

# Differential Targeting of Protein Kinase C and CaM Kinase II Signalings to Vimentin

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**Abstract.** Hydrolysis of inositol phospholipids by receptor stimulation activates two separate signaling pathways, one leading to the activation of protein kinase C (C kinase) via formation of diacylglycerol. The other is the inositol trisphosphate (IP<sub>3</sub>)/Ca<sup>2+</sup> pathway and a major downstream kinase which is activated is Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaM kinase II). To examine signaling pathways of C kinase and CaM kinase II to the cytoskeletal protein vimentin, we prepared monoclonal antibodies YT33 and MO82 which recognize the phosphorylation state of vimentin by C kinase and by CaM kinase II, respectively. Ectopic expression of constitutively active C kinase or CaM ki-

nase II in primary cultured astrocytes by microinjection of the corresponding expression vectors induced phosphorylation of vimentin at each specific phosphorylation site, followed by reorganization of vimentin filament networks. In contrast, simultaneous activation of C kinase and CaM kinase II by inositol phospholipid hydrolysis with receptor stimulation led to an exclusive phosphorylation of vimentin at the CaM kinase II site, not at the site of C kinase. These results indicate that the intracellular targeting of C kinase and CaM kinase II signalings to vimentin is regulated separately, under physiological conditions.

RECEPTOR-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>)<sup>1</sup> relays extracellular signals into the cell and two kinases, protein kinase C (C kinase) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaM kinase II) play a central role in this signaling. Hydrolysis of PIP<sub>2</sub> leads to formation of diacylglycerol and activation of inositol trisphosphate (IP<sub>3</sub>)-induced Ca<sup>2+</sup> signaling (for reviews see Michell, 1992; Berridge, 1993). C kinase and CaM kinase II, activated by diacylglycerol and Ca<sup>2+</sup>, respectively, transmit these two signalings to downstream molecules (for reviews see Nishizuka, 1984, 1986; Colbran et al., 1989; Hanson and Schulman, 1992). From the viewpoint of signaling cascade, one of the most distinctive features of kinases represented by C kinase and CaM ki-

nase II is a broad spectrum of substrate specificity in vitro (Nishizuka, 1986; Pearson and Kemp, 1991; Hanson and Schulman, 1992). This feature ensures that these kinases relay signals to divergent molecules and is, therefore, indispensable for the regulation of multiple cellular activities.

Identifying the physiological substrates of these kinases is of great importance in order to understand their intracellular signaling pathways and functions. Putative substrates have been documented based on in vitro assays in which purified proteins were phosphorylated by these kinases at a reasonable rate and stoichiometry (Nishizuka, 1986; Pearson and Kemp, 1991; Hanson and Schulman, 1992). However not all were phosphorylated in vivo. Moreover, recent studies have shown that kinases involved in cell signaling, including C kinase and CaM kinase II, are targeted to specific cellular compartments and are translocated dynamically during signaling events (for review see Inagaki et al., 1994b), thereby indicating that endogenous substrates of a kinase may be elaborately regulated in the cell and that the signaling cascade may differ according to cellular states. Therefore, to identify physiological substrates, it is essential to demonstrate that they are phosphorylated by a kinase in vivo.

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1. *Abbreviations used in this paper:* A kinase, cAMP-dependent protein kinase; CaM kinase II, Ca<sup>2+</sup>/calmodulin dependent protein kinase II; C kinase, protein kinase C; IP<sub>3</sub>, inositol trisphosphate; PGF<sub>2α</sub>, prostaglandin F<sub>2α</sub>; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine.

For this purpose, various criteria have been used (Hanson and Schulman, 1992). First, putative substrates have to be phosphorylated by a kinase *in vitro* at a reasonable rate and stoichiometry. Second, they have to be phosphorylated *in vivo* by suitable stimulations, such as phorbol ester for C kinase and Ca<sup>2+</sup> ionophores for CaM kinase II. Other kinases can be activated directly or indirectly by these stimulations. Thus, it is essential to demonstrate that phosphorylation sites of a putative protein in stimulated cells overlap those identified with the purified kinase. Two-dimensional analysis of tryptic phosphopeptides is widely used to compare the phosphorylation sites of a protein *in vitro* and *in vivo* (Griffith and Schulman, 1988; Fackler et al., 1990; Jefferson and Schulman, 1991). However, this faithful method is laborious and it is not always easy to obtain consistent data. It is also difficult to differentiate the sites of phosphorylation when multiple sites are located in the same digested peptide.

Phosphorylation state-specific antibodies which recognize the phosphorylated serine/threonine residue and its flanking sequence provide a useful tool to analyze site specific protein phosphorylation of a target protein *in vivo* (Nishizawa et al., 1991; Czernik et al., 1991; Matsuoka et al., 1992; Inagaki et al., 1994a, b). Analysis of *in vivo* substrate of a kinase is feasible using phosphorylation state-specific antibodies, if they are raised against a unique phospho-serine/threonine residue which is specifically catalyzed by the kinase *in vitro* (Tsujimura et al., 1994). In the present study, we investigated phosphorylation of the cytoskeletal protein vimentin, by C kinase and CaM kinase II in astrocytes, by preparing two monoclonal antibodies which specifically recognize site-specific phosphorylations of vimentin by C kinase and by CaM kinase II, respectively. Ectopic expression of constitutively active C kinase or CaM kinase II in astrocytes induced specific phosphorylation of vimentin at the C kinase and CaM kinase sites, respectively, suggesting that both enzymes can phosphorylate vimentin *in vivo*. In contrast, simultaneous activation of C kinase and CaM kinase II by PIP<sub>2</sub> hydrolysis with receptor stimulation led to an exclusive phosphorylation of vimentin by CaM kinase II but not by C kinase. These observations revealed the strict and separate regulation of intracellular targeting of C kinase and CaM kinase II signalings, under physiological conditions, and this regulation may account for the elaborately coordinated cellular responses mediated by these kinases.

## Materials and Methods

### Peptide Synthesis and Production of Monoclonal Antibodies

Vimentin peptides, PV33 (Cys-Ser-Tyr-Val-Thr-Thr-phosphoSer<sup>33</sup>-Thr-Arg-Thr-Tyr-Ser), V33 (Cys-Ser-Tyr-Val-Thr-Thr-Ser-Thr-Arg-Thr-Tyr-Ser), PV82 (Cys-Arg-Leu-Leu-Gln-Asp-phosphoSer<sup>62</sup>-Val-Asp-Phe-Ser-Leu), and V82 (Cys-Arg-Leu-Leu-Gln-Asp-Ser-Val-Asp-Phe-Ser-Leu) were synthesized as described previously (Tsujimura et al., 1994). Monoclonal antibodies against PV33 (YT33) and PV82 (MO82) were produced, following the method described previously (Tsujimura et al., 1994).

### Preparation of Proteins

A full-length mouse vimentin cDNA was constructed as a hybrid from pCV-877 and pV-C25 obtained from Amer. Type Culture Collection (Rock-

ville, MD). An Nde I site was engineered at the beginning of the coding segment, using oligonucleotide-directed mutagenesis. This cDNA was inserted into the Nde I-Bam HI site of plasmid pET-3a (Novagen). For bacterial expression, *E. coli* strain BL21(DE3)pLysS was transformed with this plasmid. Recombinant vimentin, C kinase, and CaM kinase II were prepared as described previously (Inagaki et al., 1985, 1987; Yamauchi and Fujisawa, 1983). Protein concentration was determined according to Bradford (1976). Vimentin (0.15 mg/ml) was phosphorylated by incubation for 40 min with 5 µg/ml C kinase, 0.1 mM ATP, 0.3 mM MgCl<sub>2</sub>, 50 µg/ml phosphatidylserine, 2.5 µg/ml diacylglycerol, 25 mM Tris-HCl (pH 7.5) at 25°C or by incubation for 40 min with 5 µg/ml CaM kinase II, 0.1 mM ATP, 0.1 mM CaCl<sub>2</sub>, 20 µg/ml calmodulin, 3 mM MgCl<sub>2</sub>, 25 mM Tris-HCl (pH 7.0) at 25°C.

### ELISA and Western Blotting

All the procedures have been described in detail elsewhere (Nishizawa et al., 1991; Yano et al., 1991a).

### Cell Preparation

Primary cultured astrocytes were prepared from the cerebral cortices of newborn rats, as described previously (Inagaki et al., 1989) and cells cultured in Eagle's minimal essential medium containing 10% FCS for three weeks were used for experiments. In some experiments, astrocytes were differentiated by incubation for 2 d with 250 µM dibutyryl cAMP in serum free medium (Trimmer et al., 1982).

### Phosphorylation of Purified Vimentin by the Soluble Fraction of Astrocytes

Cultured astrocytes were scraped, poured into tubes, sonicated in a buffer (pH 7.5) containing 20 mM Tris-HCl, 10 mM 2-mercaptoethanol, 2 mM EGTA, 0.5 mM dithiothreitol, 50 mM NaCl, 20 mM β-glycerophosphate, and 5 µg/ml leupeptin, and then centrifuged at 100,000 g for 30 min. For C kinase assay, the soluble fraction (30 µg protein/ml) was incubated for 45 min with 0.15 mg/ml vimentin in 25 mM Tris-HCl (pH 7.5), 0.3 mM MgCl<sub>2</sub>, 0.1 mM ATP, and 0.1 µM calyculin A in the presence or absence of 0.1 µg/ml 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), 50 µg/ml phosphatidylserine (PS), and 0.1 mM CaCl<sub>2</sub> at 25°C. For CaM kinase II assay, the same fraction was incubated for 15 min with 0.15 mg/ml vimentin in 25 mM Tris-HCl (pH 7.5), 3 mM MgCl<sub>2</sub>, 0.1 mM ATP, and 0.1 µM calyculin A in the presence or absence of 20 µg/ml calmodulin and 0.1 mM CaCl<sub>2</sub> at 25°C.

### Measurement of C Kinase Activity in the Soluble and Particulate Fractions

The soluble and particulate fractions of astrocytes were prepared as described in detail elsewhere (Chida et al., 1986). C kinase activity was assayed by measuring incorporation of <sup>32</sup>P from [<sup>γ</sup>-<sup>32</sup>P]ATP to histone H1 (Inagaki et al., 1985).

### Drug Application and Microinjection

Drugs were dissolved in HEPES-buffered Krebs-Ringer solution (pH 7.4) and applied to the cells. For microinjection, cells were subcultured on glass coverslips. 2 d after the seeding, plasmid DNA (1 µg/µl) for the constitutively active form of C kinaseδ, DR144/145A or CaM kinase II α subunit together with 12 µg/µl dextran-rhodamine B (MW 10,000, Molecular Probes) were injected into the nuclei of the cells. The preparation of DR144/145A has been described elsewhere (Hirai et al., 1994). The plasmid for constitutively active CaM kinase II was constructed by subcloning the cDNA for the kinase domain, α-I (Yamauchi et al., 1989), into an expression vector, pEF321 (Goshima et al., 1993).

### Immunocytochemical Procedures

For light microscopic observations, cells were fixed for 10 min with 3% formaldehyde in phosphate buffered saline (PBS), permeabilized for 15 min with 0.2% Triton X-100 in PBS, and then visualized by immunofluorescence, using YT33 (3 µg/ml) or MO82 (0.2 µg/ml) in PBS, as first antibodies. Electron microscopic observations were made according to the previously described method (Tsujimura et al., 1994), with slight modifications. Cells were fixed for 30 min with 3% formaldehyde in 2-(*N*-mor-

pholino) ethanesulfonic acid (MES) buffer (0.1 M MES [pH 6.6], 0.5 mM MgSO<sub>4</sub>, 2 mM EGTA), and then permeabilized for 2 min with 0.15% Triton X-100 in 0.1 M MES buffer. To reduce nonspecific staining they were treated with 0.5 mg/ml NaBH<sub>4</sub> in MES buffer for 20 min, followed by incubation for 1 h with 3% normal goat serum and 0.1% bovine serum albumin in MES buffer. The cells were then incubated overnight at 4°C with first antibodies MO82 (2 µg/ml) in MES buffer, and overnight at 4°C with 10-nm colloidal gold-conjugated goat anti-mouse antibodies in MES buffer. They were then fixed again with 1% glutaraldehyde and 0.5% tannic acid in 0.1 M cacodylate buffer (pH 7.4) for 30 min, followed by postfixation with 1% OsO<sub>4</sub> in the same buffer for 1 h. The cells were dehydrated with ethanol and embedded in Epon 812. Thin sections were mounted on uncoated grids and doubly stained with uracyl acetate and lead citrate. Electron micrographs were taken on a JEM2000EX microscope (JOEL, Tokyo, Japan) at an accelerating voltage of 80 kV.

## Results

### Preparation and Characterization of Monoclonal Antibodies YT33 and MO82

Vimentin is an intermediate filament protein expressed in various types of cells and is phosphorylated *in vitro* by both

C kinase and CaM kinase II. Fig. 1 A shows sites of phosphorylation by protein kinases identified in *in vitro* studies (Ando et al., 1989, 1991; Chou et al., 1991; Kusubata et al., 1992). Ser33 and Ser82 of vimentin were identified as unique phosphorylation sites specific to C kinase and CaM kinase II, respectively (Fig. 1 A), therefore, these residues serve as pertinent indicators to study phosphorylation of vimentin by C kinase and CaM kinase II. To investigate the phosphorylation of Ser33 and Ser82, we prepared monoclonal antibodies YT33 and MO82, raised against the synthetic peptides PV33 (Phospho Vimentin Ser<sub>33</sub>; Cys-Ser-Tyr-Val-Thr-Thr-phosphoSer<sup>33</sup>-Thr-Arg-Thr-Tyr-Ser) and PV82 (Phospho Vimentin Ser<sub>82</sub>; Cys-Arg-Leu-Leu-Gln-Asp-phosphoSer<sup>82</sup>-Val-Asp-Phe-Ser-Leu), respectively (Fig. 1, B and C). Fig. 1, B and C shows the specificity of YT33 and MO82 examined by ELISA. YT33 bound to PV33 but not to the unphosphorylated peptide V33 (Cys-Ser-Tyr-Val-Thr-Thr-Ser-Thr-Arg-Thr-Tyr-Ser). MO82 reacted with PV82 but not with the unphosphorylated form V82 (Cys-Arg-Leu-Leu-Gln-Asp-Ser-Val-Asp-Phe-Ser-

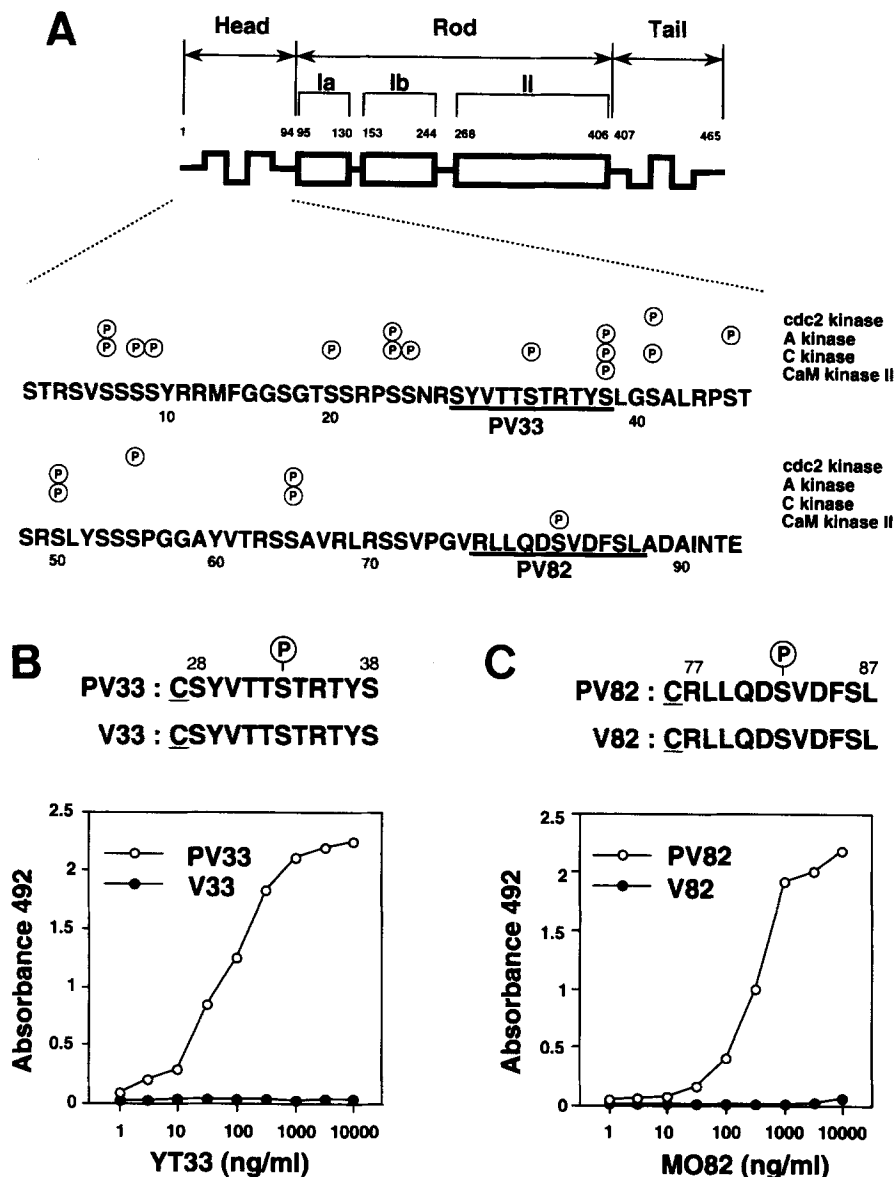
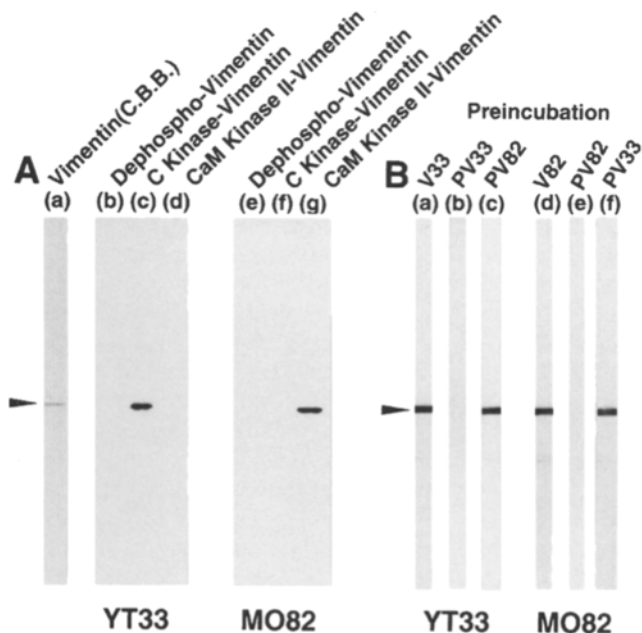


Figure 1. Specificity of monoclonal antibodies YT33 and MO82 analyzed by ELISA. (A) A map of vimentin molecule showing sites of phosphorylation by kinases identified in *in vitro* studies. The sites are indicated by P within a circle. The sites of sequences corresponding to the synthetic peptides PV33 and PV82 are underlined. (B) Amino acid sequences of the synthetic peptides PV33/V33 and specificity determination of YT33 by ELISA. (C) Amino acid sequences of the synthetic peptides PV82/V82 and specificity determination of MO82 by ELISA. (B and C) Microtiter plates were coated overnight with 60 µl (1 µg/ml) of the peptides, blocked and reacted with the antibody. The reactivity was then determined by measuring absorbance at 492 nm.

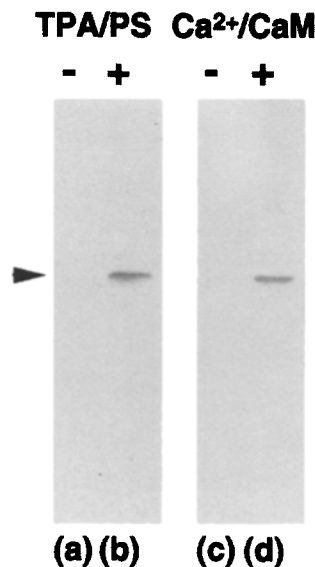


**Figure 2.** Specificity of monoclonal antibodies YT33 and MO82 analyzed by Western blotting. (A) Reactivity of YT33 and MO82 to unphosphorylated and phosphorylated vimentin. Unphosphorylated vimentin (a, b, and e) and vimentin phosphorylated at 2.2 mol phosphate/mol protein by C kinase (c and f) and vimentin phosphorylated at 0.7 mol phosphate/mol protein by CaM kinase II (d and g) were resolved by SDS-PAGE (100 ng in each lane) and transferred onto a poly vinylidene difluoride filter. Then the blots were stained with Coomassie brilliant blue (CBB, a), 3  $\mu$ g/ml YT33 (b, c, and d), or 1  $\mu$ g/ml MO82 (e, f, and g). (B) Specificity of YT33 and MO82 determined by an inhibition assay. Vimentin phosphorylated by C kinase (a, b, and c) and by CaM kinase II (d, e, and f) (100 ng in each lane) was stained with 3  $\mu$ g/ml YT33 (a, b, and c), or 1  $\mu$ g/ml MO82 (d, e, and f) preincubated with 100  $\mu$ g/ml of V33 (a), PV33 (b and f), PV82 (c and e), or V82 (d). Arrowheads indicate the site of vimentin migration.

Leu). The specificity of these antibodies was further checked by Western blotting. YT33 reacted with vimentin phosphorylated at 2.2 mol phosphate/mol protein by C kinase but not with nonphosphorylated vimentin or vimentin phosphorylated at 0.7 mol phosphate/mol protein by CaM kinase II (Fig. 2 A). On the other hand, MO82 reacted only with vimentin phosphorylated by CaM kinase II and not with nonphosphorylated vimentin or vimentin phosphorylated by C kinase (Fig. 2 A). Preincubation of YT33 with PV33 but not with V33 or PV82 inhibited the binding of YT33 to vimentin phosphorylated by C kinase (Fig. 2 B). Preincubation of MO82 with PV82 but not with V82 or PV33 prevented the reaction of MO82 with vimentin phosphorylated by CaM kinase II (Fig. 2 B). Thus, YT33 and MO82 specifically recognize the phosphorylation of vimentin at Ser33 by C kinase and at Ser82 by CaM kinase II, respectively.

#### Activities of C Kinase and CaM Kinase II in Astrocytes Detected by YT33 and MO82, Respectively

Immunocytochemical and biochemical studies revealed that astrocytes in primary culture express both C kinase and CaM kinase II (Neary et al., 1986; Masliash et al.,

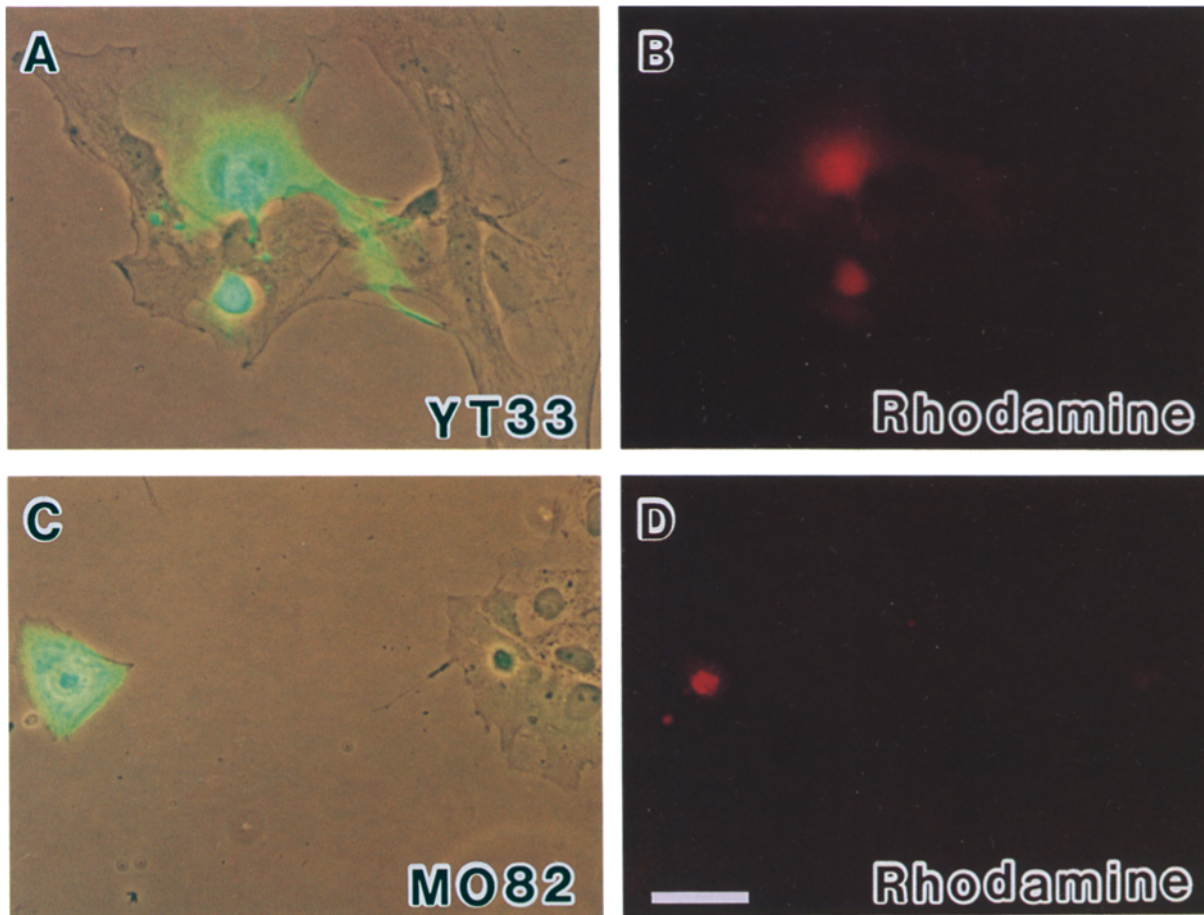


**Figure 3.** Detection of C kinase and CaM kinase II activities by YT33 and MO82 in a soluble fraction from the astrocyte lysate. Soluble fractions of astrocyte cell lysate (30  $\mu$ g protein/ml) were incubated with purified vimentin (0.15 mg/ml) in the presence (b) or absence (a) of 0.1  $\mu$ g/ml TPA and 50  $\mu$ g/ml PS. They were also incubated with vimentin in the presence (d) or absence (c) of 0.1 mM  $\text{CaCl}_2$  and 20  $\mu$ g/ml calmodulin. Western blotting was done with 3  $\mu$ g/ml YT33 (a and b) or 1  $\mu$ g/ml MO82 (c and d). The arrowhead indicates the site of vimentin migration.

1991; Yano et al., 1994). To confirm the usefulness of the present assay system, and the existence of C kinase and CaM kinase II in astrocytes, we first measured the activities of these kinases in astrocytes, using YT33 and MO82. The soluble fractions of astrocyte cell lysates (30  $\mu$ g protein/ml) were incubated with purified vimentin in the presence or absence of C kinase activators 0.1  $\mu$ g/ml TPA and 50  $\mu$ g/ml PS. Western blotting analysis with YT33 revealed the activation of endogenous C kinase that phosphorylated Ser33 of vimentin (Fig. 3). Similar results were obtained by endogenous CaM kinase II stimulated with 0.1 mM  $\text{CaCl}_2$  and 20  $\mu$ g/ml calmodulin (Fig. 3). Therefore, antibodies YT33 and MO82 can detect activities of endogenous C kinase and CaM kinase II in astrocytes, respectively.

#### Ectopic Expression of Active C Kinase or CaM Kinase II in Astrocytes Induced Phosphorylation of Vimentin at Ser33 or Ser82

To examine whether C kinase and CaM kinase II phosphorylate Ser33 and Ser82 of vimentin, respectively, in vivo, we next analyzed the phosphorylation of Ser33 and Ser82 in astrocytes which express an active form of C kinase or CaM kinase II, respectively. Plasmid DNA (1  $\mu$ g/ $\mu$ l) for the constitutively active form of C kinase $\delta$ , DR144/145A (Hirai et al., 1994) or CaM kinase II  $\alpha$  subunit (Yamauchi et al., 1989; Goshima et al., 1993) were microinjected into the nuclei of astrocytes together with 12  $\mu$ g/ $\mu$ l dextran-rhodamine. On the following day, the cells were immunocytochemically visualized, using YT33 or MO82. The cells to which the plasmid had been injected were identified by red staining of simultaneously injected dextran-rhodamine in the nuclei (Fig. 4, B and D). The staining of YT33 was specifically observed in rhodamine-positive cells, suggesting that ectopically expressed constitutively active C kinase phosphorylated Ser33 residue of vimentin in these cells (Fig. 4, A and B). Similarly only astrocytes to which the plasmid for CaM kinase II had been microinjected showed the staining of MO82 (Fig. 4, C and D). Cells expressing active C kinase were not stained with MO82, and cells expressing active CaM kinase II showed no YT33 im-



**Figure 4.** Phosphorylation of vimentin in astrocytes by ectopically expressed constitutively active forms of C kinase and CaM kinase II. (A) A fluorescent photomicrograph superimposed on the phase contrast image which shows Yt33 immunoreactive cells (green). (B) A fluorescent photomicrograph of the same frame as A, showing cells to which the plasmid DNA for constitutively active form of C kinase was injected (indicated by the red color of rhodamine). (C) A fluorescent photomicrograph superimposed on the phase contrast image showing an MO82 immunoreactive cell (green). (D) A fluorescent photomicrograph of the same frame as C, showing the cell to which the plasmid DNA for constitutively active form of CaM kinase II was injected (indicated by the red color of rhodamine). Plasmid DNA (1  $\mu\text{g}/\mu\text{l}$ ) for the constitutively active form of C kinase $\delta$ , DR144/145A (A and B) or CaM kinase II  $\alpha$  subunit (C and D) was microinjected into the nuclei of astrocytes together with 12  $\mu\text{g}/\mu\text{l}$  dextran-rhodamine. On the following day, the cells were immunocytochemically visualized using 3  $\mu\text{g}/\text{ml}$  Yt33 (A) or 0.2  $\mu\text{g}/\text{ml}$  MO82 (C). Bar, 40  $\mu\text{m}$ .

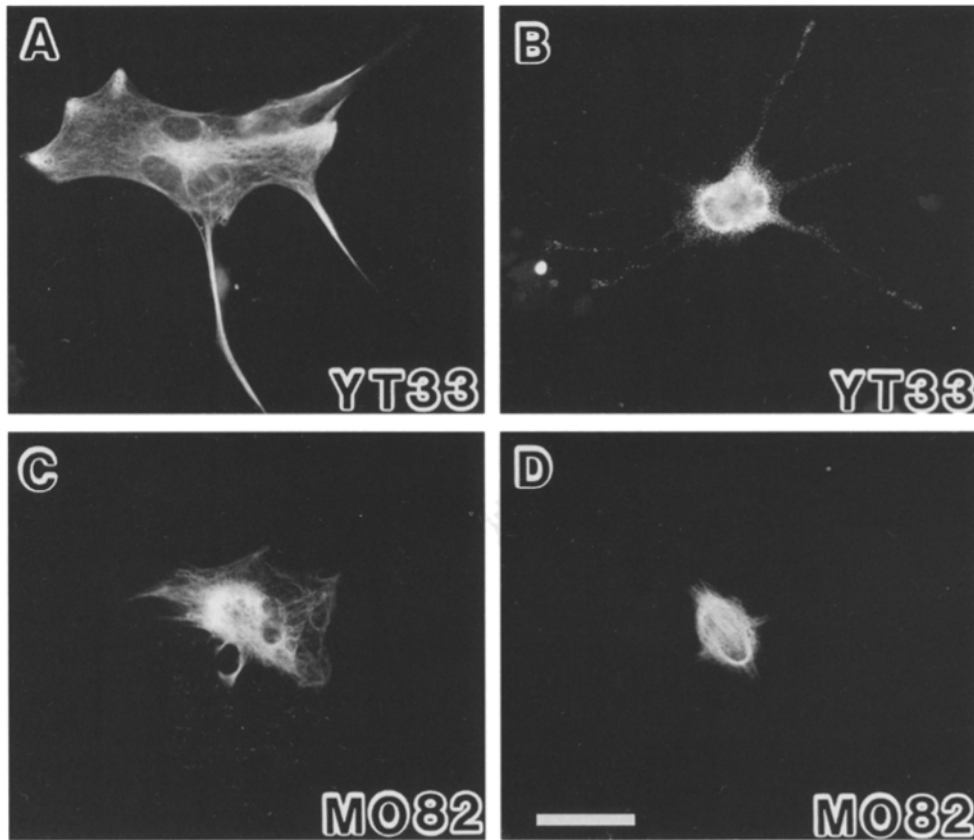
munoreactivity (data not shown). These results clearly demonstrate that C kinase and CaM kinase II selectively phosphorylate Ser33 and Ser82 residues of vimentin in the cell, respectively.

Some Yt33 or MO82 immunoreactive cells had the normal polygonal morphology of cultured astrocytes with a normal fine and well spread filament network of vimentin (Fig. 5, A and C). However, we often observed abnormal filament networks in astrocytes which expressed the active form of C kinase or CaM kinase II. In some cases, fiber bundles of phospho-vimentin completely collapsed to form a granular dot structure (Fig. 5 B). In other cases, vimentin constituted short filaments in bundles (Fig. 5 D). Astrocytes with such an abnormal vimentin filament organization were round without a normal flat morphology (Fig. 5, B and D). These results indicate that the site-specific phosphorylation of vimentin by C kinase or CaM kinase II induces dynamic changes in vimentin filament organization. Similar phosphorylation-induced reorganization and disassembly of intermediate filaments were noted in vitro (Ina-

gaki et al., 1987, 1988, 1990, 1994a; Evans, 1988; Geisler and Weber, 1988; Kitamura et al., 1989; Gonda et al., 1990; Tokui et al., 1990; Yano et al., 1991b), in fibroblasts to which the catalytic subunit of cAMP-dependent protein kinase (A kinase) had been microinjected (Lamb et al., 1989), and in mitotic cells in which cdc2 kinase activity was elevated (Chou et al., 1990; Tsujimura et al., 1994). The present observations provide further support for the notion that phosphorylation of intermediate filaments may regulate organization of filament networks.

**Activation of C Kinase and CaM Kinase II by PIP<sub>2</sub> Hydrolysis with Prostaglandin Receptor Stimulation Resulted in Phosphorylation of Vimentin by CaM Kinase II but Not by C Kinase**

We then asked whether endogenous C kinase or CaM kinase II activated by PIP<sub>2</sub> hydrolysis with receptor stimulation would phosphorylate vimentin in astrocytes. Astrocytes in primary culture are reported to express receptors



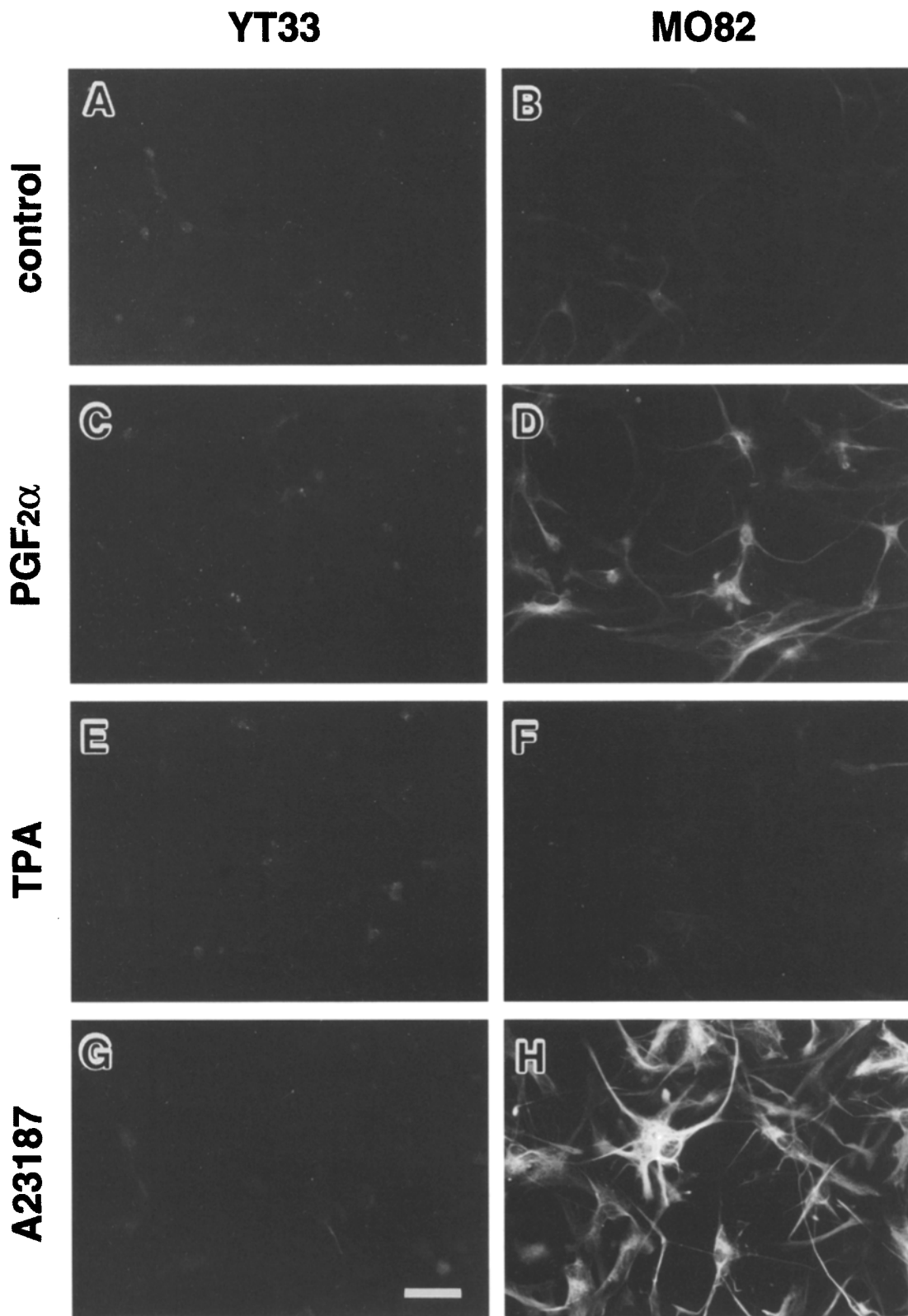
**Figure 5.** Effects of ectopically expressed constitutively active forms of C kinase and CaM kinase II on astrocyte morphology and structure of vimentin filaments. (A and B) Fluorescent photomicrographs of astrocytes into which plasmid for C kinase was microinjected, and then immunostained with TY33. (C and D) Fluorescent photomicrographs of astrocytes into which plasmid for CaM kinase II was microinjected, and then immunostained with MO82. Bar, 40  $\mu$ m.

for prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ), FP-receptors, the activation of which leads to  $PIP_2$  hydrolysis and intracellular  $Ca^{2+}$  mobilization (Kitanaka et al., 1991; Ito et al., 1992; Inagaki and Wada, 1994). After differentiation for 2 d by incubation with 250  $\mu$ M dibutyryl cAMP in serum free medium (Trimmer et al., 1982), astrocytes were stimulated with 5  $\mu$ M  $PGF_{2\alpha}$  and immunostained with YT33 and MO82. Differentiated astrocytes in the absence of stimulation showed practically no immunostaining of YT33 (Fig. 6 A) and a faint staining of MO82 (Fig. 6 B). After  $PGF_{2\alpha}$  stimulation for 20 min, astrocytes showed a remarkable increase in MO82 staining (Fig. 6 D). The increase in the immunostaining was observed diffusely in the cytoplasm (Fig. 6 D), thereby indicating that activation of CaM kinase II occurred throughout the cytoplasm. Immunoelectron microscopic observation confirmed that the epitope of MO82 was localized along the vimentin filaments (Fig. 7). On the other hand, stimulation with  $PGF_{2\alpha}$  (for 1, 3, 5, 10, or 20 min) did not increase the YT33 immunoreactivity in astrocytes (Fig. 6 C). Western blotting analysis confirmed  $PGF_{2\alpha}$ -induced phosphorylation of vimentin at Ser82 (Fig. 8 A) but not at Ser33 (data not shown). The time course of  $PGF_{2\alpha}$ -induced phosphorylation at Ser82 is shown in Fig. 8 B. The phosphorylation increased to the maximal level within the first 40 min, and then decreased gradually.  $PGF_{2\alpha}$ -induced phosphorylation at Ser82 was inhibited by a specific inhibitor of CaM kinase II, 1-[*N,O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62; Tokumitsu et al., 1990), but not by a control derivative of KN-62, *N*-[1-[*P*-(5-isoquinolinesulfonyl)benzyl]-2-(4-phenylpyperazinyl)ethyl]-5-isoquinolinesulfonamide (KN-04)

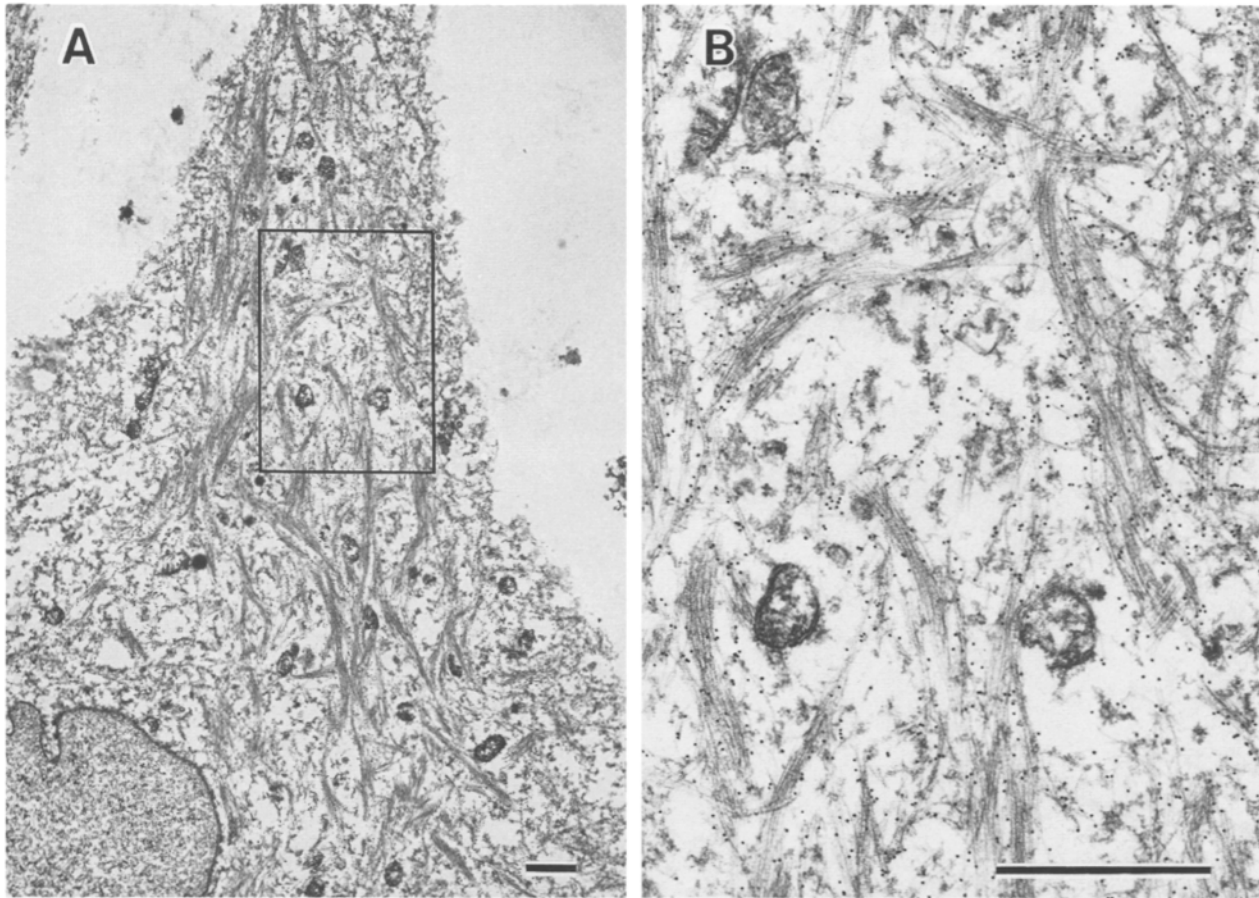
(Fig. 9). Phosphorylation of vimentin by CaM kinase II but not by C kinase was also observed in  $PGF_{2\alpha}$ -induced undifferentiated astrocytes (data not shown). These results suggest that the simultaneous activation of endogenous C kinase and CaM kinase II by  $PIP_2$  hydrolysis leads to an exclusive phosphorylation of vimentin by CaM kinase II but not by C kinase. For confirmation, we activated endogenous CaM kinase II and C kinase by a  $Ca^{2+}$  ionophore A23187 and a C kinase activator TPA, respectively. Activation of CaM kinase II by  $Ca^{2+}$  influx with 10  $\mu$ M A23187 led to a remarkable increase in MO82 immunoreactivity, consistent with the idea that the  $PGF_{2\alpha}$ -induced activation of CaM kinase II induces Ser82 phosphorylation (Fig. 6 H). Stimulation of astrocytes by 200 nM TPA indeed led to activation of endogenous C kinase, monitored by its translocation from the soluble fraction to the particulate fraction (Kraft and Anderson, 1983) (Fig. 10), but had no effect on the staining of YT33 (Fig. 6 E), thereby supporting our observation that activation of C kinase by the physiological stimulation led to no increase in Ser33 phosphorylation. Treatment of astrocytes with TPA had no apparent effect on MO82 immunoreactivity (Fig. 6 F). A23187 had no effect on YT33 immunoreactivity (Fig. 6 G). Thus, the present data indicate that the hydrolysis of  $PIP_2$  leads to differentially targeted signalings of C kinase and CaM kinase II to vimentin, under physiological conditions.

## Discussion

In the present study, using phosphorylation state-specific



*Figure 6.* Fluorescent photomicrographs showing effects of PGF<sub>2α</sub>, TPA, and A23187 on the phosphorylation of vimentin at Ser33 and Ser82 in astrocytes. After differentiation by incubation with 250 μM dibutyryl cAMP in serum free medium for 2 d, astrocytes were stimulated with buffer alone (*A* and *B*), 5 μM PGF<sub>2α</sub> for 20 min (*C* and *D*), 200 nM TPA for 20 min (*E* and *F*), or 10 μM A23187 for 40 min, and then immunostained with 3 μg/ml YT33 (*A*, *C*, *E*, and *G*) or 0.2 μg/ml MO82 (*B*, *D*, *F*, and *H*). Bar, 40 μm.



**Figure 7.** Immunogold localization of the MO82 epitope. (A) An immunoelectron photomicrograph showing vimentin filament network in an astrocyte. (B) A photograph at higher magnification of the area indicated by a rectangle in A, which reveals the localization of MO82 immunoreactivity along with the vimentin filaments. After differentiation, astrocytes were stimulated with 10  $\mu\text{M}$  A23187 for 40 min and stained with 0.2  $\mu\text{g/ml}$  MO82. The electron photomicrographs were taken, as described in Materials and Methods. A similar localization of the MO82 epitope was observed in astrocytes stimulated by 5  $\mu\text{M}$   $\text{PGF}_{2\alpha}$  for 40 min. Bar, 1  $\mu\text{m}$ .

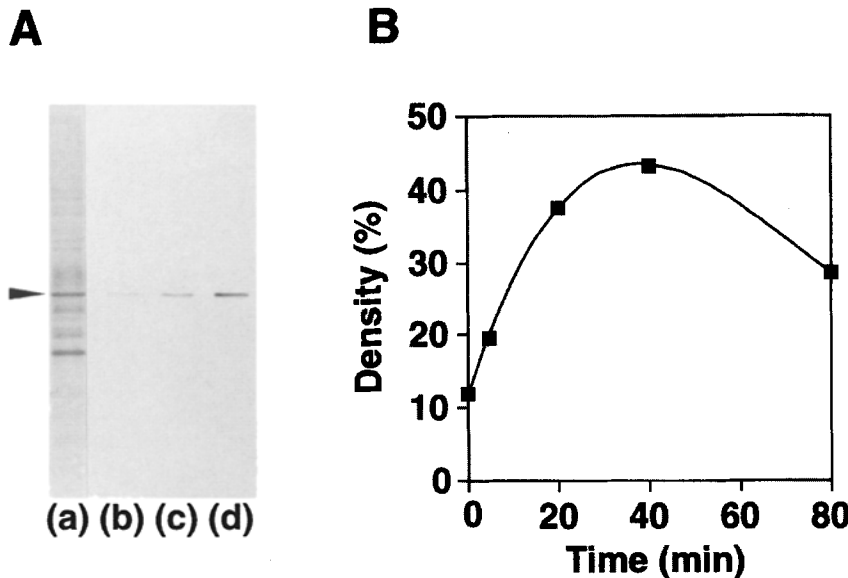
antibodies, we have shown that activation of C kinase and CaM kinase II in primary cultured astrocytes by  $\text{PIP}_2$  hydrolysis led to an exclusive phosphorylation of vimentin by CaM kinase II, not by C kinase.

Identification of the physiological substrates of CaM kinase II and C kinase has received much attention in order to better understand the signaling cascade initiated by receptor-mediated  $\text{PIP}_2$  hydrolysis. However, only a limited number of molecules have been identified as physiological substrates (Nishizuka, 1986; Azzi et al., 1992; Hanson and Schulman, 1992). Several studies were reported on vimentin phosphorylation by CaM kinase II. Purified CaM kinase II introduced 2 mol of phosphate into 1 mol of purified vimentin in vitro (Ando et al., 1991).  $\text{Ca}^{2+}$ /calmodulin-dependent phosphorylation of vimentin was shown in subcellular fractions of Sertoli cells (Spruill et al., 1983). Tsuda et al. (1988) noted increases in vimentin phosphorylation in aortic vascular smooth muscle cells, by angiotensin II or a  $\text{Ca}^{2+}$  ionophore, ionomycin. Yano et al. (1994) reported that stimulation of primary cultured astrocytes by glutamate led to the activation of CaM kinase II and to the phosphorylation of vimentin. By two-dimensional analysis of tryptic phosphopeptides, they found that some tryptic phosphopeptides of vimentin in the stimulated astrocytes

matched those identified by in vitro phosphorylation with CaM kinase II. However, the data did not show the complete overlap of the phosphorylation sites in vitro and vivo, therefore it was difficult to conclude that vimentin was phosphorylated directly by the kinase. The monoclonal antibody MO82, we prepared here, proved to be a powerful tool for investigating phosphorylation of vimentin by CaM kinase II. It recognized phosphorylation of Ser82 of vimentin, a unique phosphorylation site specific to CaM kinase II (Fig. 1), and specifically bound to vimentin phosphorylated by purified CaM kinase II (Fig. 2). MO82 detected in vitro activation of endogenous CaM kinase II in astrocytes (Fig. 3). Moreover, it detected in vivo phosphorylation of vimentin by introduced CaM kinase II (Fig. 4). The present data obtained with MO82 (Figs. 6 and 8) clearly showed that vimentin in astrocytes is directly phosphorylated by CaM kinase II activated by  $\text{PIP}_2$  hydrolysis.

MO82 is also useful to investigate distribution of cytoplasmic CaM kinase II activities in various types of cells. Because the vimentin filament network is distributed diffusely throughout the cytoplasm in most cultured cells (Schnitzer et al., 1981; Klymkowsky et al., 1989), it serves as a pertinent substrate to locate kinase activities in the cytoplasm (Tsujimura et al., 1994; Inagaki et al., 1994a, b).





**Figure 8.** Western blot analysis of vimentin phosphorylation at Ser82 in astrocytes by  $\text{PGF}_{2\alpha}$  and A23187. (A) After differentiation, astrocytes were stimulated by buffer alone (a and b), 5  $\mu\text{M}$   $\text{PGF}_{2\alpha}$  for 20 min (c), or 10  $\mu\text{M}$  A23187 for 40 min (d). Lysates of  $1.8 \times 10^3$  cells were loaded in the lanes, resolved by SDS-PAGE, and transferred onto a nitrocellulose filter, and then the blots were stained with Coomassie brilliant blue (a), or 1  $\mu\text{g/ml}$  MO82 (b, c, and d). The site of vimentin migration is indicated by the arrowhead. (B) Time course of  $\text{PGF}_{2\alpha}$ -induced Ser82 phosphorylation. The density of MO82 immunoreactivity was measured by densitometer and expressed as % of the density of the staining obtained with 40 min stimulation of 10  $\mu\text{M}$  A23187.

Using several phosphorylation state-specific antibodies for glial fibrillary acidic protein, another intermediate filament protein, we identified kinase activities localized in the cleavage furrow of mitotic cells (Nishizawa et al., 1991; Matsuoka et al., 1992; Inagaki et al., 1994a, b). The present results showed that the activation of CaM kinase II occurred diffusely in the cytoplasm of astrocytes in response to maximal concentration (5  $\mu\text{M}$ ) of  $\text{PGF}_{2\alpha}$  (Fig. 6 D). This is reasonable because maximal concentration of  $\text{PGF}_{2\alpha}$  induces  $\text{Ca}^{2+}$  mobilization throughout the cytoplasm of astrocytes (Ito et al., 1992). Recent studies showed that submaximal concentration of ligands evoked  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  signaling in localized areas of glial (Inagaki et al., 1991) and pancreatic (Thorn et al., 1993; Kasai et al., 1993) cells. Such localized  $\text{Ca}^{2+}$  signaling possibly regulates cellular activities in a defined area of the cell. Therefore, it will be of interest to determine if localized  $\text{Ca}^{2+}$  signaling activates CaM kinase II in a localized area of the cell.

It has been also unclear whether C kinase phosphorylates vimentin in vivo. Purified C kinase was seen to incorporate 4.6 mol (Ando et al., 1989) or 1.5 mol (Geisler et al., 1989) of phosphate into 1 mol of vimentin in vitro. Phorbol ester induced vimentin phosphorylation in membrane fractions of neutrophils (Huang et al., 1988). Phorbol ester-induced phosphorylation of vimentin in vivo was observed in macrophages (Ciesielski-Treska et al., 1991), astrocytes (Harrison and Mobley, 1991), endothelial cells (Stasek et al., 1992), retina (Williams et al., 1994), and fibroblasts (Bertrand et al., 1994). However, two-dimensional analysis of tryptic phosphopeptides showed that phosphorylation sites of vimentin in stimulated cells did not completely match those identified by in vitro analysis (Harrison and Mobley, 1991; Mobley and Combs, 1992). Furthermore, phorbol ester did not induce phosphorylation of vimentin in vascular smooth muscle cells (Tsuda et al., 1988) or in thyroid epithelial cells (Deery, 1993). The data we obtained here using the monoclonal antibody YT33 (Fig. 6) suggest that protein kinase C does not directly phosphorylate vimentin in astrocytes, under physiological conditions. One could argue that YT33 may not detect the phosphorylation of vimentin by C kinase if Ser33 is a mi-

nor site for the phosphorylation by C kinase. However, this is unlikely since YT33 showed high sensitivity in detecting vimentin phosphorylation by C kinase. While vimentin has 11 known sites of phosphorylation for C kinase (Fig. 1 A), YT33 could detect vimentin phosphorylated at only 2.2 mol phosphate/mol protein (Fig. 2 A) and 0.3 mol phosphate/mol protein (data not shown). It is notable that the tryptic vimentin peptides which are not phosphorylated by C kinase in vivo were phosphorylated in phorbol ester-stimulated cells (Harrison and Mobley, 1991). Therefore, vimentin may be phosphorylated by unidentified kinases which are activated by C kinase/TPA, directly or indirectly, in these cells.

It is intriguing to compare the data on C kinase-induced vimentin phosphorylation, obtained under different conditions. (a) C kinase in the soluble fraction of astrocytes phosphorylated vimentin in vitro. (b) Ectopically expressed constitutively active C kinase phosphorylated vimentin in astrocytes. (c) Endogenous C-kinase activated by  $\text{PIP}_2$  hydrolysis or TPA did not phosphorylate vimentin in astrocytes. Why is vimentin phosphorylated by the endogenous C kinase in vitro and by the constitutively active form in vivo but not by the endogenous C kinase in vivo? One possible explanation is the compartmentalized distribution of C kinase in the cell. Accumulating evidence suggests that isozymes of C kinase are targeted to specific cellular compartments and that the cell signaling is accompanied by their intracellular translocation (for review see Inagaki et al., 1994b). If C kinase is kept apart from vimentin during the signaling under physiological conditions, vimentin would not be phosphorylated. On the other hand, in the cases of in vitro experiments and the introduction of exogenous constitutively active kinase to cells, normal intracellular organization of C kinase is disrupted. In such cases, C kinase would encounter vimentin. Astrocytes investigated in the present study (type-1 astrocytes) express  $\alpha$ -isozyme of C kinase but not  $\beta$ -isozyme (Masliah et al., 1991), however recent studies reported immunocytochemical evidence that  $\beta$ -isozyme of C kinase is associated with vimentin filaments in BHK, SW13, CEM, U937, HL60, and RBL cells (Murti et al., 1992; Spudich et al., 1992). In the cases of

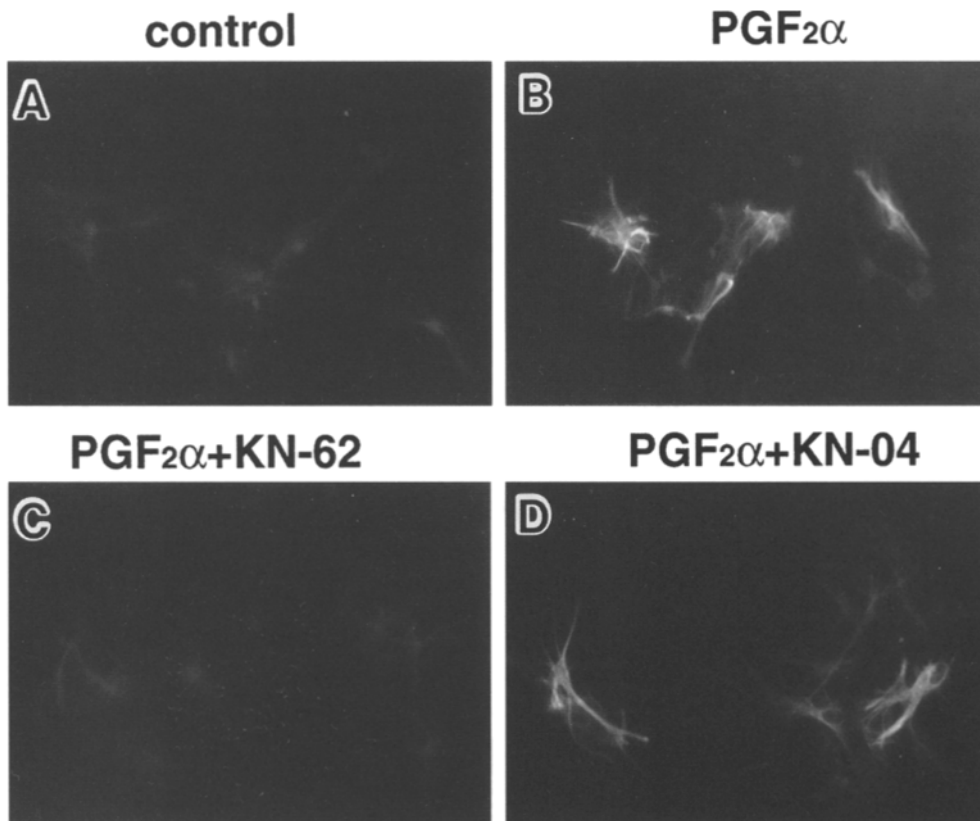


Figure 9. Fluorescent photomicrographs showing effects of KN-62 and KN-04 on  $\text{PGF}_{2\alpha}$ -induced phosphorylation of vimentin at Ser82 in astrocytes. After differentiation by incubation with 250  $\mu\text{M}$  dibutyryl cAMP in serum free medium for 2 d, astrocytes were pretreated with buffer alone (A and B), 3  $\mu\text{M}$  KN-62 (C), or 3  $\mu\text{M}$  KN-04 (D) for 30 min, stimulated by buffer alone (A) or 5  $\mu\text{M}$   $\text{PGF}_{2\alpha}$  (B, C, and D) for 20 min, and then immunostained with 0.2  $\mu\text{g}/\text{ml}$  MO82.

these cells,  $\beta$ -isozyme may phosphorylate vimentin under physiological conditions. It is notable that ectopically expressed C kinase phosphorylated vimentin, which is not phosphorylated under physiological conditions. Introduction of a gene into cells in culture and tissue is in wide use to analyze functions of a gene. The present results suggest that care has to be taken when interpreting such studies, as an introduced kinase may phosphorylate unphysiological substrates. In this context, the present results do not rule out the possibility that C kinase might phosphorylate vimentin, under pathological conditions. Abnormal activation of C kinase might lead to the phosphorylation of vimentin.

The present study revealed the missing link between receptor operated  $\text{PIP}_2$  hydrolysis and the phosphorylation of vimentin. Accumulating reports also support the idea that vimentin phosphorylation may be regulated by the cAMP/A kinase pathway (Browning and Sanders, 1981; Gard and Lazarides, 1982; Coca-Prados, 1985; McCarthy et al., 1985; Inagaki et al., 1987; Lamb et al., 1989), although there is no evidence that A kinase directly phosphorylates vimentin under physiological conditions. Therefore it appears evident that the phosphorylation of vimentin is under the regulation of cell signaling. Numbers of observations strongly suggest that phosphorylation of vimentin may regulate its morphological organization. In vitro studies reported that site-specific phosphorylation of vimentin induced disassembly of filament structure (Inagaki et al., 1987, 1988; Evans, 1988). In vivo experiments demonstrated that increase in vimentin phosphorylation by microinjection of the catalytic subunit of A kinase (Lamb et al., 1989), mitotic arrest with colcemid treatment (Franke et al., 1982;

Chou et al., 1990; Tsujimura et al., 1994), or treatment with phosphatase inhibitors (Eriksson et al., 1992) led to the collapse of vimentin filaments into bundles and granular dot structures. In this study, we observed a similar reorganization of vimentin in astrocytes into which constitutively active form of C kinase or CaM kinase II was introduced. We did not see a dramatic reorganization of vimentin filament network when vimentin was phosphorylated by endogenous CaM kinase II (Fig. 6, D and H). Probably, in

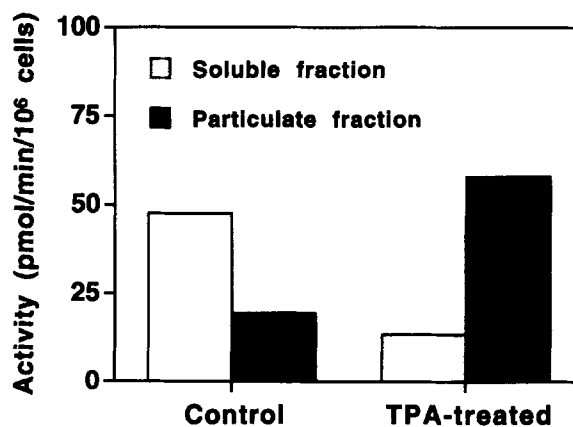


Figure 10. TPA-induced translocation of C kinase in astrocytes. After differentiation by incubation with 250  $\mu\text{M}$  dibutyryl cAMP in serum free medium for 2 d, astrocytes were stimulated by 200 nM TPA or buffer alone for 20 min. The soluble and particulate fractions of astrocytes were prepared and C kinase activity in each fraction was assayed by measuring incorporation of  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to histone H1.

these conditions, the level of vimentin phosphorylation was not enough to induce the collapse of filaments. We consider therefore that receptor-mediated cell signaling induces submaximal level of vimentin phosphorylation which leads not to a dramatic change but to a more minute and coordinated reorganization of vimentin filament network.

In conclusion, we examined here the signalings of C kinase and CaM kinase II to vimentin, using phosphorylation state-specific antibodies YT33 and MO82. These antibodies proved to be powerful tools for investigating vimentin phosphorylation by these kinases, under physiological conditions. Evidence was obtained for a separately regulated targeting of C kinase and CaM kinase II signalings to vimentin, in astrocytes. Although both signalings are activated by a common event, receptor-mediated PIP<sub>2</sub> hydrolysis, each signaling is likely to play a distinctive role in regulating cellular activities. Differential targeting of C kinase and CaM kinase II signalings may account for the elaborately coordinated cellular responses mediated by the kinases.

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