Kasahara K, Kartasova T, Ren X-Q, Ikuta T, Chida K, Kuroki T. 1993. Hyperphosphorylation of keratins by treatment of okadaic acid of BALB/MK-2 mouse keratinocytes. J Biol Chem 268:23531–23537.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 27:680–685.

Lai Y-K, Lee W-C, Chen K-D. 1993. Vimentin serves as phosphate sink during the apparent activation of protein kinases by okadaic acid in mammalian cells. J Cell Biochem 53:161–168.

Lee W-C, Yu J-S, Yang S-D, Lai Y-K. 1992. Reversible hyperphosphorylation and reorganization of vimentin intermediate filaments by okadaic acid in 9L rat brain tumor cells. J Cell Biochem 50:1–16.

Lee W-C, Lee Y-C, Perng M-D, Chen C-M, Lai Y-K. 1993. Induction of vimentin modification and vimentin-HSC72 association by withangulatin A in 9L rat brain tumor cells. J Cell Biochem 52:253–265.

Liauw S, Steinberg RA. 1996. Dephosphorylation of catalytic subunit of cAMP-dependent protein kinase at Thr-197 by a cellular protein phosphatases and by purified protein phosphatase 2A. J Biol Chem 271:258–263.

McCright B, Rivers AM, Audlin S, Verishup DM. 1996. The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. J Biol Chem 271:22081–22089.

Meisinger J, Patel S, Vellody K, Bergstrom R, Benefield J, Lozano Y, Young MR. 1997. Protein phosphatase-2A association with microtubules and its role in restricting the invasiveness of human head and neck squamous cell carcinoma cells. Cancer Lett 111:87–95.

Mills JC, Lee VM-Y, Pittman RM. 1998. Activation of a PP2A-like phosphatase and dephosphorylation of tau protein characterize onset of the execution phase of apoptosis. J Cell Sci 111:625–636.

Mumby MC, Walter G. 1993. Protein serine/threonine phosphatases: structure, regulation and functions in cell growth. Physiol Rev 73:673–697.

Napolitano EW, Liem RKH, Pachter JS, Chin SSM. 1985. β-internexin, a ubiquitous intermediate filament associated protein. J Cell Biol 101:1323–1331.

Ohkura H, Yanagida M. 1991. S. pombe gene sds22+ essential for a midmitotic transition encodes a leucine-rich repeat protein that positively modulates protein phosphatase-1. Cell 64:149–157.

Okubo S, Ito M, Takashiba Y, Ichikawa K, Miyahara M, Shimizu H, Konishi T, Shima H, Nagao M, Hartshorne DJ. 1994. A regulatory subunit of smooth muscle myosin bound phosphatase. Biochem Biophys Res Commun 200:429–434.

Saito T, Shima H, Osawa Y, Nagao M, Hemmings BA, Kishimoto T, Hisagana S. 1995. Neurofilament-associated protein phosphatase 2A: its possible role in preserving neurofilaments in filamentous states. Biochemistry 34:7376–7384.

Sontag E, Numbhakdi-Craig V, Blom GS, Mumbym MC. 1995. A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J Cell Biol 128:1131–1144.

Sontag E, Nunbhakdi-Craig V, Lee G, Bloom GS, Mumby MC. 1996. Regulation of the phosphorylation state and microtubule-binding activity of Tau by protein phosphatase 2A. Neuron 17:1201–1207.

Sontag E, Sontag JM, Carcia A. 1997. Protein phosphatase 2A is a critical regulator of protein kinase C zeta signaling targeted by SV40 small t to promote cell growth and NF-kappa B activation. EMBO J 16:562–571.

Strack S, Westphal RS, Colbran RJ, Ebner FF, Wadzinski BE. 1997. Protein serine/threonine phosphatase 1 and 2A associate with and dephosphorylate neurofilaments. Mol Brain Res 49:15–28.

Toivola DM, Goldman RD, Garrod DR, Eriksson JE. 1997. Protein phosphatases maintain the organization and structural interactions of hepatic keratin intermediate filaments. J Cell Sci 110:23–33.

Veeranna KT, Sheety KT, Link WT, Jaffe H, Wang J, Pant HC. 1995. Neuronal cyclin-dependent kinase-5 phosphorylation sites in neurofilament protein (NF-H) are dephosphorylated by protein phosphatase 2A. J Neurochem 64:2681–2690.

Weizsaecker M, Deen DF, Rosenblum ML, Hoshino T, Gutin PH, Baker M. 1981. The 9L rat brain tumor: description and application of an animal model. J Neurol 224: 183–192.

Wisniewski T, Goldman JE. 1998. Alpha β-crystallin is associated with intermediate filaments in astrocytoma cells. Neurochem Res 23:386–392.

Yamashita K, Yasuda H, Pines J, Yasumoto K, Nishitani H, Ohtsubo M, Hunter T, Sugimura T, Nishimoto T. 1990. Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induce a premature mitosis-like state in BHK21 cells. EMBO J 9:4331–4338.

Yatsunami J, Fujiki H, Suganuma M, Yoshizawa S, Eriksson JE, Olson MOJ, Goldman RD. 1991. Vimentin is hyperphosphorylated in primary human fibroblasts treated with okadaic acid. Biochem Biophys Res Commun 177:1165–1170.

Zhu DM, Tekle E, Chock PB, Huang CY. 1996. Reversible phosphorylation as a controlling factor for sustaining calcium oscillations in HeLa cells: involvement of calmodulin-dependent kinase II and a calyculin A-inhibitable phosphatases. Biochemistry 35:7214–7223.