Protein Phosphatase 2A Inhibitors, Phoslactomycins. Effects on the Cytoskeleton in NIH/3T3 Cells[1]

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Received September 9, 1998; accepted February 12, 1999

Protein phosphorylation is a key regulatory mechanism of the organization and dynamics of the actin cytoskeleton during cell motility, differentiation, and cytokinesis. The level of protein phosphorylation is dependent on the relative activities of both protein kinases and protein phosphatases. In this paper, we examined the effect of phoslactomycins (PLMs) on the regulation of the cytoskeleton of NIH/3T3 fibroblasts. Treatment of cells with PLM-F (10 μM) induced actin filament depolymerization after 4 h. This effect was reversible and actin filaments were reformed 1 h after removal of the inhibitors. As PLM-F had no effect at all on polymerization of purified actin *in vitro*, it is thought that PLMs induce actin depolymerization through an indirect mechanism. An *in vitro* assay showed PLMs inhibited protein phosphatase 2A at lower concentrations (IC_{50} 4.7 μM) than protein phosphatase 1. An *in situ* phosphorylation assay also revealed that PLM-F treatment stimulated the phosphorylation of intracellular vimentin. These results suggest that phoslactomycins are protein phosphatase 2A-specific inhibitors and that protein phosphatase 2A is involved in regulation of the organization of the actin cytoskeleton.

Key words: actin, protein phosphatase 1, protein phosphatase 2A, site-specific phosphorylated antibodies, vimentin.

Protein phosphorylation is involved in the regulation of components of the cytoskeleton, namely, microfilaments, microtubules, and intermediate filaments. Changes in the organization of actin filaments are involved in membrane ruffling, motility, and transformation. This regulation is achieved, in part, by phosphorylation of either actin itself (*1, 2*) or actin-associated proteins such as fragmin, myosin, or heat shock protein 25/27 (*3-6*). It has also been shown that protein phosphorylation is a principal mechanism in the regulation of intermediate filament polymerization through the N-terminal domain (*7-10*).

To date, many papers have appeared describing the relationship between the regulation of the cytoskeleton and the role of protein kinases. In contrast, the biological role of protein phosphatases in this regulation has received little attention, partly because there are few specific inhibitors for protein phosphatase (PP)-1, 2A, 2B, and 2C (*11*). Okadaic acid and calyculin A are potent inhibitors for both PP1 and PP2A, with a preference for PP2A (*12-14*). On the other hand, tautomycin is a different type inhibitor that inhibits PP1 more strongly than PP2A (*15, 16*). In general, the substrate specificities of these inhibitors differ in their IC_{50} values for PP1 and PP2A by only about 100-fold or less. This difference is not sufficient for investigating the biological meaning of protein phosphatases in cultured cells since both PP1 and PP2A are equally inhibited by these inhibitors at the concentrations normally used for cell treatments.

Phosphazomycins (*17, 18*), phoslactomycins (*19, 20*), and phospholine (*21, 22*) were isolated as antifungal antibiotics from the soil bacteria species *Streptomyces*. Leustroducsins were reported to be inducers of a colony-stimulating factor in bone marrow stromal cells (*23, 24*). These compounds commonly contain an α,β-unsaturated δ-lactone, an amino group, a phosphate ester, and a cyclohexane ring, but have a different substituents bound to the cyclohexane ring. We investigated the effects of phoslactomycins (PLMs) on the organization of the cytoskeleton in murine fibroblast NIH/3T3 cells. This report also shows that phoslactomycins exert cytotoxic effects by inhibiting protein dephosphorylation.

[1] This work was supported by a Grant for Multibioprobe (RIKEN), a Grant from the Ministry of Education, Science, Sports and Culture of Japan, and the Grant MPG215 awarded to G.M.

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Abbreviations: EDTA, ethylenediamine-*N,N,N',N'*-tetraacetic acid; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; IC_{50}, inhibitory concentration 50%; PLM, phoslactomycin; PP1, protein phosphatase type-1; PP2A, protein phosphatase type-2A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Cell Culture, Chemicals, and Fluorescence Microscopy—Murine fibroblast NIH/3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For morphological studies, the cells were examined under a phase contrast microscope after exposure to or removal of PLMs. For microinjection and fluorescence microscopy studies, the cells were seeded at a low density on acid-washed glass coverslips and grown for 24 h at 37°C. Protein phosphorylation levels were analyzed in a confluent culture of cells on 35-mm plastic cell culture dishes. Stock solutions of the inhibitors used in this paper were prepared in methanol.

For F-actin staining, cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline, and treated with 0.1 μg/ml rhodamine phalloidin (Molecular Probes, OR). *In vitro* actin assembly assays using the fluorescence Prodan-labeled G-actin were performed as described (*25*).

Protein Phosphatase Assay In Vitro—Protein phosphatase type-1 (PP1; from rabbit skeletal muscle) and protein phosphatase type-2A (PP2A; from human red blood cells) were purchased from Upstate Biotechnology (NY). *In vitro* protein phosphatase assays were carried out at 30°C for 10 min using 0.05 unit/60 μl of the enzyme and ^{32}P-labeled phosphorylase (Gibco), which was prepared from phosphorylase kinase and γ-[^{32}P]ATP. The reaction was terminated by the addition to each sample of 4.5 volumes 20% trichloroacetic acid and chilling on ice. After centrifugation, the clear supernatant was collected and the amount of radioactivity released as $^{32}P_i$ was determined using a liquid scintillation counter, Packard TRICURVE 2000.

Dual-specificity phosphatase, VHR (*26*), was overexpressed in *Escherichia coli* BL21 (DE3) using pET-3a (Stratagene) and purified as described (*27*). Phosphatase assays were performed at 30°C using 10 mM pNPP as a substrate in 0.2 ml reaction solution (50 mM succinate, 1 mM EDTA, 150 mM NaCl, pH 6.7). After incubation for 3 min, phosphatase reactions were terminated by adding 1 ml of 1 N NaOH, and the increases in absorbance at 405 nm of the product *para*-nitrophenolate were measured (*28*).

The cDNA of PTP-S2, a protein tyrosine phosphatase, was cloned by R.S. Reddy and G. Swarup (*29*). The plasmid to express the GST-PTP-S2 fusion protein was constructed by inserting of the full-length PTP-S2 cDNA into pGEX1 vector. Phosphatase assays were performed at 30°C using 10 mM pNPP as a substrate in 0.2 ml reaction solution (100 mM sodium acetate, 1 mM EDTA, 0.2% NP-40, pH 6.0). After incubation for 10 min, phosphatase activity was determined as above.

Phosphorylation in Intact Cells—[^{32}P]Orthophosphate (0.2 mCi) (ICN Pharmaceuticals, CA) was added to cells at the same time as the drugs. The reaction was stopped by washing with ice-cold Stop buffer (10 mM HEPES, 100 mM NaF, 10 mM EDTA, and 80 mM sucrose, pH 8.0). The cells were harvested by centrifugation at 1,000 $\times g$ for 5 min and homogenized with a sonicator (Handy Sonic, Tomy Seiko, Tokyo). After centrifugation at 20,000 $\times g$ for 5 min, the soluble fractions were analyzed by SDS-PAGE and the ^{32}P-phosphorylated proteins were visualized by autoradiography.

Immunoprecipitation, Isolation, and Immunoblotting of Vimentin—The immunoprecipitation and isolation of vimentin from cell extracts was performed as described (*30*). Immunoblotting of vimentin with site-specific phosphorylated vimentin antibodies was performed as below. After the cells were treated with one of the drugs for 15 min, they were lysed with lysis buffer [50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM vanadate, 1 mM dithiothreitol, 0.1% Tween-20, 1% Nonidet P-40, 10% glycerol, and proteinase inhibitors, pH 7.5] and homogenized with a sonicator. The nuclei were removed by centrifugation, and proteins in the supernatant fraction were electrophoresed and blotted onto a polyvinylidene fluoride membrane. The membrane had been treated with antibodies against site-specific phosphorylated vimentin [4H4, 4A4 (*31*), MO82, YT33 (*32*), and TM50 (*33*)]. The phosphorylated vimentin was detected using the SuperSignal Substrate (Pierce, Rockford), and the phosphorylation levels were quantified with a densitometer (Molecular Dynamics).

RESULTS

Effects of Phoslactomycins on Actin Filaments in NIH/3T3 Cells—Phoslactomycins (PLMs, Fig. 1) were origi-

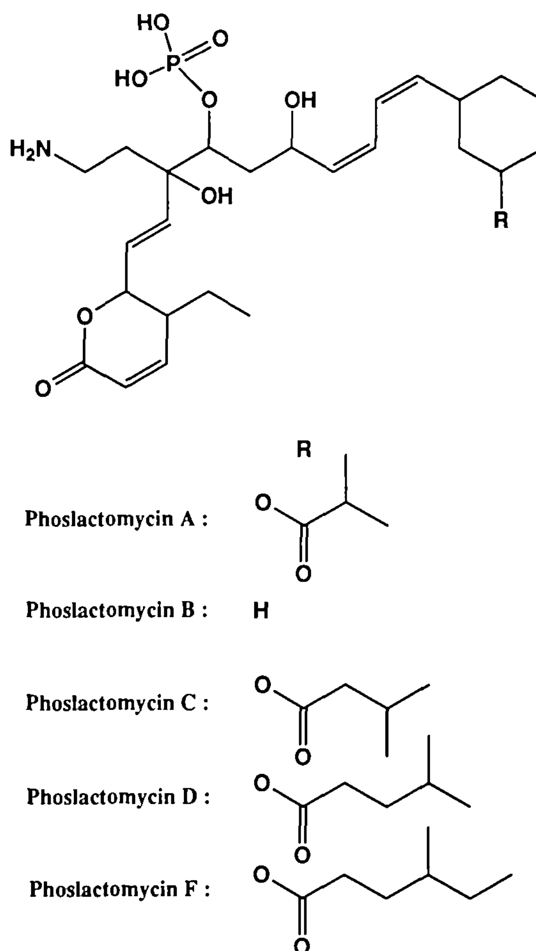

Fig. 1. **Structures of phoslactomycins.**

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nally isolated as antifungal antibiotics and found to induce morphological changes in various mammalian cells. To elucidate the mechanism of its action, we investigated the effect of PLMs on the organization of the actin cytoskeleton of NIH/3T3 cells using rhodamine phalloidin staining. Whereas control cells showed brightly labeled, actin stress fibers that often spanned the length of the cell (Fig. 2A), actin filaments were largely absent in cells treated with 10 μM PLM-F for 24 h (Fig. 2B). These effects were reversible and the reorganization of the actin cytoskeleton occurred 3 h after removal of the inhibitor (Fig. 2C). These effects of PLM-F were time-dependent. More detailed time-course studies revealed that the inhibitor required at least 4 h to completely break down the actin cytoskeleton. On the other hand, a partial reorganization of the actin cytoskeleton was observed within 1 h of removing the PLM-F (data not shown). These effects were also observed with the other derivatives tested. PLM-A, B, C, E induced the disassembly of actin filaments at exposures of 10 μM for 4 h (Fig. 3). However, PLM-F had no direct effects on polymerization processes nor did it induce the depolymerization of filamental actin *in vitro* (data not shown).

PLMs Inhibit Protein Phosphatase 2A and Induce the Phosphorylation of Vimentin In Situ—Actin polymerization/depolymerization is known to be influenced by phosphorylation. So we examined the effects of PLM-F on protein phosphatases *in vitro* using glycogen phosphorylase, a well-characterized substrate of PP1 and PP2A. Surprisingly, PLM-F showed only PP2A-specific inhibitory activity (Fig. 4). PLM-F caused a 50% inhibition of PP2A activity (IC_{50}) at 4.7 μM (*ca.* 3 μg/ml) but, at the same concentration, PLM-F did not inhibit PP1 activity at all. PLM-F did not inhibit PP1, dual-specificity phosphatase VHR, or tyrosine phosphatase PTP-S2 at all, even at 200 μM. PP2A-specific inhibitory activity was also observed for other derivatives, PLM-A, B, C, and E, which differ only in the side chains on the cyclohexane ring (Table I).

We next determined the effect of PLM-F on the overall profile of protein phosphorylation in NIH/3T3 cells. In this experiment, we used PLM-F at 40 μM to inhibit PP2A but not PP1. SDS-PAGE autoradiograms of the soluble fractions of the control and PLM-F-treated fibroblasts are shown in Fig. 5A. A potent protein phosphatase inhibitor, calyculin A, elevated the phosphorylation level of many proteins (Fig. 5A, lane 5). On the other hand, PLM-F increased the phosphorylation levels of proteins with molecular masses about 65- and 58-kDa (Fig. 5A, lane 3, arrowhead). Immunoprecipitation and partial purification indicated the phosphorylated 58-kDa protein to be vimentin (Fig. 5A, lane 4, arrowhead). To determine the phosphorylation sites on vimentin, we used site-specific phosphorylated vimentin antibodies (Fig. 5B). Total cell lysates

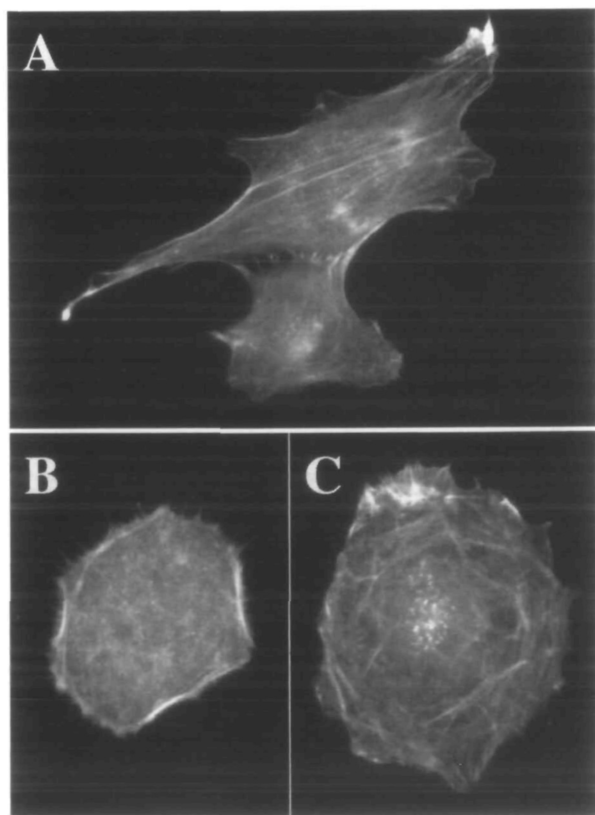

Fig. 2. **PLM-F induces the actin filament breakdown in NIH 3T3 cells.** The cells were fixed and stained with rhodamine-phalloidin. (A) Control cells. (B) The cells treated with 10 μM PLM-F for 24 h. (C) The cells at 3 h after removal of PLM-F from (B).

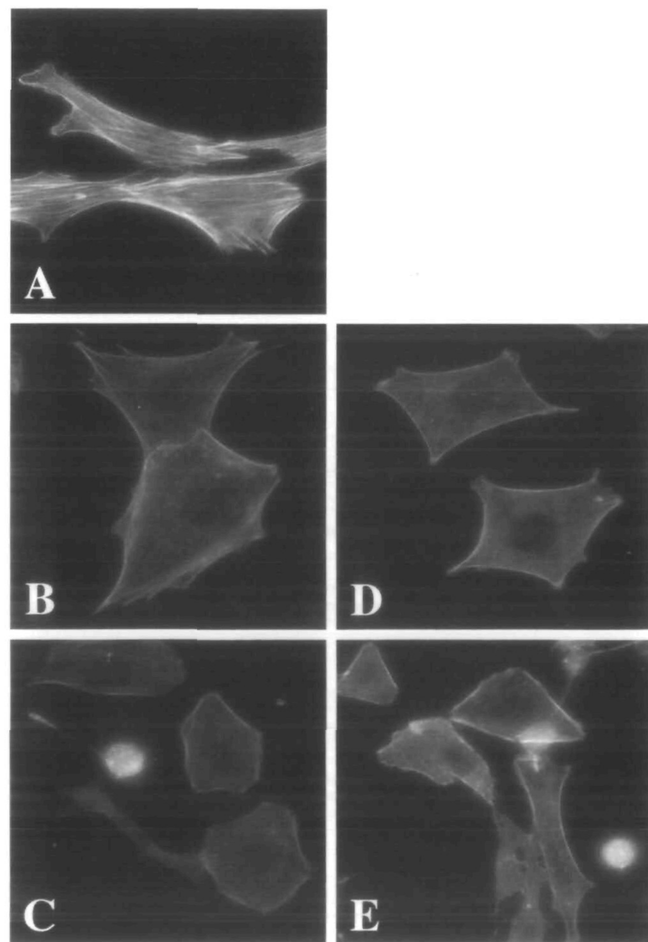

Fig. 3. **PLMs induces the actin filament breakdown in NIH/3T3 cells.** The cells were fixed and stained with rhodamine-phalloidin. (A) Control cells. (B–E) The cells treated for 4 h with 10 μM PLM-A, B, C, and D, respectively.

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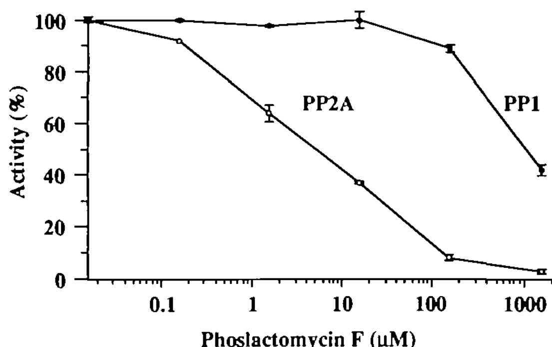

Fig. 4. **Inhibition of protein phosphatases by PLM-F.** The activity of PP1 (●) and PP2A (○) in the presence of different concentrations of PLM-F is expressed as a percentage of the activity measured without added PLM-F. Bars show mean±deviation obtained from two independent experiments.

of control and phosphatase inhibitor-treated cells were electrophoresed, blotted on membranes, and detected with antibodies. 4H4 is a control antibody that recognizes both phosphorylated and non-phosphorylated vimentin. YT33, TM50, 4A4, and MO82 recognize phosphorylated vimentin at Ser33, Ser50, Ser55, and Ser82, respectively. Calyculin A treatment stimulated the phosphorylation of Ser55 (4A4) and Ser82 (MO82) of vimentin up to 171 and 130% of control (4H4), respectively. On the other hand, only the phosphorylation of Ser82 (MO82) was specifically enhanced (to 164% of control) by treatment with PLM-F (Fig. 5B). The *in situ* phosphorylation levels of Ser50 and Ser33 remained unchanged by treatment with these phosphatase inhibitors.

DISCUSSION

In this paper, we studied the effects of PLMs on the actin cytoskeleton of live NIH/3T3 cells. Exogenously added PLM-F induced actin depolymerization and its effects were

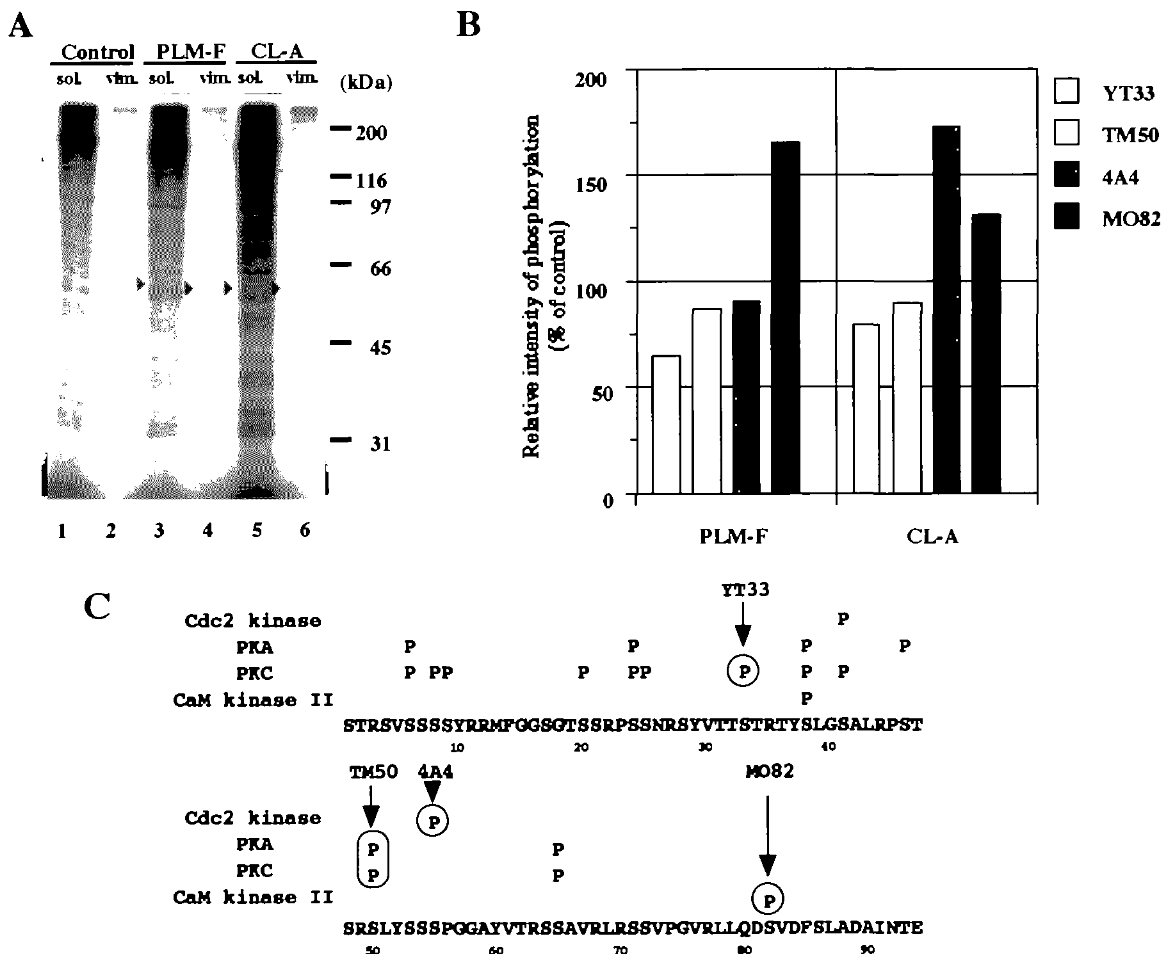

Fig. 5. **Effect of PLM-F on protein phosphorylation in NIH/3T3 cells.** (A) SDS-PAGE autoradiograms patterns of soluble fractions (lanes 1, 3, and 5) and vimentin partial purification fractions (lanes 2, 4, and 6). Lanes 1 and 2, control cells; lanes 3 and 4, 40 μM PLM-F treated cells; lanes 5 and 6, 0.5 μM calyculin A treated cells. (B) Relative intensity of phosphorylated vimentin at Ser33, Ser50, Ser55, and Ser82 of 40 μM PLM-F treated cells. After immunoblotting of the control and PLM-F treated cells using mouse monoclonal antibodies against site-specific phosphorylated vimentin, 4H4, 4A4, MO82, TY50, and YT33. Phosphorylation levels of vimentin were quantified with Scanning Imager (Molecular Dynamics). (C) Phosphorylation sites of vimentin head domain (1-94). Phosphorylation sites by Cdc2 kinase, PKA, PKC, and Ca^{2+}/calmodulin kinase II are shown by P. Recognition sites by site-specific phosphorylated vimentin, 4A4, MO82, TY50, and YT33 are shown by arrows.

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reversible (Fig. 2). According to a time-course observation, the disappearance of the actin filaments required at least 4 h, but its reorganization after removal of the drug was observed after 1 h. The breakdown of the cytoskeleton by PLM-F was much slower than that by cytochalasin B, which depolymerizes the actin filaments in cells within several minutes (unpublished data). Moreover, PLM-F had no effects on polymerization processes nor did it induce the depolymerization of filamental actin *in vitro* (data not shown). These results suggest that PLM-F induces actin disassembly by an indirect mechanism.

Actin polymerization/depolymerization is known to be regulated by phosphorylation (*1-6*), so we performed *in vitro* protein phosphatase assay using Ser/Thr phosphatase type-1 and -2A, tyrosine phosphatase PTP-S2, and dual-specificity phosphatase VHR. Surprisingly, PLM-F selectively inhibited PP2A activity. As shown in Fig. 4, the IC_{50} value of PLM-F for PP2A was 4.7 μM, whereas concentrations up to 200 μM of PLM-F did not inhibit PP1, VHR, or PTP-S2 activity. The IC_{50} value for PP1 was estimated to be about 1,250 μM, but we could not determine the exact IC_{50} value because of the low solubility of PLM-F in the assay solution (data not shown).

An *in situ* phosphorylation assay also revealed an enhanced phosphorylation of p58, which was identified as vimentin by immunoprecipitation and partial purification (Fig. 5A). Recently, Ho and Roberge reported that fostriecin, a PLM-F analog, stimulates vimentin hyperphosphorylation *in situ* (*30*), and our finding is coincident with their results. Vimentin is an intermediate filament and its polymerization/depolymerization cycle is regulated by phosphorylation at the N-terminal domain (*8, 34*). PLM-F induced the hyperphosphorylation of vimentin Ser82 (Fig. 5B), which is phosphorylated by Ca^{2+}/calmodulin kinase II (*32*), but we did not observe any vimentin depolymerization by indirect immunofluorescence microscopy (data not shown). These results suggest that the polymerization/depolymerization of vimentin is not influenced by the phosphorylation of Ser82. It has been reported that fostriecin induces vimentin hyperphosphorylation primarily at two sites that are phosphorylated by protein kinase C *in vitro* (*30*). As Ser82 of vimentin is phosphorylated by Ca^{2+}/calmodulin kinase II and not by protein kinase C *in vivo* and *in situ* (*32*), protein kinase C phosphorylation sites might be dephosphorylated by PP1. We could not rule out the possibility that other sites on vimentin might be recognition sites for PP2A, because we used only 4 kinds of antibodies.

There are many PLM derivatives with different side chains on the cyclohexane ring. We investigated the effects of PLM-A, B, C, and E on both actin filaments *in situ* and the phosphatase *in vitro*. We could not find any differences in the effects of the derivatives (Fig. 3 and Table I). These results strongly suggest that the PP2A-specific inhibitory activity of PLMs derives from their fundamental structure, an α,β-unsaturated δ-lactone, an amino group, a phosphate ester, and a cyclohexane ring.

There are several reports that okadaic acid, calyculin A, and tautomycin induce morphological changes in cells through the hyperphosphorylation of several proteins, including 440-kDa protein, myosin heavy/light chain, and vimentin (*35, 36*). However, there is the possibility that these effects might result from the complete loss of various

TABLE I. **The IC_{50} values of PLMs for protein phosphatases.**

	IC_{50} (μM)			
	PP1	PP2A	VHR	PTP-S2
PLM-A	>1000	3.7	>200	>200
PLM-B	>1000	5.8	>200	>200
PLM-C	>1000	4.9	>200	>200
PLM-D	>1000	4.0	>200	>200
PLM-F	>1000	4.7	>200	>200

phosphatase activities, since the inhibition specificity of these drugs for each phosphatase is low. It is therefore necessary to exploit specific PP1 or PP2A inhibitors in order to elucidate the function of PP1 or PP2A *in situ*. In this context, fostriecin, an analog of phoslactomycins, has been reported to be a good inhibitor of PP2A *in vitro* (*37, 38*). PLMs (IC_{50} 3.7-5.8 μM) are not as potent inhibitors as fostriecin [IC_{50} 40 (*38*), 3.2 nM (*37*)], however, the specificity for PP2A is satisfactory. The significant difference between PLMs and fostriecin is the presence of an amino group in the PLM structure. The amino group may decrease the inhibitory activity toward PP2A through intramolecular hydrogen bond formation with the phosphate ester. However, it is noteworthy that PLM-F shows specific inhibition toward PP2A (IC_{50} 4.7 μM) compared with PP1 (IC_{50} > 1000 μM).

We thank Dr. Jie Cui, Ifeaka Mordi, and Manfred Heidecker for useful discussions.

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