Mitosis-specific Phosphorylation of Vimentin by Protein Kinase C Coupled with Reorganization of Intracellular Membranes

Yasushi Takai,*§ Midori Ogawara,* Yasuko Tomono,‡ Chiharu Moritoh,‡ Shinobu Imajoh-Ohmi,‖ Osamu Tsutsumi,§ Yuji Taketani,§ and Masaki Inagaki*

*Department of Neurophysiology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173, Japan;
‡Division of Molecular and Cell Biology, Shigei Medical Research Institute, Okayama 701-02, Japan;
§Department of Gynecology and Obstetrics, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan;
‖The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108, Japan

Abstract. Using two types of anti-phosphopeptide antibodies which specifically recognize vimentin phosphorylated by protein kinase C (PKC) at two distinct PKC sites, we found that PKC acted as a mitotic vimentin kinase. Temporal change of vimentin phosphorylation by PKC differed from changes by cdc2 kinase. The mitosis-specific vimentin phosphorylation by PKC was dramatically enhanced by treatment with a PKC activator, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), while no phosphorylation of vimentin by PKC was observed in interphase cells treated with TPA. By contrast, the disruption of subcellular compartmentalization of interphase cells led to vimentin phosphorylation by PKC. Cytoplasmic and nuclear membranes are fragmented and dispersed in the cytoplasm and some bind to vimentin during mitosis. Thus, targeting of activated PKC, coupled with the reorganization of intracellular membranes which contain phospholipids essential for activation, leads to the mitosis-specific phosphorylation of vimentin. We propose that during mitosis, PKC may phosphorylate an additional subset of proteins not phosphorylated in interphase.

PROTEIN kinase C (PKC)[1] is a phospholipid-dependent serine/threonine kinase which plays a key role in signal transduction (Nishizuka, 1988, 1992). Recent studies suggest that PKC may function in regulating specific facets of the cell cycle. PKC inhibitors such as H7 and calphostin C inhibit cell cycle progression in G1 phase (Usui et al., 1991; Miñana et al., 1992). In primitive eukaryote *Saccharomyces cerevisiae*, a PKC-related gene, *PKC1*, was proposed to be essential for cell growth and the G2-M transition of yeast (Levin et al., 1990; Levin and Bartlett-Heubusch, 1992). Cells overexpressing the δ subspecies are arrested at the G2/M phase by treatment with a phorbol ester (Watanabe et al., 1992). However, there is a small number of direct target substrates for PKC involved in progressing the cell cycle. For example, βII PKC translocates to the nucleus during the G2/M phase of cell cycle, leading to the phosphorylation of a cytoskeletal protein lamin B which, since lamin B is part of the nuclear lamina, might influence stability of the nuclear lamina structure (Goss et al., 1994).

We reported that intermediate filaments (IFs) are pertinent in vitro PKC substrates (Inagaki et al., 1987*a*, 1988, 1990; Gonda et al., 1990; Yano et al., 1991*b*). During mitosis, IFs undergo dynamic change in organization accompanied with phosphorylation. It has been reported that the reorganization of cytoplasmic IFs during mitosis is mediated by phosphorylation of vimentin by cdc2 kinase (Chou et al., 1990; Matsuoka et al., 1992; Tsujimura et al., 1994), but it has also been reported that multiple kinases are responsible for the phosphorylation of vimentin during mitosis, which may function in conjunction with cdc2 kinase in mediating IF reorganization (Evans, 1989; Chou et al., 1990). However, only cdc2 kinase has been identified as a mitotic vimentin kinase.

To investigate the pathway of signaling between PKC and vimentin, we recently developed the monoclonal antibody YT33 which recognizes the phosphorylated state of vimentin by PKC at its specific site of phosphorylation, Ser33 (Ogawara et al., 1995). Using antibody YT33, we found that in differentiated astrocytes, endogenous PKC activated by phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis or a phorbol ester did not lead to phosphorylation of vimentin by PKC. Because ectopic expression of constitutively active PKC induced Ser33-phosphorylation of vimentin and reorganization of its filament networks, we considered that activated PKC is kept apart from vi-

Address all correspondence to Masaki Inagaki, Laboratory of Biochemistry, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya, Aichi 464, Japan. Tel.: 81 52 762 6111. Fax.: 81 52 763 5233.

The present address of M. Inagaki is Laboratory of Biochemistry, Aichi Cancer Center Research Institute, Nagoya, Aichi 464, Japan

1. *Abbreviations used in this paper*: A kinase, cAMP-dependent protein kinase; CaM kinase II, Ca^{2+}-calmodulin-dependent protein kinase II; 2-ME, 2-mercaptoethanol; PI, propidium iodide; PKC, protein kinase C; PS, phosphatidylserine.

mentin during signaling in differentiated cells. However, PKC signaling to vimentin during the cell cycle remains to be investigated.

In the present study, we used the antibody YT33 and the newly developed antibody TM50 which recognize the phosphorylated states of vimentin by PKC at distinct phosphorylation sites. We report here that PKC acts as an in vivo vimentin kinase specifically during mitosis. The compartmentalization between activated PKC and vimentin filaments in mitotic cells differs from that in interphase cells. We discuss the putative mechanism in which PKC phosphorylates a cytoskeletal protein vimentin specifically during mitosis. The spatial change of activated PKC may be regulated by reorganization of intracellular membranes which supply PKC with phospholipids indispensable for activation.

Materials and Methods

Preparation of Proteins

Recombinant vimentin was prepared from *E. coli* as described previously (Ogawara et al., 1995). The catalytic subunit of cAMP-dependent protein kinase (A kinase), Ca^{2+}-calmodulin–dependent protein kinase II (CaM kinase II), PKC, and cdc2 kinase were prepared as described previously (Beavo et al., 1974; Yamauchi and Fujisawa, 1983; Inagaki et al., 1985, 1987*b*; Kusubata et al., 1992). Protein concentrations were measured according to Bradford (1976) with bovine serum albumin as standard. Purified vimentin (0.15 mg/ml) was phosphorylated by incubation with 5 μg/ml of the catalytic subunit of A kinase, 5 μg/ml of PKC, 0.5 μg/ml of cdc2 kinase, or 5 μg/ml of CaM kinase II, as described previously (Tsujimura et al., 1994).

Peptide Synthesis

Vimentin peptides, PV6 (Cys-Ser-Thr-Arg-Ser-Val-phosphoSer^{6}-Ser-Ser-Ser-Tyr-Arg), PV24 (Cys-Thr-Ser-Ser-Arg-Pro-phosphoSer^{24}-Ser-Asn-Arg-Ser-Tyr), PV33 (Cys-Ser-Tyr-Val-Thr-Thr-phosphoSer^{33}-Thr-Arg-Thr-Tyr-Ser), V33 (Cys-Ser-Tyr-Val-Thr-Thr-Ser^{33}-Thr-Arg-Thr-Tyr-Ser), PV41(Cys-Thr-Tyr-Ser-Leu-Gly-phosphoSer^{41}-Ala-Leu-Arg-Pro-Ser), PV50 (Cys-Pro-Ser-Thr-Ser-Arg-phosphoSer^{50}-Leu-Tyr-Ser-Ser-Ser), V50 (Cys-Pro-Ser-Thr-Ser-Arg-Ser^{50}-Leu-Tyr-Ser-Ser-Ser), PV55 (Ser-Leu-Tyr-Ala-Ser-phosphoSer^{55}-Pro-Gly-Gly-Val-Tyr-Cys), PV65 (Cys-Tyr-Val-Thr-Arg-Ser-phosphoSer^{65}-Ala-Val-Arg-Leu-Arg), and PV82 (Cys-Arg-Leu-Leu-Gln-Asp-phosphoSer^{82}-Val-Asp-Phe-Ser-Leu) were synthesized as described previously (Tsujimura et al., 1994).

Preparation of Mouse-Monoclonal Antibodies 4A4 and YT33

Mouse-monoclonal antibodies against PV55 (4A4) and PV33 (YT33) were prepared as described previously (Matsuoka et al., 1992; Tsujimura et al., 1994; Ogawara et al., 1995).

Production of Rat-Monoclonal Antibody TM50

Phosphopeptide PV50 was conjugated to keyhole limpet hemocyanin and injected into female Jcl:Wister rats via hind footpads. The enlarged medial iliac lymph nodes from the injected rats were used for cell fusion after the method described in detail elsewhere (Kishiro et al., 1995).

Cell Preparation

U251 human astrocytoma cells and MDBK bovine kidney cells were cultured in Dulbecco's modified Eagle's medium containing 10% FCS.

Immunofluorescence Microscopy and Western Blotting

Immunofluorescence microscopy was performed according to the previously described method (Nishizawa et al., 1991), with slight modifications. Cells grown on cover glasses were fixed for 10 min with 3.7% formaldehyde in PBS, permeabilized for 20 min with 0.5% Triton X-100 in PBS, and then visualized by immunofluorescence, using YT33 (3 μg/ml) or TM50 (3 μg/ml) in PBS, as first antibodies. All the procedures for Western blotting have been described in detail elsewhere (Nishizawa et al., 1991; Yano et al., 1991*a*).

Preparation of Interphase and Mitotic Cell Lysates for Western Blotting

Cell lysates of interphase and mitotic cells for Western blotting were prepared according to the previously described method (Tsujimura et al., 1994) with slight modifications. Just before cells reached confluence, 10 ng/ml colcemid was added to the culture media, and 12 h later the mitotic cells were collected by gently shaking the culture flasks. For the collection of interphase cells, cells were cultured to reach confluence and a small number of mitotic cells were removed by gently shaking the flasks, and then cells adhering to the bottom of the flasks were used as the interphase cells. After washing with cold PBS, cell pellets were dissolved in SDS sample buffer, which contained 0.2 mg/ml Bromophenol blue, 10% glycerol, 100 mM 2-mercaptoethanol (2-ME), 2% SDS, and 62.5 mM Tris-HCl (pH 6.8), with brief sonication.

Preparation of the Cytosol and Membrane Fractions of U251 Cells

The cytosol and membrane fractions of U251 cells were prepared according to the previously described method (Chida et al., 1986) with slight modifications. Interphase and mitotic U251 cells were prepared as described above. After incubation in the presence or absence of 200 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 2.0×10^6 cells were washed with cold PBS and suspended in buffer A consisting of 2 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′,-tetraacetic acid (EGTA), 50 μg/ml leupeptin, 50 mM 2-ME and 25 mM Tris-HCl (pH 7.5). The cells were then homogenized and centrifuged at 100,000 *g* for 30 min. The supernatant was subjected to chromatography on a DEAE-cellulose (DE-52; Whatman) column (0.6 × 1.0 cm) equilibrated with buffer A. The fraction eluted with 0.4 M NaCl was used as the preparation of the cytosol fraction. The crude membrane fraction (100,000 *g* precipitate) was solubilized by treatment with buffer A containing 1.0% Triton X-100 at 4°C for 30 min and centrifuged at 100,000 *g* for 30 min. The supernatant was subjected to chromatography on a DEAE-cellulose column (0.6 × 1.0 cm) equilibrated with buffer A. The column was washed with 5 ml of buffer A without Triton X-100. The fraction eluted with 0.4 M NaCl was used as the preparation of the membrane fraction.

Assay of Protein Kinase C

PKC activity was assayed by measuring incorporation of ^{32}P from [γ-^{32}P]ATP to histone H1 according to the previously described method (Inagaki et al., 1985) with slight modifications. Each fraction (5 μl) was incubated at 25°C for 5 min with 0.5 mg/ml histone H1 (Boehringer Mannheim Corp., Indianapolis, IN) in a reaction mixture containing, in a final volume of 25 μl, 10 μM [γ-^{32}P]ATP, 0.1 μM calyculin A, 1 mM EGTA or 0.1 mM $CaCl_2$, 2 mM $MgCl_2$ and 25 mM Tris-HCl (pH 7.5), in the presence or absence of 200 nM TPA and 50 μg/ml phosphatidylserine (PS). The reaction was stopped by adding 25 μl of cold 300 mM H_3PO_4 and the mixture was spotted onto a P81 phosphocellulose paper. The papers were washed successively three times for 5 min in 75 mM H_3PO_4 and once briefly in ethanol, dried, and then counted in a liquid scintillation counter. Activity of PKC was defined as the difference between the ^{32}P incorporation in the presence and absence of $CaCl_2$, TPA, and PS.

Phosphorylation of Purified Vimentin by the Cytosol and Membrane Fractions of U251 Cells

Each fraction was incubated at 25°C for 30 min with 0.15 mg/ml vimentin as an exogenous substrate in a reaction mixture containing 1.0 mM ATP, 0.1 μM calyculin A, 1.2 mM $MgCl_2$ and 25 mM Tris-HCl (pH 7.5), in the presence or absence of 200 nM TPA, 50 μg/ml PS, and 0.1 mM $CaCl_2$. The reaction was stopped by adding equal volume of SDS sample buffer.

Phosphorylation of Endogenous Vimentin in the Whole Cell Lysates of U251 Cells

Interphase and mitotic U251 cells with or without TPA treatment were suspended and sonicated in a buffer consisting of 0.5 mM dithiothreitol, 20 mM β-glycerophosphate, 50 μg/ml leupeptin, 10 mM 2-ME, 2 mM PMSF and 25 mM Tris-HCl (pH 7.5). The whole cell lysates (0.6 mg protein/ml) containing endogenous vimentin were incubated at 25°C for 30 min in a reaction mixture containing 1.0 mM ATP, 0.1 μM calyculin A, 1.2 mM $MgCl_2$, 2 mM PMSF, and 25 mM Tris-HCl (pH 7.5). The reactions were stopped by adding an equal volume of SDS sample buffer.

Results

Is PKC a Mitotic Vimentin Kinase?

We recently developed the monoclonal antibody YT33 against a synthetic phosphopeptide PV33 (Phosphorylated Vimentin Ser33; Cys-Ser-Tyr-Val-Thr-Thr-phosphoSer[33]-Thr-Arg-Thr-Tyr-Ser, residues 28-38 of vimentin) (Ogawara et al., 1995). Vimentin-Ser33 has been identified as a PKC phosphorylation site in vitro (Ando et al., 1989).

The reactivity of the antibody YT33 to nonphosphorylated vimentin and phosphorylated vimentin by various protein kinases was analyzed by Western blotting. Recombinant mouse nonphosphorylated vimentin and vimentin phosphorylated by cAMP-dependent protein kinase, PKC, cdc2 kinase, and Ca^{2+}-calmodulin–dependent protein kinase II were resolved by SDS-PAGE, and blots were stained with the antibody YT33 (Fig. 1 *A*). The antibody YT33 reacted only with vimentin phosphorylated by PKC and did not react with nonphosphorylated vimentin or vimentin phosphorylated by other protein kinases. Next, we examined the sensitivity of the antibody YT33 to phosphorylated vimentin by PKC (Fig. 1 *B*). While vimentin has 11 known sites of phosphorylation for PKC (Ando et al., 1989), the antibody YT33 showed sensitivity high enough to detect vimentin phosphorylated at 0.3 mol phosphate/mol vimentin by PKC. We also examined the specificity of the antibody YT33 to vimentin phosphorylated at Ser33 (Fig. 1 *C*). It was neutralized by preincubation exclusively with a phosphopeptide PV33 but not with a peptide V33 (Cys-Ser-Tyr-Val-Thr-Thr-Ser[33]-Thr-Arg-Thr-Tyr-Ser) or other phosphopeptides such as PV6, PV24, PV41, PV50, and PV65 which were designed to represent vimentin phosphorylated at known PKC sites such as Ser6, Ser24, Ser41, Ser50, and Ser65, respectively.

To observe the in vivo phosphorylation of vimentin-Ser33 residues during the cell cycle, U251 human astrocytoma cells and MDBK bovine kidney cells were stained with the YT33 antibody. As we noted previously in primary cultured astrocytes (Ogawara et al., 1995), U251 and MDBK cells in interphase showed no YT33 immunoreactivity. On the other hand, YT33 immunostained an intricate mesh of vimentin filament in the entire cytoplasm of mitotic cells (Fig. 2, *A* and *B*). These results suggest that vimentin-Ser33 is phosphorylated specifically during mitosis.

To confirm the mitosis-specific phosphorylation of vimentin-Ser33, Western blots were prepared. Whole cell lysates of interphase and mitotic U251 and MDBK cells were stained with the YT33 antibody. As shown in Fig. 2, *C* and *D*, prominent immunoreactive bands at 57 kD were visible in lysates obtained from mitotic cell fractions of both U251 and MDBK cells. Since vimentin migrates at a position corresponding to a molecular mass of 57 kD, the results shown in Fig. 2, *C* and *D* provide strong evidence that the appearance of immunoreactivities with use of the antibody YT33 in cell staining truly represents the presence of vimentin-phosphoSer33 residues during mitosis.

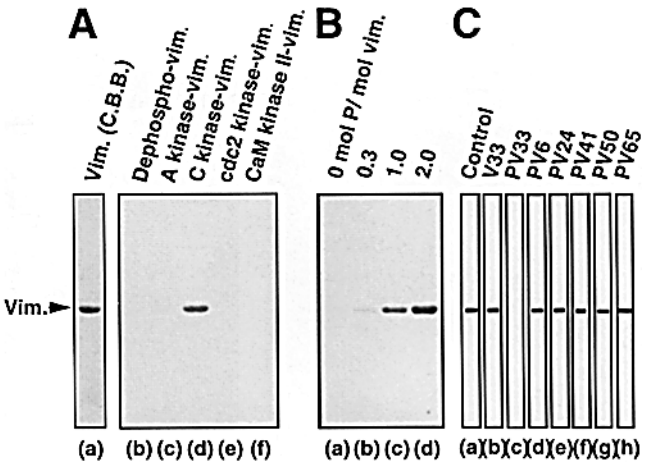

Figure 1. Immunoreactivity of the antibody YT33 analyzed by Western blotting. (*A*) Recombinant vimentin was phosphorylated by cAMP-dependent protein kinase (*A kinase-vim.*) (*c*), protein kinase C (*C kinase-vim.*) (*d*), cdc2 kinase (*e*), or Ca^{2+}-calmodulin–dependent protein kinase II (*CaM kinase II-vim.*) (*f*), respectively. 0.75 μg of the protein was loaded in each lane and stained with the antibody YT33 (1 μg/ml). The same amount of nonphosphorylated vimentin was loaded in lanes (*a*) and (*b*) and stained with Coomassie brilliant blue and the antibody YT33, respectively. (*B*) Sensitivity of the antibody YT33 was assayed. 0.75 μg of vimentin phosphorylated by protein kinase C at 0.3 (*b*), 1.0 (*c*), and 2.0 (*d*) mol phosphate/mol vimentin were loaded in each lane and stained with the antibody YT33 (1 μg/ml). Nonphosphorylated vimentin was loaded in lane (*a*). (*C*) Specificity of the antibody YT33 was assayed. 0.75 μg of recombinant vimentin phosphorylated by protein kinase C was loaded in each lane and stained with the antibody YT33 (1 μg/ml) after absorption with synthetic peptides (50 μg/ml of *V33* (*b*), *PV33* (*c*), *PV6* (*d*), *PV24* (*e*), *PV41* (*f*), *PV50* (*g*) or *PV65* (*h*)). Lane (*a*) shows the reactivity of YT33 after preincubation with PBS. The position of vimentin is indicated (*arrowhead*).

Temporal Change of Vimentin-Ser33 Phosphorylation by PKC during Mitosis

We then examined the temporal change of phosphorylation-dephosphorylation of vimentin-Ser33 observed in mitotic U251 cells. Ser33-phosphorylation was started at metaphase and maintained throughout anaphase as shown in Fig. 3 *A*. Similar results were obtained when MDBK cells were stained (data not shown). These patterns of staining differed from those of a monoclonal antibody 4A4 which specifically recognizes cdc2 kinase–phosphorylated vimentin (Fig. 3 *B*; Tsujimura et al., 1994). Thus, PKC may act as a mitotic vimentin kinase on a time schedule that differs from vimentin phosphorylation by cdc2 kinase.

Antibody Specific to Phosphorylated Vimentin at a Distinct PKC Site, Ser50

To determine whether or not PKC is a mitotic vimentin kinase, we developed another monoclonal antibody TM50

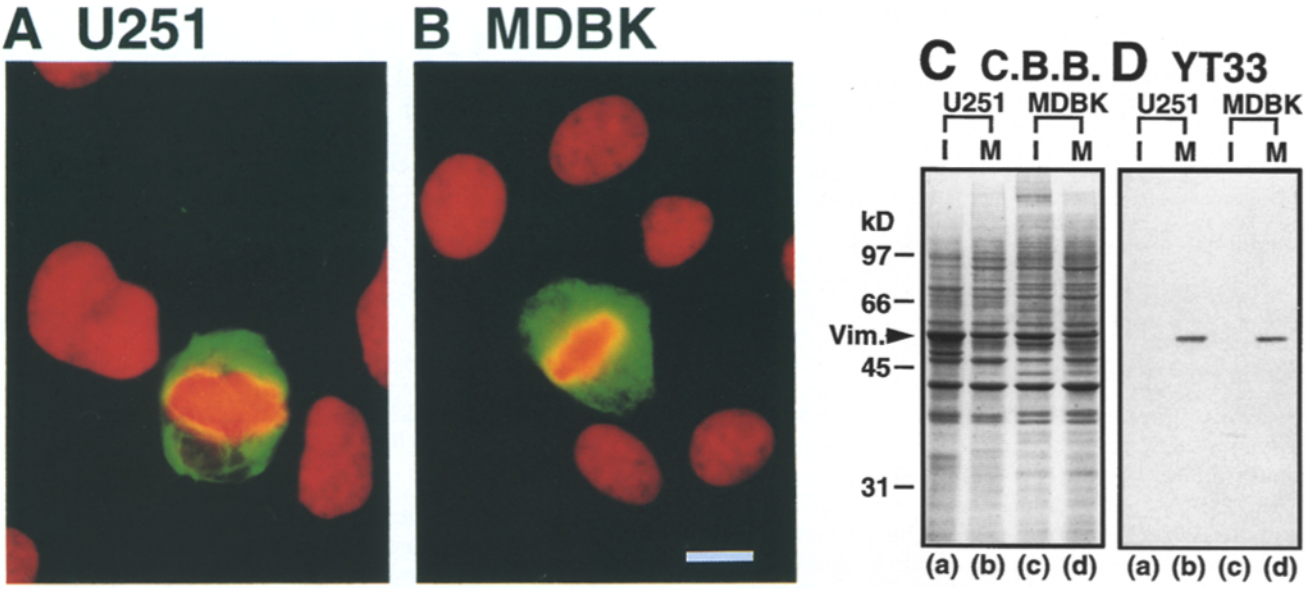

Figure 2. (*A* and *B*) Immunofluorescence micrographs of U251 human astrocytoma cells (*A*) and MDBK bovine kidney cells (*B*) stained with the antibody YT33 (3 μg/ml). Propidium iodide was mixed in the embedding solution to visualize chromosomes (*red*). The bar represents 10 μm. (*C* and *D*) Western blotting of interphase and mitotic U251 and MDBK cells. The same amount of cell lysates obtained from ~5×10^4 cells was loaded in each lane and stained with Coomassie brilliant blue (*C*) and the antibody YT33 (*D*). The position of vimentin is indicated (*arrowhead*). *I*, interphase cells; *M*, mitotic cells. The markers used were phosphorylase B (M_r 97,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 43,000), and carbonic anhydrase (M_r 31,000).

which specifically recognizes the Ser50-phosphorylated state of vimentin. Vimentin-Ser50 is another site of phosphorylation for PKC in vitro (Ando et al., 1989). Western blotting indicated that the antibody TM50 reacted with phosphorylated vimentin by PKC but not with nonphosphorylated vimentin, and that it was neutralized by preincubation with a phosphopeptide PV50 (Cys-Pro-Ser-Thr-Ser-Arg-phosphoSer^{50}-Leu-Tyr-Ser-Ser-Ser) but not with

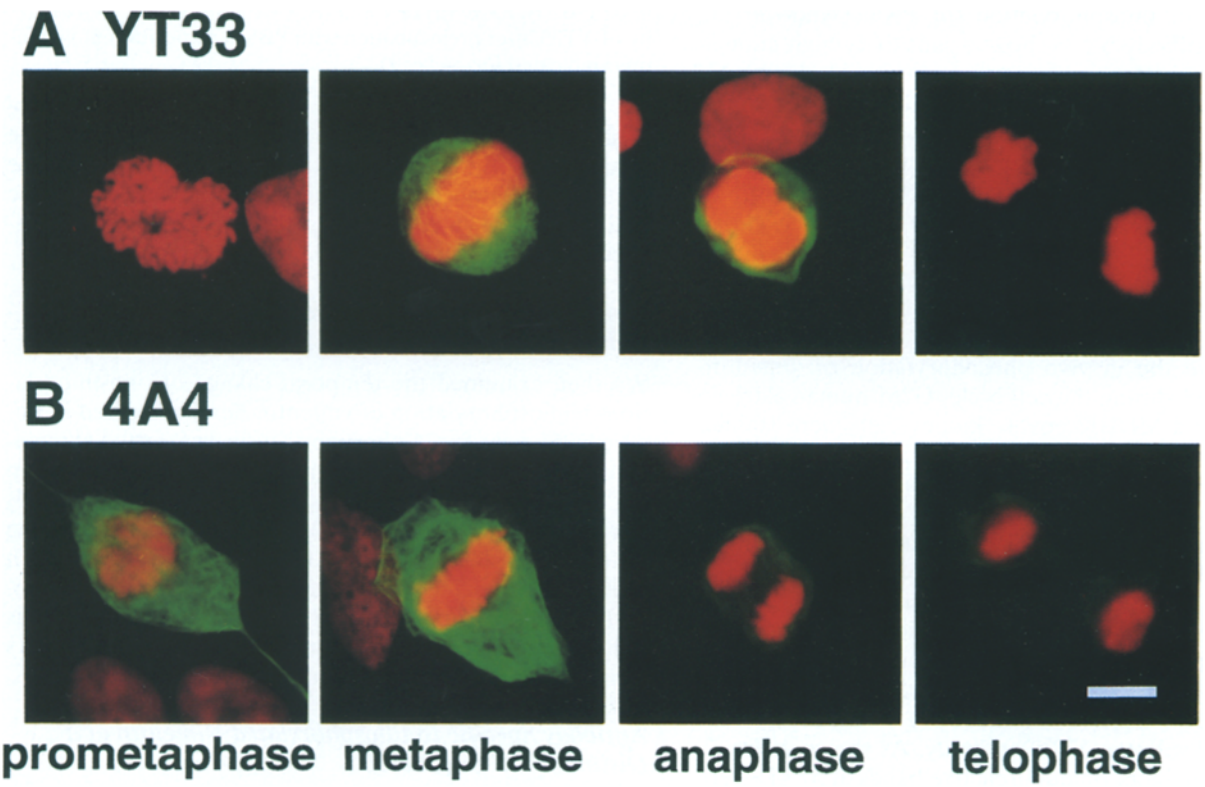

Figure 3. Immunofluorescence micrographs of mitotic U251 cells stained with the antibody YT33 (*A*) or 4A4 (*B*). The bar represents 10 μm.

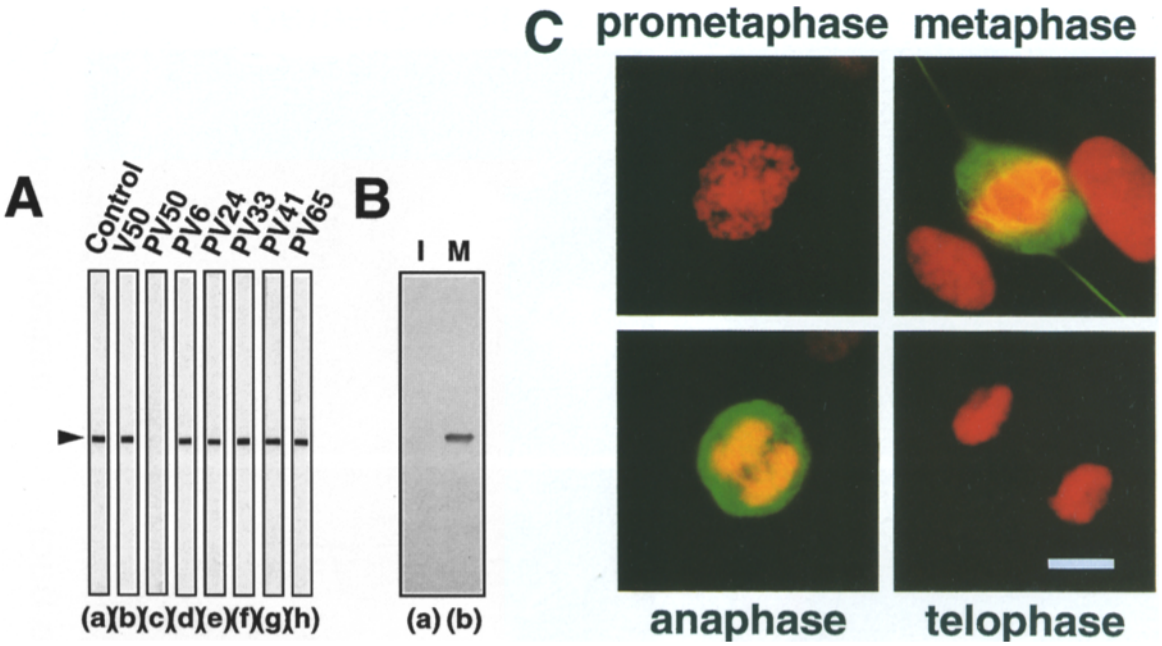

Figure 4. Immunoreactivity of the antibody TM50 revealed by Western blotting (*A* and *B*) and immunofluorescence micrography (*C*). (*A*) 0.75 μg of recombinant vimentin phosphorylated by protein kinase C was loaded in each lane and stained with the antibody TM50 (1 μg/ml) after absorption with synthetic peptides (50 μg/ml of *V50* (*b*), *PV50* (*c*), *PV6* (*d*), *PV24* (*e*), *PV33* (*f*), *PV41* (*g*) or *PV65* (*h*)). Lane (*a*) shows the reactivity of TM50 after preincubation with PBS. (*B*) Western blotting of interphase (*a*) and mitotic (*b*) U251 cells. The same amount of cell lysates was loaded in each lane and stained with the antibody TM50. (*C*) Immunofluorescence micrographs of mitotic U251 cells stained with the antibody TM50 (3 μg/ml). The bar represents 10 μm.

a peptide V50 (Cys-Pro-Ser-Thr-Ser-Arg-Ser^{50}-Leu-Tyr-Ser-Ser-Ser) or a phosphopeptide such as PV6, PV24, PV33, PV41, and PV65 (Fig. 4 *A*).

In Western blotting using whole cell lysates and TM50, an immunoreactive band at the position corresponding to that of vimentin was visible in the mitotic but not in the interphase cell lysate (Fig. 4 *B*). Immunofluorescence micrography using TM50 revealed that another PKC site Ser50 on vimentin was also phosphorylated, specifically during mitosis. Furthermore, temporal change of Ser50-phosphorylation during mitosis was much the same as that of Ser33-phosphorylation (Fig. 4 *C*). These results strongly suggest that PKC is a mitotic vimentin kinase.

Mitosis-specific Enhancement by TPA of Vimentin Phosphorylation by PKC

To further elucidate whether the mitosis-specific Ser33- and Ser50-phosphorylation of vimentin was carried out by PKC, we asked whether or not the PKC-activator, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) would enhance these types of vimentin phosphorylation. As shown in Fig. 5, the mitosis-specific Ser33-phosphorylation was significantly enhanced by treatment with TPA (*large arrows*). On the other hand, TPA had no effect on vimentin phosphorylation by PKC in interphase U251 cells (*small arrows*), findings consistent with our previous results using differentiated astrocytes (Ogawara et al., 1995). Similar results were obtained when MDBK cells instead of U251 cells were stained and also when the antibody TM50 was used instead of YT33 (data not shown). Western blotting revealed about three times the degree of enhancement of mitosis-specific Ser33-phosphorylation by TPA treatment (Fig. 6, *A* and *B*). Interphase cells were not stained with the antibody YT33 even when U251 cells were treated with a phosphatase inhibitor calyculin A (10 nM) alone or with calyculin A and TPA (data not shown). After 20–30 min in the presence of calyculin A, an increasing number of the cells showed a more rounded morphology as described previously (Eriksson et al., 1992), but interphase cells were not stained with the antibody YT33 (data not shown). These results suggest that PKC can phosphorylate vimentin specifically in mitotic cells and not in interphase cells, even in case of TPA and/or phosphatase inhibitor treatment.

Subcellular Localization of PKC Activity in Interphase and Mitotic Cells

To investigate mechanism(s) involved in the mitosis-specific vimentin phosphorylation by PKC, we examined whether PKC is activated when cells enter mitosis. Because PKC is redistributed from the cytosol to the membrane fraction when activated by PIP_2 hydrolysis or phorbol esters (Kraft and Anderson, 1983), we checked the subcellular localization of PKC activity in interphase and mitotic U251 cells. PKC activity of each subcellular fraction was assayed by measuring incorporation of phosphate into histone H1 (Fig. 7 *A*). In interphase U251 cells, ~70% of the PKC activity was found in the cytosol fraction and 30% in the membrane fraction. In mitotic U251 cells, the subcellular localization of PKC activity was similar to that in interphase cells.

Next, we wanted to determine if PKC is activated when

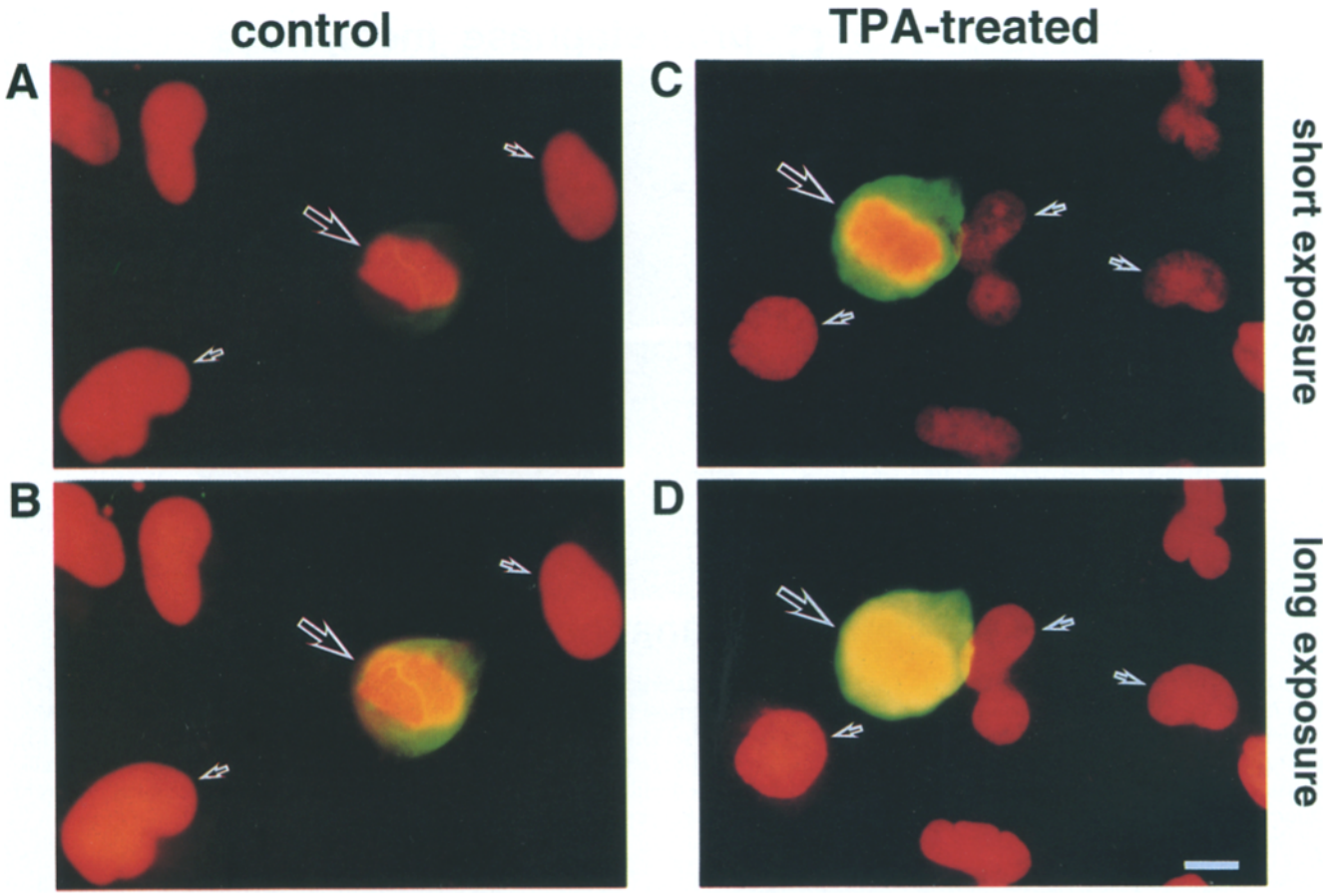

Figure 5. Effects of TPA on the phosphorylation of vimentin at Ser33 residues in U251 cells. Cells were treated with buffer alone (*A* and *B*) or 200 nM TPA for 30 min (*C* and *D*), stained with antibody YT33 (3 μg/ml), and photographed in short (*A* and *C*, 1.6 s) or long (*B* and *D*, 6.4 s) exposure time. Note that Ser33-phosphorylation in mitotic U251 cells (*large arrows*) was significantly enhanced by TPA, while no Ser33-phosphorylation was observed in interphase U251 cells (*small arrows*), even when treated with TPA. The bar represents 10 μm.

interphase and mitotic cells are treated with TPA. By treatment with 200 nM TPA, the PKC activity in the cytosol fraction of both interphase and mitotic cells disappeared almost completely while the activity in the membrane fraction increased to the level almost equal to the total PKC activity seen in untreated cells (Fig. 7 *A*).

Instead of histone H1, we incubated purified vimentin as an exogenous substrate with each fraction, which was depleted of endogenous vimentin and membrane phospholipid, in the presence or absence of PKC activators such as TPA, phosphatidylserine (PS) and $CaCl_2$, and then assayed the activity for Ser33-phosphorylation of vimentin by Western blotting using the YT33 antibody. No Ser33-phosphorylation was detected when vimentin was incubated in the absence of PKC activators (Fig. 7 *B*; *upper panel*). In the presence of PKC activators, vimentin-Ser33-phosphorylation demonstrated almost the same subcellular localization of the activity as demonstrated by histone H1-phosphorylation (Fig. 7 *B*; *lower panel*). These results support the possibility that PKC is the only protein kinase in interphase and mitotic cell lysates which phosphorylates vimentin-Ser33.

Subcellular localization of PKC activity assayed by measuring phosphorylation of histone H1 and vimentin-Ser33

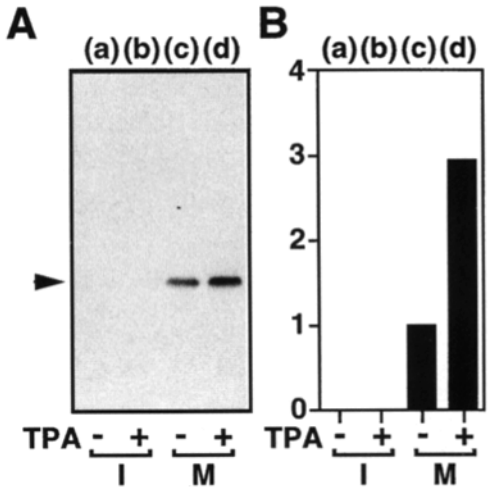

Figure 6. Western blotting of interphase (*a* and *b*) and mitotic (*c* and *d*) U251 cells with (*b* and *d*) or without (*a* and *c*) treatment of TPA. (*A*) Same amount of cell lysates obtained from ~5 × 10^4 cells was loaded in each lane and stained with the antibody YT33. (*B*) The density of each blot in *A* was measured by densitometer and expressed as ratio to the density of the blot from mitotic cells without treatment.

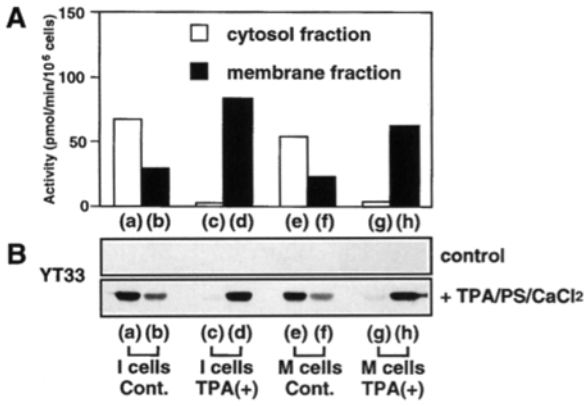

Figure 7. PKC activity in the cytosol and membrane fractions of U251 cells assayed by phosphorylation of histone H1 (*A*) or vimentin (*B*). The cytosol (*a*, *c*, *e*, and *g*) and membrane (*b*, *d*, *f*, and *h*) fractions were obtained from 2.0×10^6 interphase (*a–d*) and mitotic (*e–h*) U251 cells with (*c*, *d*, *g*, and *h*) or without (*a*, *b*, *e*, and *f*) TPA treatment. Each fraction was incubated with 0.5 mg/ml histone H1 (*A*) or 0.15 mg/ml vimentin (*B*). (*B*) 1.875 μg of the vimentin samples phosphorylated in the presence (*lower panel*) or absence (*upper panel*) of TPA, PS, and $CaCl_2$ were loaded for each lane and stained with the antibody YT33.

suggests that PKC redistribution does not occur when cells enter mitosis and that PKC is redistributed and activated by TPA treatment in both interphase and mitotic cells. In other words, the mitosis-specific vimentin phosphorylation by PKC is not coupled with PKC redistribution.

Disruption of Subcellular Compartmentalization of Interphase Cells Leads to Vimentin Phosphorylation by PKC

Interphase and mitotic U251 cells with or without TPA treatment were sonicated and whole cell lysates were prepared. After the incubation of whole cell lysates in the presence of ATP, Ser33-phosphorylation of endogenous vimentin was detected in the whole cell lysates prepared from interphase cells as well as in those from mitotic cells (Fig. 8). Moreover, a significantly higher level of Ser33-phosphorylation of vimentin was observed in the sample prepared from TPA-treated interphase cells, compared to untreated interphase cells. Similar results were obtained when mitotic cells were used. These results indicate that the disruption of subcellular compartmentalization of interphase cells leads to vimentin phosphorylation by PKC.

Discussion

In the present study, we presented evidence for the in vivo phosphorylation of vimentin by PKC specifically during mitosis, using two types of phosphorylated state-specific antibodies (Figs. 1–4). Furthermore, we demonstrated that TPA-induced enhancement of vimentin phosphorylation by PKC is also mitosis-specific (Figs. 5 and 6). In addition to these observations, we tried to inhibit Ser33- and Ser50-phosphorylation of vimentin during mitosis using PKC inhibitors such as H7 and calphostin C. However, it was impossible to obtain evidence for inhibition of the mitosis-specific Ser33- or Ser50-phosphorylation by PKC inhibitors because they prevented cells from entering mitosis (Usui et al., 1991; Miñana et al., 1992).

Why vimentin phosphorylation by PKC is mitosis-specific has to be addressed. Subcellular localization of PKC in mitotic cells is similar to that in interphase cells, and TPA induces PKC redistribution from the cytosol to the membrane fraction in both interphase and mitotic cells (Fig. 7). Since the disruption of subcellular compartmentalization of interphase cells leads to vimentin phosphorylation by PKC (Fig. 8), we propose mechanisms for the mitosis-specific phosphorylation of vimentin by PKC outlined in Fig. 9. In interphase cells (Fig. 9 *A*), the majority of PKCs are distributed in the cytosol fraction while some PKCs are distributed in the membrane fraction. When interphase cells are treated with TPA (Fig. 9 *B*), most PKCs in the cytosol fraction are redistributed to the membrane fraction. The membrane-bound PKCs cannot encounter and phosphorylate vimentin because of structural organization of intracellular membranes such as nuclear envelopes (NE), endoplasmic reticulums (ER), and Golgi apparatus. When cells enter mitosis (Fig. 9 *C*), however, structure of the intracellular membranes disassemble into

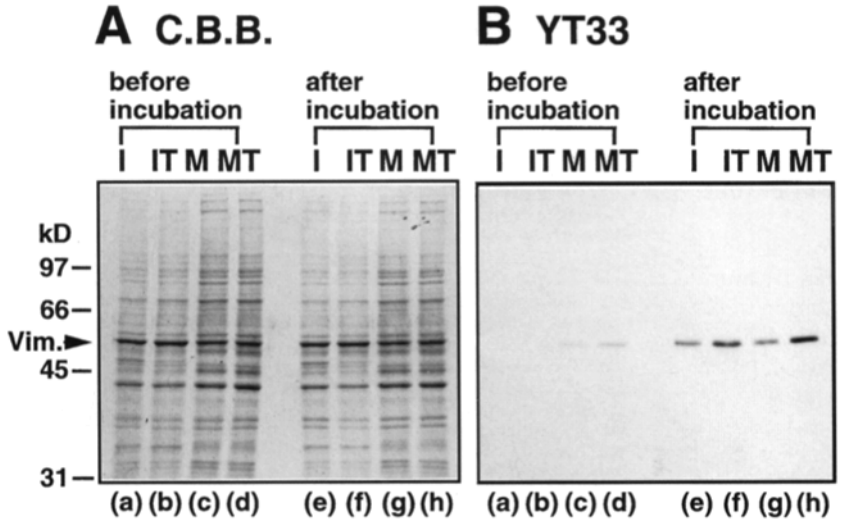

Figure 8. PKC activity in the whole cell lysates of U251 cells assayed by phosphorylation of endogenous vimentin. Interphase (*a*, *b*, *e*, and *f*) and mitotic (*c*, *d*, *g*, and *h*) cells with (*b*, *d*, *f*, and *h*) or without (*a*, *c*, *e*, and *g*) TPA treatment were sonicated and whole cell lysates were prepared. The same amount of each lysate (~$1–2 \times 10^4$ cells) was loaded for each lane before (*a–d*) or after (*e–h*) the incubation with ATP, and then stained with Coomassie brilliant blue (*A*) or the antibody YT33 (*B*). Endogenous vimentin in the mitotic cell lysates was phosphorylated at Ser33 without the incubation (*c* and *d*).

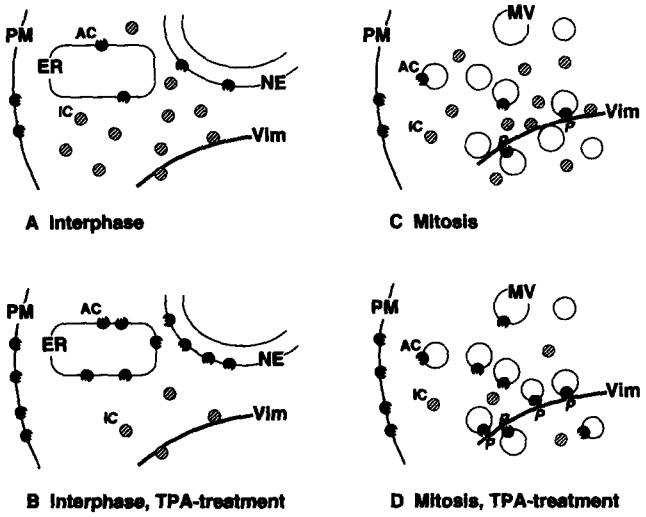

Figure 9. Schematic representation of the mechanism of mitosis-specific phosphorylation of vimentin filament by protein kinase C. Activated protein kinase C (*AC*) was assumed to be almost membrane-bound whereas inactive protein kinase C (*IC*) to be almost cytosolic. *ER*, endoplasmic reticulum; *MV*, membrane vesicles; *NE*, nuclear envelope; *PM*, plasma membrane; *Vim*, vimentin filament. *P* denotes the phosphorylation of vimentin filament by protein kinase C.

the form of vesicles (MV, membrane vesicles) (Zeligs and Wollman, 1979; Gerace and Blobel, 1980). Thus, the MV-bound PKCs do encounter and phosphorylate vimentin. In TPA-treated mitotic cells (Fig. 9 *D*), an increased number of MV-bound PKCs encounter and phosphorylate vimentin. In other words, the mitosis-specific vimentin phosphorylation by PKC may be caused by mitosis-specific targeting of activated PKC to vimentin coupled with reorganization of intracellular membranes.

It has been reported that vimentin filaments bind under in vitro conditions to vesicles reconstituted from total cellular lipids and phospholipid mixtures (Traub et al., 1986). Vimentin is also known to form a filament basket around lipid globules present in differentiating adipocytes. The IF basket is "sandwiched" between the surface of the globule and a flat, fenestrated membrane cisterna (Franke et al., 1987). It was also reported that vimentin filaments of mitotically arrested cells are associated with nuclear membrane vesicles and that their binding to vimentin is modulated by phosphorylation and dephosphorylation (Maison et al., 1993). These vimentin-membrane interactions support our proposal (Fig. 9) for mechanisms by which PKC exerts activity to phosphorylate vimentin specifically during mitosis.

Like the vimentin phosphorylation, phosphorylation of other PKC substrates may possibly be regulated by mitosis-specific targeting coupled with the reorganization of intracellular membranes. Therefore, our proposal also implies that during mitosis, PKC may phosphorylate an additional subset of substrates not phosphorylated in interphase.

Phosphorylated 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., 1994*a*,*b*). Analysis of an in vivo substrate of a kinase is feasible using phosphorylated state-specific antibodies, if they are raised against unique phospho-serine/threonine residues which are specifically catalyzed by the kinase in vitro (Tsujimura et al., 1994; Ogawara et al., 1995). In the present study, we obtained evidence for the in vivo phosphorylation of vimentin by PKC in the cell cycle, the visualization of which was made feasible by using two monoclonal antibodies which specifically recognize vimentin phosphorylated at distinct PKC sites. Such findings suggest the spatiotemporal distribution of PKC activity in the cytoplasm during mitosis coupled with reorganization of intracellular membranes, events not recognized by means of anti-PKC antibodies or cell lines overexpressing or lacking PKC.

Because vimentin is localized only in the cytoplasm, detection of PKC activity in the membrane and nucleus is difficult using these antibodies. However, as we have shown, they are powerful tools to monitor the spatiotemporal organization of PKC activity in the cytoplasm. Analysis of the functions of activated PKC in the cytoplasm during mitosis is an area for further exploration.

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