Phosphorylation of Vimentin in Mitotically Selected Cells. In Vitro Cyclic AMP-independent Kinase and Calcium-stimulated Phosphatase Activities

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Abstract. The phosphorylation of the intermediate filament protein vimentin was examined under in vitro conditions. Cell cytosol and Triton-insoluble cytoskeleton preparations from nonmitotic and mitotically selected mouse L-929 cells exhibited vimentin kinase activity that is apparently cAMP and Ca^{2+} independent. The level of vimentin kinase activity was greater in preparations from mitotically selected cells than nonmitotic cells. Addition of Ca^{2+} to mitotic cytosol decreased net vimentin phosphorylation. Dephosphorylation experiments indicated that there is phosphatase activity in these preparations which is stimulated by addition of Ca^{2+}. Fractionation of cytosol from nonmitotic cells on DEAE-Sephacel and phosphocellulose revealed a single major vimentin kinase activity (peak I). Fractionation of cytosol from mitotically selected cells yielded a similar activity (peak I) and an additional vimentin kinase activity (peak II) that was not found in nonmitotic preparations. Based on substrate specificity and lack of inhibition to characteristic inhibitors, the semipurified peak I and II vimentin kinase activities appear to be cAMP-independent enzymes that are distinct from casein kinases I and II. Phosphopeptide mapping studies indicated that both peak I and peak II vimentin kinases phosphorylate tryptic peptides in the NH_2-terminal region of vimentin that are phosphorylated in intact cells. Electron microscopic examination of reconstituted vimentin filaments phosphorylated with both semipurified kinases indicated that phosphorylation induced filament disassembly.

These experiments indicate that the increased phosphorylation of vimentin during mitosis may be catalyzed by a discrete cAMP-independent protein kinase. In addition, preparations from mitotic cells exhibited a Ca^{2+}-stimulated phosphatase activity, suggesting that Ca^{2+} may play a regulatory role in vimentin dephosphorylation during mitosis.

INTERMEDIATE filaments are abundant structures found in the cytoplasm of eukaryotic cells. Based on differences in protein subunit structure, it appears that there are at least five major classes of intermediate filaments (35). The most widely distributed class of intermediate filaments in cells of mesenchymal origin are composed of a single subunit protein called vimentin (52, 55).

A common feature of all intermediate filaments is that they are phosphorylated (35). Two-dimensional gel analysis of proteins from ^{32}P-labeled cells has revealed that the subunit proteins of intermediate filaments are among the most prominent phosphoproteins in the cytoplasm (8, 9, 15, 19, 41, 42). The physiological significance of intermediate filament phosphorylation is not known. However, the phosphate turns over more rapidly than the protein itself, suggesting that phosphorylation serves a regulatory function (8, 17, 37).

The organization of intermediate filaments is changed in cells that round during mitosis. Unlike other cytoskeletal structures, which are largely dissolved when cells undergo mitosis, intermediate filaments are retained but appear to be dramatically reorganized. Early in mitosis, the characteristic wavy filament bundles form a cagelike structure around the developing mitotic spindle (1, 4, 23, 29, 59). This change in filament organization is temporally associated with a site-specific increased level of filament phosphorylation (6, 9, 12, 17, 54, 56, 58).

Reports from a number of laboratories have indicated that vimentin filaments may be the substrate for multiple protein kinases. In some cells, the phosphorylation of vimentin is sensitive to cAMP or agents which increase cAMP levels (7, 20, 41-43). Vimentin is an in vitro substrate for cAMP-dependent kinases (42, 43), and this phosphorylation has recently been shown to produce in vitro filament disassembly (30, 31). However, in other cell types, vimentin phosphorylation is apparently insensitive to exogenous cAMP (8, 15, 20). There are also reports that vimentin phosphorylation may be calcium/calmodulin dependent (50, 51) and an in vitro substrate for protein kinase C (30, 31). Although vimentin may be phosphorylated by a variety of protein kinases in interphase cells of different types, virtually nothing is known about the kinase activity involved in mitosis-specific changes in intermediate filament protein phosphorylation.

Changes in protein phosphorylation have been postulated to be important events in the initiation of mitosis (10, 11, 36, 38). Protein phosphorylation may also play an important role in the modulation of cytoplasmic organization (47). The phosphorylation of intermediate filaments may be directly related to changes in filament structure and an indication of an important regulatory event during mitosis. To identify the mechanisms responsible for the change in vimentin phosphorylation which occurs during mitosis, and to identify potential regulatory molecules which may be involved in this process, vimentin phosphorylation and dephosphorylation experiments were conducted in vitro. These studies indicate that the phosphorylation of vimentin in both interphase and mitotic L-929 cells is primarily cAMP independent. The increased phosphorylation of vimentin observed in intact mitotic cells is associated with increased kinase activity in vitro. Partial purification of vimentin kinase activities indicates that this increased phosphorylation is associated with a distinct mitosis-specific protein kinase activity. In addition, there is a measurable, Ca^{2+}-stimulated phosphatase activity that also appears to be increased in mitotic preparations.

Materials and Methods

Cell Culture

Mouse L-929 cells, hamster BHK-21 cells, and human HeLa cells were grown in monolayer culture as previously described (16). In experiments involving radiolabeling of intact cells, the cultures were labeled with 0.5 mCi/ml ^{32}P-inorganic phosphate (carrier-free, ICN Pharmaceuticals, Inc., Irvine, CA) for 2 h in medium containing 1% the normal phosphate concentration, or 100 μCi/ml of [^{35}S]methionine (10 Ci/mmol, New England Nuclear, Boston, MA) for 4 h in methionine-free medium. In studies involving mitotic cells, populations enriched in mitotic and nonmitotic L-cells were produced by double thymidine block followed by the mitotic selection procedure of Terasima and Tolmach (53), as previously described (12). Some experiments were performed using material from cells treated with 0.1 μg/ml nocodazole followed by mitotic selection.

Preparation of Cytosol Fractions and Triton-insoluble Cytoskeletons

To produce cytosol fractions, cells were rinsed with TBS and scraped from the culture dish. The cell suspension was centrifuged at 500 *g* and the cells resuspended in a solution containing 50 mM Hepes, pH 7.0, 0.5 μg/ml leupeptin, 1 mM EGTA, and 0.5 mM PMSF. The cells were lysed by nitrogen cavitation at 500 psi. In some experiments a 20% homogenate of mouse skeletal muscle was prepared in the same buffer using a virtis homogenizer. The lysate was centrifuged at 105,000 *g* for 30 min. The resulting supernatant was used as a cytosol fraction for kinase assays.

Triton-insoluble cytoskeletons were made essentially as described by Zackroff and Goldman (57). Confluent monolayers of cells were rinsed with TBS and lysed in a solution (4 ml/10-cm dish) containing 0.6 M KCl, 0.5 mg/ml DNAse I, 1% Triton X-100, 10 mM $MgCl_2$, and 0.5 μg/ml leupeptin. The cell lysate was centrifuged at 3,000 *g* for 5 min and the resulting precipitate rinsed three times in TBS.

In Vitro Phosphorylation

[γ-^{32}P]ATP was produced by the method of Johnson and Walseth (32) from carrier-free [^{32}P]phosphoric acid. The purity and yield of all [γ-^{32}P]ATP preparations were determined by ascending chromatography on polyethyleneimine-cellulose in 0.75 M potassium phosphate, pH 3.5. [γ-^{32}P]GTP was purchased from ICN Pharmaceuticals, Inc. Stock solutions of [γ-^{32}P]nucleotide triphosphates were diluted 1:10,000 and 0.1 and 0.2 aliquots were dried on 1 × 1 cm strips of Whatman Inc. (Clifton, NJ) ET-31 filter paper. ^{32}P-radioactivity was determined for duplicate samples in 4.0 ml Ecolume (ICN Pharmaceuticals, Inc.) by scintillation counting. Nucleotide triphosphate concentrations of nonradioactive stock solutions were determined by UV-spectral analysis of diluted samples. Substrate vimentin was purified from Triton-insoluble cytoskeletons as described previously (12). Assays of vimentin phosphorylation in cytosol preparations were routinely conducted using ~10–20 μg of gel-purified vimentin in 50–100 μl of a solution of 150 mM NaCl, 20 mM Hepes, pH 7.0, 0.5 μg/ml leupeptin, and 50 μM ATP containing 5–50 μCi [γ-^{32}P]ATP. Unless noted, these assays were conducted for 15 min at room temperature. The assays were terminated with the addition of an equal volume of a solution containing 0.14 M Tris-HCl, 22.3% glycerol, 6% SDS, and 0.001% bromophenol blue. Phosphorylated proteins were then separated on 7.5% SDS-polyacrylamide gels (33). Conditions for gel electrophoresis, staining, and destaining of gels have been described previously (16). The stained vimentin band was excised from the gel and placed in a scintillation vial containing 1.0 ml of 0.1% SDS, 0.05 M ammonium bicarbonate for 16–18 h. Radioactivity was determined by Cerenkov counting or by scintillation counting after addition of 4.0 ml Ecolume (ICN Pharmaceuticals, Inc.). In studies involving ^{32}P- and ^{35}S-labeled samples, gel pieces were incubated in absolute methanol for 1 h, dried, and rehydrated in 1 M sodium salicylate for 20 min. The gel pieces were then transferred to vials with 4.0 ml Aquasol (New England Nuclear) and the ^{32}P/^{35}S radioactivity determined by scintillation counting.

DEAE-Sephacel Chromatography

Mitotic and nonmitotic cytosol preparations were produced in 0.02 M Hepes, pH 7.8, containing 1 mM EDTA, 0.5 μg/ml leupeptin, and 0.5 mM PMSF as described above. 3–5 ml of cytosol, representing material from 40 10-cm semiconfluent culture dishes, was mixed with 2 ml (settled volume) of DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in 0.02 M Hepes, pH 7.8, 1 mM EDTA. The slurry was placed in a small glass column and washed with 5 ml of the same buffer. Retained protein was eluted with a 60-ml gradient of 0–0.4 M KCl in 0.01 M Hepes, pH 7.8. Preliminary studies indicated that salt gradients to 1.0 M KCl produced no additional fractions with vimentin kinase activity. Approximately 1.0-ml fractions were collected and conductivity determined with a YSI model 31 conductivity bridge. 20–40 μl samples of each fraction were assayed for protein kinase activity using 2.5 mg/ml vimentin, 3 mg/ml β-casein (Sigma Chemical Co., St. Louis, MO), and 2 mg/ml calf thymus histone (type II, Sigma Chemical Co.), respectively, as substrates. Assays were conducted in 60 μl of 0.02 M Hepes, pH 7.8, 5 mM $MgCl_2$, 0.67 mM EGTA, and 50 μM ATP containing 500–1,000 dpm/pmol [γ-^{32}P]ATP. Assays for histone kinase also contained 0.1 mM 3′5′ cAMP. The assays were carried out for 15 min at room temperature and terminated with the addition of 1.0 ml of cold 95% ethanol. Samples were then centrifuged at 12,000 *g* for 5 min at 4°C. The supernatant was removed and the precipitated protein air dried. The precipitates were resuspended in 50 μl of a solution containing 0.14 M Tris-HCl, 22.3% glycerol, 6% SDS, and 0.001% bromophenol blue. Phosphorylated proteins were separated on SDS-polyacrylamide gels (33). Vimentin kinase and casein kinase assays were separated on 7.5% and 10% acrylamide gels, respectively. Histone kinase assays were separated on 10% acrylamide gels with separation gels that contained 6 M urea and bis-acrylamide rations of 1:10 (wt/wt). After electrophoresis, the gels were stained, destained, and dried onto filter paper (16). The gels were radioautographed on Kodak XAR x-ray film at −70°C. The vimentin, β-casein, and histone H-1 bands were then excised from the gel, placed in scintillation vials, and rehydrated in 0.1% SDS for 16–18 h. ^{32}P-radioactivity from individual gel slices was determined in 4.0 Econolume (ICN Pharmaceuticals, Inc.) by scintillation counting.

Phosphocellulose Chromatography

Pooled fractions from DEAE-Sephacel chromatography were dialyzed against 0.1 M NaCl, 0.2 M Hepes, pH 7.0, for 3–5 h. The dialyzed samples were then applied to a 0.7 × 2 cm phosphocellulose (Whatman Inc., P-11) column equilibrated in the same buffer. The column was washed with 10 ml of this buffer and retained protein was then eluted with a 0.1–0.8-M NaCl gradient in 0.02 M Hepes, pH 7.0. 1.0-ml fractions were collected and assayed for protein kinase activity using vimentin, casein, and histone (with cAMP) as substrates as described above.

Peptide Mapping and Phosphoamino Acid Analysis

Vimentin was prepared from ^{32}P-labeled double thymidine-blocked (nonmitotic) cells and cells mitotically harvested after release from the second thymidine block as previously described (12). In vitro ^{32}P-labeled vimentin was eluted from stained gel slices in 0.05 M ammonium bicarbonate, 0.1% SDS. The labeled protein was precipitated in 10% trichloracetic acid, the precipitate rinsed twice with ethanol/ether at 1:1 (vol/vol), and dried under

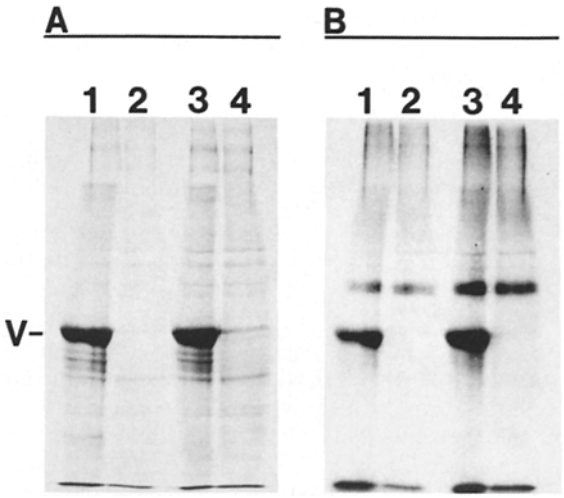

Figure 1. In vitro phosphorylation of vimentin by mitotic and nonmitotic cytosol preparations. Cells were thymidine blocked (nonmitotic), or mitotically selected after release from the thymidine block (mitotic). Crude cytosols were prepared. The preparations were incubated for 20 min with [γ-^{32}P]ATP in the presence or absence of 20 μg of gel-purified vimentin and the proteins analyzed on 7.5% SDS–polyacrylamide gels. The protein staining pattern (*A*) and ^{32}P-radioautograph (*B*) are shown for nonmitotic (lanes *1* and *2*) and mitotic (lanes *3* and *4*) preparations with (lanes *1* and *3*) and without (lanes *2* and *4*) added vimentin (*V*).

a stream of nitrogen. ^{32}P-labeled vimentin was resuspended in 100 μl of a solution containing 0.05 M ammonium bicarbonate, 0.5% SDS, and 10 μg L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin (Sigma Chemical Co.) for 4 h at 37°C. An additional 10 μg of trypsin was then added and the sample incubated at 37°C for 20 h. The tryptic digests were diluted with an equal volume of 2% trifluoroacetic acid (TFA),[1] and immediately analyzed by HPLC. Vimentin tryptic peptides were separated by reversed-phase chromatography on a 4.6 mm × 12.5 cm Partisphere C_8 column (Whatman Inc.). All analyses were performed with a gradient HPLC system (Gilson Medical Electronics, Middleton, WI). The column was run at room temperature with a flow rate of 1 ml/min. Samples were applied to the column and run in aqueous 0.1% TFA for 2 min. Retained material was eluted with a gradient of 0–40% acetonitrile, 0.1% TFA for 38 min, then 40–80% acetonitrile, 0.1% TFA for 15 min. 0.5-ml fractions were collected and ^{32}P levels determined by Cerenkov counting. An initial peak of unretained material was found in all samples. This material cochromatographed on polyethyleneimine-cellulose and coelectrophoresed on thin layer cellulose at pH 3.5 with [^{32}P]phosphate. A second, poorly retained radioactive peak was found in samples from in vitro phosphorylation experiments. This material cochromatographed with [γ-^{32}P]ATP on PEI-cellulose and was concluded to represent unincorporated ATP.

In experiments involving proteolysis at lysine residues, purified ^{32}P-labeled vimentin was resuspended in 50 μl of a solution containing 0.05% SDS, 0.05 M ammonium bicarbonate, pH 8.0. 0.3 U of lysine-specific endoprotease (Calbiochem-Behring Corp., La Jolla, CA) in 10 μl of the same buffer was added. The digestion was carried out for 4 h at 37°C. An additional 0.15 U of lysine-specific protease was added, and the mixture incubated for an additional 4–5 h at 37°C. The resulting peptides were analyzed on 15% SDS gels or by HPLC as previously described (13).

Phosphoamino acids were produced by partial hydrolysis of [^{32}P]vimentin in 6 N HCl at 105°C for 90 min and analyzed on thin layer cellulose by electrophoresis at pH 3.5 at 1,000 V for 1 h. Authentic unlabeled phosphoamino acid standards were included in each sample.

Phosphorylation-induced Filament Disassembly

Triton-insoluble cytoskeletons were prepared as described above and the protein separated on 7.5% SDS gels with the addition of 0.5 mM thioglycolic acid to the cathode running buffer. The vimentin band was visualized by soaking the gel briefly in 1 M KCl. The band was excised and the protein electroeluted in 0.02 M Tris acetate, pH 8.8, containing 1 mM DTT. Typical protein concentrations were 2–3 mg/ml. Filaments were reconstituted by dialysis in 0.15 M NaCl, 0.2 M Tris-HCl, pH 7.0, 1 mM $MgCl_2$, and 1 mM DTT for 6–8 h. The solution was centrifuged at 3,000 *g* for 5 min and the precipitated filaments recovered. The catalytic subunit of cAMP-dependent kinase was purified from bovine heart for use in control experiments (2, 3). Purified vimentin filaments were phosphorylated essentially under conditions described by Inagaki et al. (31). A solution containing 5 μM vimentin, 0.1 mM ATP, 30 mM Hepes, pH 7.0, 20 mM NaCl, and 1 mM $MgCl_2$, was incubated with or without kinase preparation for 45 min at 25°C. The resulting solution was examined for filament content in electron microscopy by negative staining with 2% uranyl acetate on carbon-coated collodion film grids.

Results

Cytosol and Triton-insoluble Cytoskeleton Preparations from Mitotic and Nonmitotic Cells Exhibit Vimentin Kinase Activity

Crude cytosol preparations from L-cells blocked with thymidine (nonmitotic) or cells released from the thymidine block and mitotically selected (mitotic index >80%) were used to examine the phosphorylation of purified vimentin. Vimentin is not found in significant amounts in a soluble form (29, 44). Therefore, gel-purified vimentin was added to the reactions as a substrate. Analysis of the proteins phosphorylated after addition of [γ-^{32}P]ATP (Fig. 1) demonstrated that both mitotic and nonmitotic preparations exhibited apparent vimentin kinase activity. As shown in Table I, this activity was stimulated by the addition of Mg^{2+}, but the rate of phosphorylation was slightly reduced when cAMP was included in the reaction. Addition of Ca^{2+} or Ca^{2+} and phosphatidylserine had little or no effect on the ability of nonmitotic cytosol preparations to phosphorylate vimentin. However, addition of Ca^{2+} to mitotic cytosol preparations resulted in an apparent reduction in vimentin phosphorylation.

Vimentin is a major component of what has been termed the Triton-insoluble cytoskeleton (57). As shown in Fig. 2, Triton-insoluble cytoskeletons also contain an endogenous kinase activity which will phosphorylate vimentin under in vitro conditions. The endogenous activity in both mitotic and nonmitotic cytoskeleton preparations, given in Table II, re-

Table I. In Vitro Phosphorylation of Vimentin by Crude Cytosol Preparations

Addition	% ^{32}P-Incorporation	
	Nonmitotic	Mitotic
None	100 ± 18	100 ± 14
1 mM EDTA	52 ± 10	79 ± 13
5 mM Mg^{+2}	487 ± 66	430 ± 53
5 mM Mg^{+2} + 1 mM cAMP	293 ± 32	284 ± 42
5 mM Ca^{+2}	96 ± 20	30 ± 5
5 mM Ca^{+2} + 1 mM PS	121 ± 11	38 ± 6

Cells were thymidine blocked (nonmitotic) or mitotically selected after release from the thymidine block (mitotic). Crude cytosols were prepared and incubated with ~50 μg of gel-purified vimentin and 50 μM [γ-^{32}P]ATP for 20 min. The proteins were electrophoresed on SDS–polyacrylamide gels as shown in Fig. 1, the vimentin-containing band excised from the gel and ^{32}P-radioactivity determined. Preparations phosphorylated without added vimentin served as blank values. Values represent the average of triplicate determinations ± SD. *PS*, phosphatidylserine.

1. *Abbreviation used in this paper*: TFA, trifluoroacetic acid.

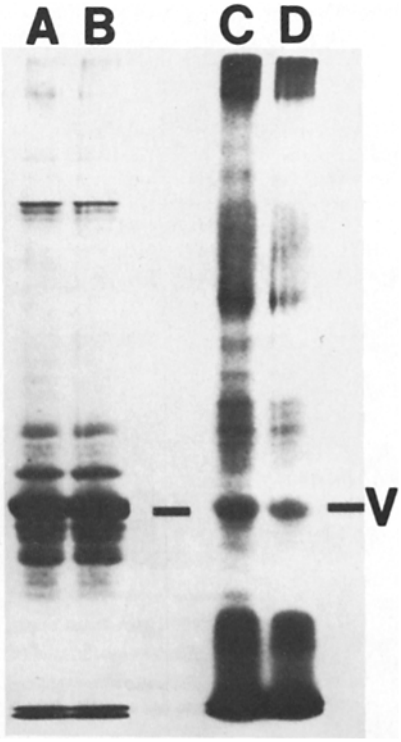

Figure 2. In vitro phosphorylation of Triton-insoluble cytoskeletons from mitotic and nonmitotic cells. Cells were thymidine blocked (nonmitotic), or mitotically selected after release from the thymidine block (mitotic). Triton-insoluble cytoskeletons were prepared, incubated with [γ-^{32}P]ATP for 20 min, and the proteins analyzed on 7.5% SDS-polyacrylamide gels. The protein staining pattern of mitotic (*A*) and nonmitotic (*B*) preparations and the ^{32}P-radioautographs of mitotic (*C*) and nonmitotic (*D*) are shown for material from similar cell numbers. The position of vimentin is shown (*V*).

quired a divalent cation such as Mg^{2+} and was not stimulated by the addition of cAMP. Addition of Ca^{2+} or Ca^{2+} and phosphatidylserine had little effect on the apparent phosphorylation of endogenous vimentin in either mitotic or nonmitotic cytoskeletons. Surprisingly, addition of NaF, a commonly used phosphatase inhibitor, resulted in decreased phosphorylation in both mitotic and nonmitotic preparations.

Table II. In Vitro Phosphorylation of Vimentin in Cytoskeletal Preparations

Addition	% ^{32}P-Incorporation	
	Nonmitotic	Mitotic
None	100 ± 11	100 ± 18
1 mM EDTA	31 ± 6	54 ± 10
5 mM Mg^{+2}	407 ± 69	574 ± 86
5 mM Mg^{+2} + 1 mM cAMP	345 ± 59	428 ± 69
5 mM Ca^{+2}	102 ± 17	85 ± 13
5 mM Ca^{+2} + 1 mM PS	152 ± 29	89 ± 16
5 mM Mg^{+2} + 10 mM NaF	53 ± 10	59 ± 10

Triton-insoluble cytoskeletons were produced from nonmitotic and mitotically selected cells, phosphorylated, and electrophoresed as described in Fig. 2. The vimentin-containing bands were excised from the gel and ^{32}P-radioactivity determined. Values represent the average of four determinations ± SD.
PS, phosphatidylserine; *NaF*, phosphatase inhibitor.

Table III. Specific Radioactivity of Vimentin Phosphorylated in Mitotic and Nonmitotic Triton-insoluble Cytoskeletal Preparations

Preparation	^{32}P cpm	^{35}S cpm	^{32}P/^{35}S
Mitotic	7,668 ± 843	235 ± 21	32.6
Nonmitotic	3,038 ± 364	249 ± 19	19.2

Cells were labeled with [^{35}S]methionine and Triton-insoluble cytoskeletons were prepared as described in Table II. Values represent the average of triplicate determinations ± SD.

Similar results were obtained with preparations from untreated cells compared with cells mitotically selected after treatment with colcemid (data not shown). These results indicate that vimentin kinase in L-929 cell cytosol and cytoskeleton preparations is apparently cAMP independent and not related to C-type (40) or Ca^{2+}/calmodulin-dependent (46) protein kinases.

A comparison of cytosol and cytoskeleton preparations containing approximately similar concentrations of vimentin indicated that cytosol preparations from either nonmitotic or mitotic cells contained approximately four times as much apparent vimentin kinase activity as the corresponding cytoskeleton prepared from a similar number of cells (data not shown). However, it is difficult to directly compare the relative distribution of vimentin kinase activity on this basis. The substrate vimentin in Triton-insoluble cytoskeletons is nearly all in an insoluble filamentous form, while the vimentin added to cytosol preparations has been denatured and then partially reassembled in vitro.

Mitotic Preparations Have a Higher Level of Vimentin Kinase Activity Than Nonmitotic Preparations

In intact cells, vimentin appears to be more highly phosphorylated during mitosis than during interphase (6, 9, 15, 17, 56). Studies were conducted to determine if this quantitative difference is reflected during in vitro phosphorylation. Cells were labeled with [^{35}S]methionine and Triton-insoluble cytoskeletons were prepared from mitotic and nonmitotic cells. As shown in Table III, when ^{35}S-labeled Triton-insoluble cytoskeletons were in vitro phosphorylated under identical conditions, mitotically enriched cytoskeletons exhibited

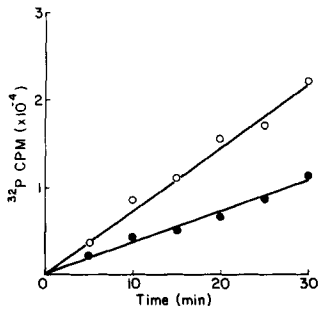

Figure 3. Time course of vimentin phosphorylation by mitotic and nonmitotic cytosol preparations. Purified vimentin was phosphorylated in vitro for the indicated times and subjected to gel electrophoresis as described in Fig. 1. After electrophoresis, the vimentin band was excised and incorporated radioactivity determined. Each reaction contained 10 μg of cytosol protein and 15 μg of purified vimentin. Mitotic cytosol (○); nonmitotic cytosol (●).

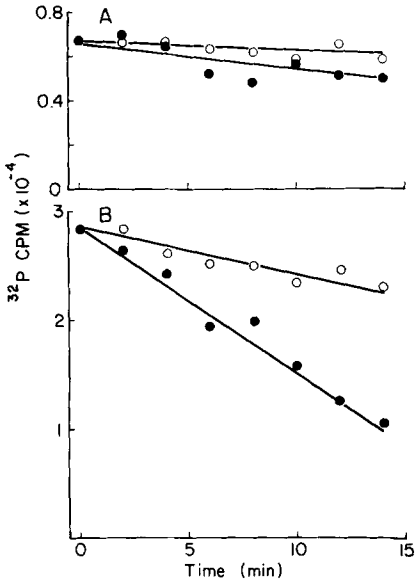

Figure 4. Time course of vimentin dephosphorylation by mitotic (*B*) and nonmitotic (*A*) cytosol preparations. Purified vimentin was phosphorylated as described in Fig. 1. After a 20-min incubation, unlabeled ATP was added to a final concentration of 1 mM and the incubations continued for the indicated times. The preparations were subjected to gel electrophoresis, the vimentin band was excised, and ^{32}P-radioactivity determined. Each reaction contained 10 μg of cytosol protein and 15 μg of purified vimentin. Dephosphorylation in the presence of 1 mM EDTA (○) and 5 mM Ca^{2+} (●) is shown.

higher rates of phosphorylation than cytoskeletons from nonmitotic cells. Similarly, time course experiments were conducted with cytosol preparations. Fig. 3 shows the phosphorylation of purified vimentin by similar amounts of cytosol protein from nonmitotic and mitotically selected cells. Cytosol from mitotically selected cells phosphorylated exogenously added, purified vimentin in a time-dependent manner at approximately twice the apparent rate of nonmitotic cytosol. Considerable variation was observed between individual experiments in the absolute rate of vimentin phosphorylation. However, in all experiments mitotic cytoskeletons and cytosol preparations were two to four times more active in vimentin kinase activity than nonmitotic preparations when assayed under similar conditions.

Cytosol Preparations Exhibit a Vimentin Phosphatase Activity

In an effort to understand the apparent inhibitory effect of Ca^{2+} on vimentin phosphorylation in mitotic cytosol preparations (Table I), phosphorylation-dephosphorylation studies were conducted as shown in Fig. 4. Gel-purified vimentin was added to mitotic cytosol preparations and phosphorylated for 20 min in the presence of Mg^{2+}. Unlabeled ATP was then added and the dephosphorylation allowed to proceed for 15 min in the presence of Ca^{2+} or EDTA. Fig. 4 *B* shows the time-dependent loss of labeled phosphate from vimentin in the presence of 1 mM unlabeled ATP. The rate of loss of phosphate was stimulated by the addition of Ca^{2+} compared with reactions conducted in the presence of EDTA.

Table IV. Effect of Ca^{2+} on Dephosphorylation of ^{35}S-Labeled Vimentin by Mitotic Cytosol

	^{32}P cpm	%	^{35}S cpm	%	^{32}P/^{35}S
Phosphorylation	12,861	100	811	100	15.9
Dephosphorylation 1 mM EDTA	11,189	87	746	92	15.0
Dephosphorylation 1 mM Ca^{2+}	2,829	22	640	79	4.4

Cytosol was prepared from mitotically selected cells after treatment with colcemid. Gel-purified vimentin, isolated from [^{35}S]methionine-labeled cells, was added and phosphorylated in the presence of 1 mM Mg^{2+} and 50 μM [γ-^{32}]ATP for 20 min. Unlabeled ATP was then added to a final concentration of 1.0 mM and dephosphorylation was allowed to proceed for 20 min. Values represent the average of triplicate determinations.

Similar experiments with nonmitotic cytosol preparations exhibited little or no apparent increase in dephosphorylation in the presence of Ca^{2+} (Fig. 4 *A*). Cultured cells contain a Ca^{2+}-activated protease which may be responsible for the endogenous degradation of vimentin (39). This activity may be increased in some cells during cell division (5). To determine if the apparent Ca^{2+} loss of vimentin phosphate was due to phosphatase or protease activity, phosphorylation-dephosphorylation experiments were conducted with purified ^{35}S-labeled vimentin. As shown in Table IV, there is a loss of ^{35}S-labeled vimentin during in vitro dephosphorylation in the presence of Ca^{2+}. However, a comparison of the ^{32}P to ^{35}S radioactivity demonstrated that the specific ^{32}P radioactivity of vimentin was decreased in the presence of Ca^{2+}. These experiments show that there is protease activity in these preparations, but the effect of Ca^{2+} was primarily due to phosphatase activity.

Partial Purification of Two Vimentin Kinase Activities from Mitotic and Nonmitotic Cells by DEAE-Sephacel and Phosphocellulose Chromatography

To determine if the increased kinase activity detected in mitotic cytosol preparations is due to modulation of interphase kinase activity or perhaps a mitotic-specific enzyme, cytosol preparations from nonmitotic and mitotically selected cells were fractionated on DEAE-Sephacel. Column fractions were then assayed for protein kinase activity using purified vimentin as a substrate. In addition, to examine whether vimentin kinase activity is separable from other well-characterized enzymes, the column fractions were also assayed for kinase activity using other protein substrates. Since the DEAE chromatographic properties of cAMP-dependent kinases and the cAMP-independent casein kinases I and II have been extensively described (25, 26, 28), the column fractions were assayed for protein kinase activity using histone with cAMP, and β-casein as substrates. As shown in Fig. 5, DEAE-Sephacel chromatography of nonmitotic cytosol resulted in a single major broad peak of protein kinase activity using vimentin as a substrate. Similar chromatography of mitotic cytosol produced a single sharp peak with a broad shoulder of vimentin kinase activity that eluted at similar KCl concentrations as the activity detected in nonmitotic cells. When these fractions were assayed using either histone plus cAMP or β-casein as substrates, two distinct peaks of activity were detected. These may correspond to type I and II cAMP-dependent kinases, and casein kinases I and II. Comparison of the elution profiles indicated that vimentin kinase activity in

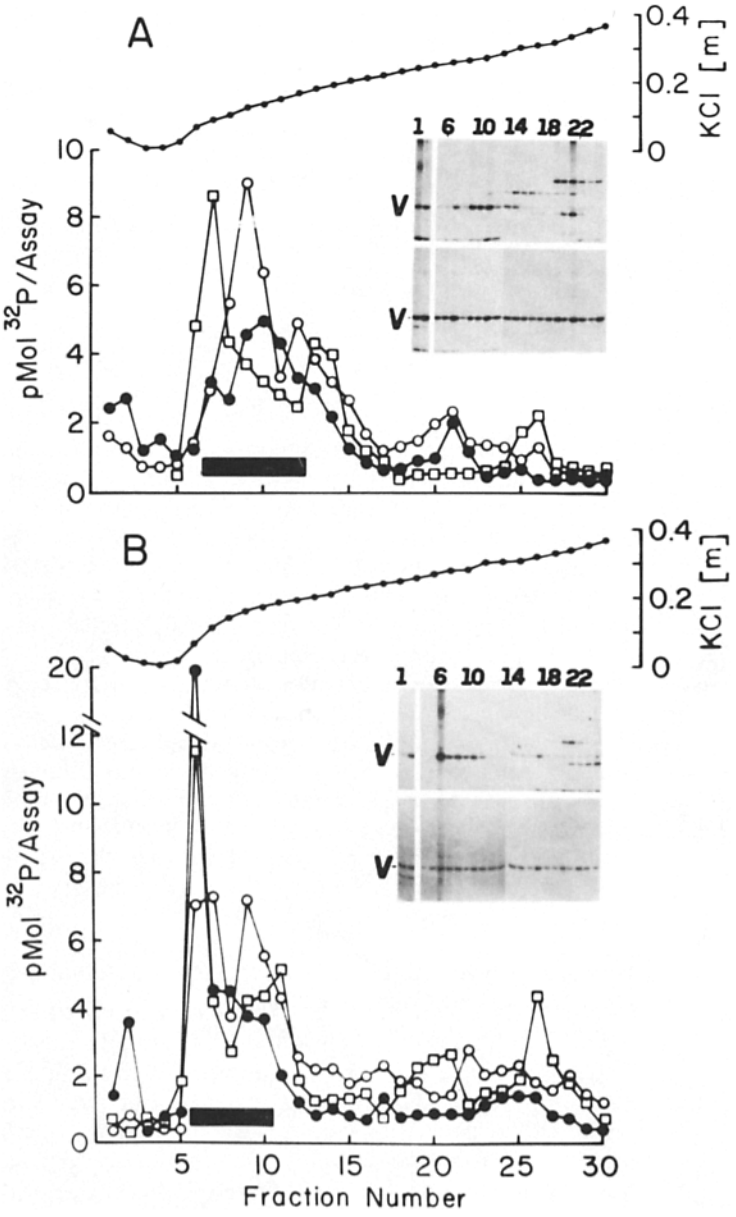

Figure 5. DEAE-Sephacel chromatography of nonmitotic and mitotic protein kinase activities. Cytosol preparations from nonmitotic (*A*) and mitotically selected (*B*) L-929 cells were adsorbed to and eluted from DEAE-Sephacel as described in Materials and Methods. Collected fractions were assayed for protein kinase activity using vimentin (●), β-casein (○), or histone and cAMP (□) as substrates. The insets show SDS gel radioautograph (*top inset*) and protein stain (*bottom inset*) of relevant fractions assayed for vimentin kinase activity. The position of vimentin (*V*) is indicated. Fractions that were pooled for subsequent phosphocellulose chromatography are indicated with a solid bar.

nonmitotic fractions did not precisely coelute with either casein kinase or histone kinase activities. However, the sharp peak of vimentin kinase activity detected in mitotic fractions was not separated from the major peak of cAMP histone kinase activity. Because vimentin kinase activity was poorly resolved from other kinase activities on DEAE-Sephacel, the fractions containing the major peak of vimentin kinase activity were pooled and rechromatographed on phosphocellulose.

Fig. 6 shows the elution profile for vimentin kinase from DEAE-Sephacel fractions rechromatographed on phosphocellulose columns. Comparison of the elution profiles for mitotic and nonmitotic preparations show that both contain a peak of vimentin kinase activity that elutes from phosphocellulose between 0.2 and 0.35 M NaCl (peak I). While this represents the only detectable activity in nonmitotic preparations, a second peak of vimentin kinase activity was detected in mitotic preparations, which eluted between 0.45 and 0.6 M NaCl. Assay of the column fractions for casein kinase activity indicated a single detectable peak of casein kinase in both mitotic and nonmitotic preparations that was not well resolved from the mitotic-specific vimentin kinase peak II. The elution characteristics of this casein kinase activity are similar to those described for casein kinase I (25, 26). Column fractions were also assayed for histone plus cAMP kinase activity. While the bulk of this activity was not retained by phosphocellulose, there was detectable histone kinase activity which coeluted with the peak I vimentin kinase activity in both mitotic and nonmitotic preparations. This is in apparent agreement with reports that cAMP-dependent kinases are not retained by phosphocellulose under these conditions (25, 26). Assay of column fractions with vimentin

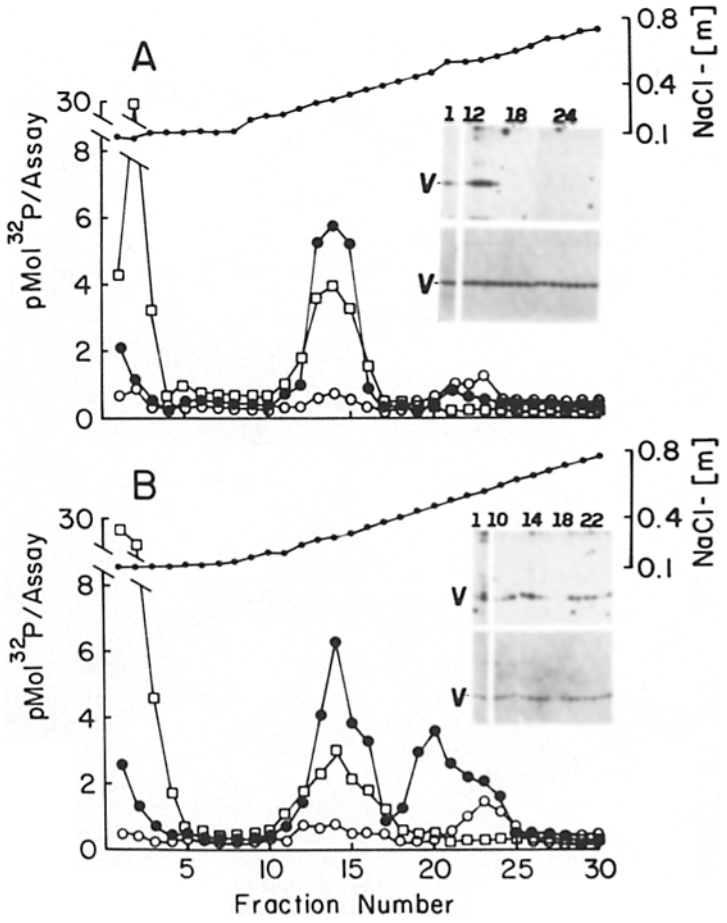

Figure 6. Phosphocellulose chromatography of nonmitotic and mitotic protein kinase activities. DEAE-Sephacel fractions (Fig. 5) with vimentin kinase activity from either nonmitotic (*A*) or mitotic (*B*) cytosol preparations were pooled and chromatographed on phosphocellulose columns as described in Materials and Methods. Collected fractions were assayed for protein kinase activity using vimentin (●), β-casein (○), or histone and cAMP (□) as substrates. The insets show the SDS gel radioautograph (*top inset*) and protein stain (*bottom inset*) of the relevant fractions assayed for vimentin kinase activity. The position of vimentin (*V*) is indicated.

and [γ-^{32}P]GTP indicated that neither peak I nor peak II vimentin kinase activities could effectively use GTP as a phosphate donor (data not shown).

Addition of cAMP had no effect on either peak I or peak II vimentin kinase activities (Table V). Similarly, addition of the heat-stable Walsh inhibitor of cAMP-dependent kinase had no effect on either semipurified vimentin kinase activity (Table V). Heparin, a characteristic inhibitor of casein kinase II, also had no apparent effect on either peak I or peak II vimentin kinase activities (Table V).

Table V. Effect of cAMP, Walsh Inhibitor, and Heparin on Semipurified Vimentin Kinase Activities

	Vimentin kinase activity (pmol/min per mg protein)		
Additions	Peak I Nonmitotic	Peak I Mitotic	Peak II
Control	3.06	7.25	25.0
1 mM cAMP	3.14	7.21	18.0
10 μg/ml Walsh inhibitor	3.10	7.26	26.0
1 μg/ml Heparin	3.22	7.20	23.0

Peak I and peak II vimentin kinase active fractions from phosphocellulose chromatography were assayed for vimentin kinase activity. Assays contained 0.05 M Hepes, pH 7.0, 5 mM $MgCl_2$, 10 μg vimentin, 50 μm ATP (950 dpm [γ-^{32}P]ATP/pmol), and either 2.9 μg (nonmitotic peak I), 1.2 μg (mitotic peak I), or 0.3 μg (mitotic peak II) semipurified cytosol protein. Values represent duplicate determinations.

Comparison of Vimentin Phosphorylation In Vitro with the Phosphorylation of Vimentin in Intact Cells

Experiments involving in vitro phosphorylation may not accurately reflect phosphorylation in intact cells. To determine if the sites phosphorylated under in vitro conditions were similar to those phosphorylated in vivo, phosphoamino acid and phosphopeptide mapping studies were carried out. In intact L-cells, phosphoserine is the only amino acid detectable in purified vimentin (12). Phosphoamino acid analysis of vimentin labeled in vitro with either peak I or peak II vimentin kinase preparations from phosphocellulose chromatography indicated that phosphoserine was the only detectable phosphoamino acid (data not shown).

Fig. 7 shows a reversed-phase HPLC analysis of tryptic phosphopeptides for vimentin isolated from nonmitotic and mitotic ^{32}P-labeled L-cells, compared with vimentin phosphorylated in vitro with peak I and peak II vimentin kinase preparations. Tryptic digests of vimentin isolated from ^{32}P-labeled nonmitotic cells produced at least six phosphorylated peptides that could be separated by reversed-phase chromatography (Fig. 7 *A*). While similar analysis of vimentin from labeled mitotic cells produced a nearly identical pattern of six tryptic phosphopeptides (Fig. 7 *B*), two of these peptides are reproducibly more highly phosphorylated than the coeluting peptides for vimentin from nonmitotic cells. Analysis of vimentin phosphorylated in vitro with peak I kinase produced multiple phosphopeptides (Fig. 7 *C*), with five individual labeled peptide species being reproducibly obtained.

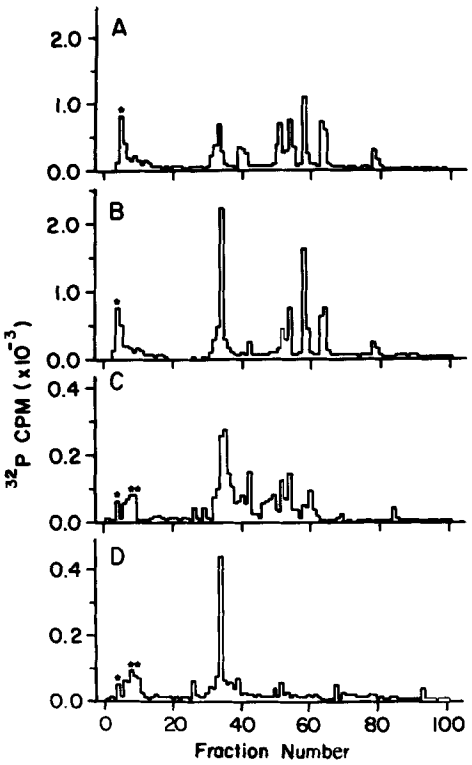

Figure 7. Tryptic phosphopeptide analysis of vimentin phosphorylated in intact cells and phosphorylated in vitro with semipurified vimentin kinases. Reversed-phase HPLC analysis was performed on vimentin isolated from ^{32}P-labeled nonmitotic cells (*A*), ^{32}P-labeled mitotically selected cells (*B*), phosphorylated in vitro with phosphocellulose peak I kinase (*C*), and phosphorylated in vitro with phosphocellulose peak II kinase (*D*). Each of the phosphopeptide maps was produced from ~50 μg of gel-purified vimentin. Radioactive fractions containing free $^{32}PO_4$ (*) and unincorporated [γ-^{32}P]ATP (**) are indicated.

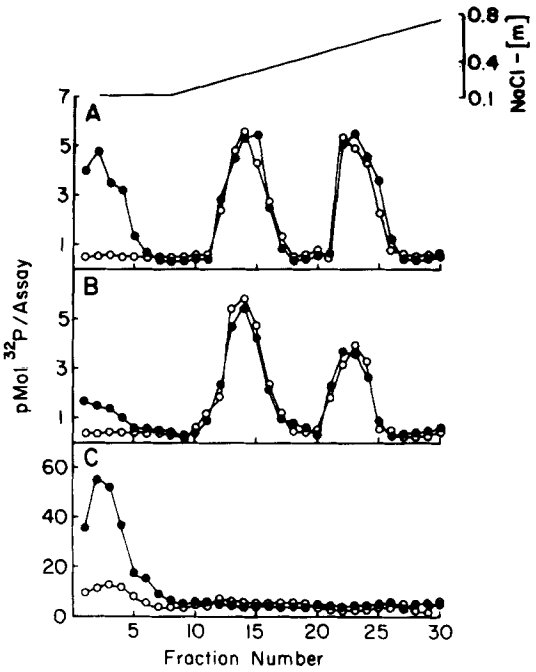

Figure 8. Phosphocellulose chromatography of HeLa, BHK-21, and mouse skeletal muscle preparations assayed for vimentin kinase activity. Cytosols from mitotically selected HeLa cells (*A*), BHK-21 cells (*B*), or skeletal muscle (*C*) were fractionated on DEAE-Sephacel columns. The main active fractions were pooled and rechromatographed on phosphocellulose under the same conditions as shown in Fig. 6. Collected fractions were assayed for kinase activity using vimentin as a substrate in the presence (○), or absence (●) of 1 μg of the heat-stable Walsh inhibitor.

Comparison with the phosphopeptides obtained from in vivo-labeled vimentin showed that four of the five in vitro-labeled peptides coeluted with peptides labeled in intact cells. The major peptide phosphorylated in vitro by peak I kinase was found to coelute with one of the two peptides that were more highly phosphorylated in mitotic cells. In contrast to the multiple sites phosphorylated by peak I kinase, vimentin phosphorylated by peak II kinase yielded a single major tryptic phosphopeptide (Fig. 7 *D*). This phosphopeptide coeluted with the major peptide phosphorylated by peak I kinase and one of the two peptides found to be more highly phosphorylated in intact cells in a mitotic-specific manner. Individual ^{32}P-labeled peaks coeluting from in vitro- and in vivo-labeled samples were recovered, dried under nitrogen, resuspended on 0.1% TFA, and mixed. All coeluting peaks were found to rechromatograph as single ^{32}P-labeled species in mixing experiments (data not shown). It is interesting to note that there appear to be vimentin peptides that are phosphorylated in intact cells that are not phosphorylated in vitro by either peak I or peak II activities, including one of the two peptides that exhibits increased phosphorylation in mitotic cells.

Hamster and Human Cell Lines Have Similar cAMP-independent Vimentin Kinase Activities

In an effort to evaluate if similar vimentin kinase activities are found in other cells during mitosis, cytosol preparations from mitotically selected hamster BHK-21 and human HeLa cells were fractionated on DEAE-Sephacel columns and the main peak of vimentin kinase activity rechromatographed on phosphocellulose. As shown in Fig. 8, both BHK-21 and HeLa cell preparations contained vimentin kinase activities that were chromatographically similar to peak I and peak II vimentin kinase from L-cells. In addition, there were variable amounts of vimentin kinase activity in the unretained fractions. This activity was inhibitable with the heat-stable Walsh inhibitor and we conclude probably represents cAMP-dependent kinase activity (see below). Similar preparations from unselected BHK-21 and HeLa cells lacked a detectable peak II kinase activity (data not shown), indicating that nonmitotic cells lack this vimentin kinase activity.

One of the unexpected findings of fractionation and assay of L-cell preparations for vimentin kinase activity was the apparent lack of cAMP-dependent kinase vimentin phosphorylation. A control experiment was carried out to evaluate the possibility that the fractionation conditions used in these studies did not detect cAMP-dependent kinase activity. Skeletal muscle is reported to be a tissue source where cAMP-dependent kinase is the primary activity that phosphorylates intermediate filament protein (43). As shown in Fig. 8 *C*, when cytosol from mouse skeletal muscle was chro-

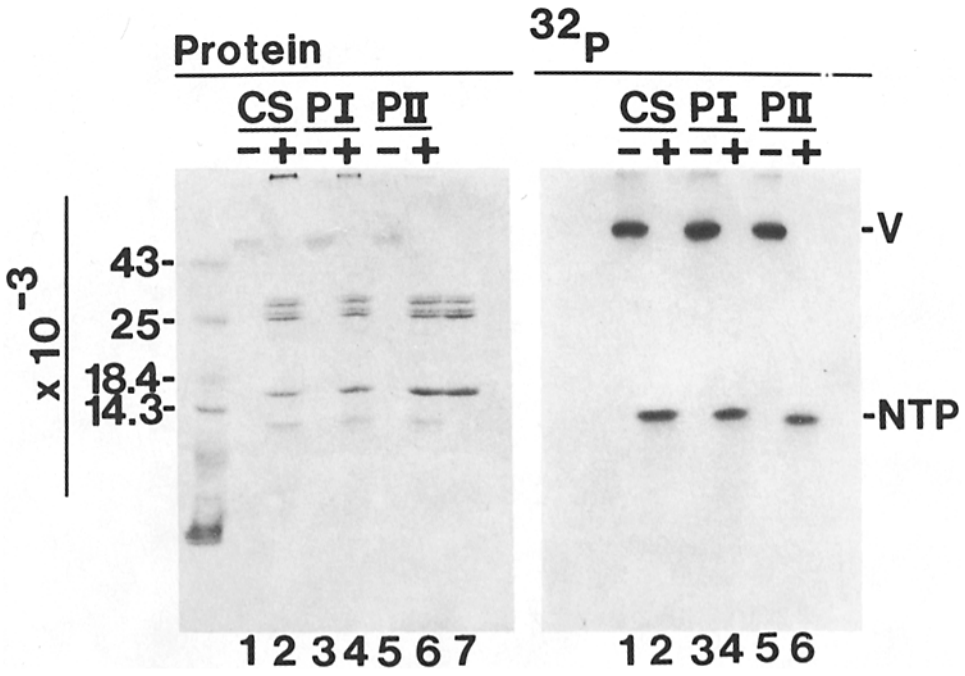

Figure 9. Lysine-specific protease digestion of vimentin phosphorylated with semipurified L-cell cAMP-independent kinases and purified catalytic subunit of cAMP-dependent kinase. The figure shows the protein stain and autoradiograph of a 15% SDS gel of purified vimentin phosphorylated with catalytic subunit of cAMP-dependent kinase (lanes *1* and *2*), peak I kinase (lanes *3* and *4*), and peak II kinase (lanes *5* and *6*), incubated with (lanes *2*, *4*, and *6*) or without (lanes *1*, *3*, and *5*) lysine-specific protease. The peptide composition of the protease alone is also shown (lane *7*). The position of undigested vimentin (*V*), the NH_2-terminal peptide (*NTP*), and protein molecular weight standards are shown.

matographed on DEAE-Sephacel and the vimentin kinase active fractions rechromatographed on phosphocellulose, the main peak of activity was in the unretained fraction. This activity was inhibited with the heat-stable Walsh inhibitor, and we conclude that this represents cAMP-dependent kinase activity. None of the column fractions exhibited increased vimentin kinase activity in the presence of exogenous cAMP, which is in agreement with the observations of O'Connor et al. (42, 43), indicating that cAMP effects were observed only in concentrated samples.

Vimentin Kinase Activities from Mitotic and Nonmitotic Cells Phosphorylate the NH_2-Terminal Region of Vimentin and Induce Filament Disassembly

There is accumulating evidence that phosphorylation of particular regions or domains of intermediate filaments can directly affect filament structure. We have reported that the sites of vimentin phosphorylation in interphase and mitotic cells are located within a 12-kD NH_2-terminal domain (13). As described previously, digestion of vimentin with lysine-specific protease results in a 12-kD NH_2-terminal fragment and a variety of smaller fragments derived from the remainder of the molecule (13, 22). Analysis of the peptides after lysine-specific protease digestion of vimentin phosphorylated in vitro with peak I and peak II preparations is shown in Fig. 9. A positive control, digestion of vimentin phosphorylated with the catalytic subunit of cAMP-dependent kinase which phosphorylates within the 12-kD NH_2-terminal fragment (14), was also included. The only detectable phosphorylated peptide after in vitro phosphorylation with all three enzymes was the 12-kD NH_2-terminal fragment.

Inagaki et al. (30, 31) have demonstrated that in vitro phosphorylation of vimentin filaments with purified cAMP-dependent kinase induces filament disassembly. This apparently results from phosphorylation at sites within the NH_2-terminal "head" domain of the vimentin filament subunit (14). Since the sites modified by the cAMP-independent kinase activities from L-cells appear to be located in the same region of the molecule, experiments were performed to determine if this phosphorylation would also affect filaments. Reconstituted vimentin filaments were phosphorylated with both kinase preparations under conditions that resulted in ~0.8–1.0 mol phosphate/mol vimentin. The assembly state of the filaments was then examined in negative stain electron microscopy. Similar filament preparations were also phosphorylated with the purified catalytic subunit of cAMP-dependent kinase as a positive control for filament disassembly. Fig. 10 shows that phosphorylation of vimentin filaments with all three enzymes resulted in a loss of assembled filaments. Deletion of ATP from the reactions preserved assembled filaments. These results indicate that the enzymes from both mitotic and nonmitotic cells modify the NH_2-terminal region of the molecule and can induce filament disassembly in vitro.

Discussion

The reorganization of intermediate filaments in cells during mitosis represents a major change in cytoplasmic organization that is associated with an increase in filament phosphorylation (6, 9, 15, 17, 54, 56). The present studies indicate that this increased phosphorylation in intact cells is reflected in increased in vitro kinase activity. We have not conducted in vitro studies with preparations from cells in all phases of the cell cycle, and therefore cannot strictly assign a mitosis-specific role to the observed differences between nonmitotic and mitotically selected preparations. However, the increased rate of vimentin phosphorylation observed when gel-purified vimentin is added to mitotic cytosol preparations compared with cytosol prepared from nonmitotic cells suggests that the change in vimentin phosphorylation is

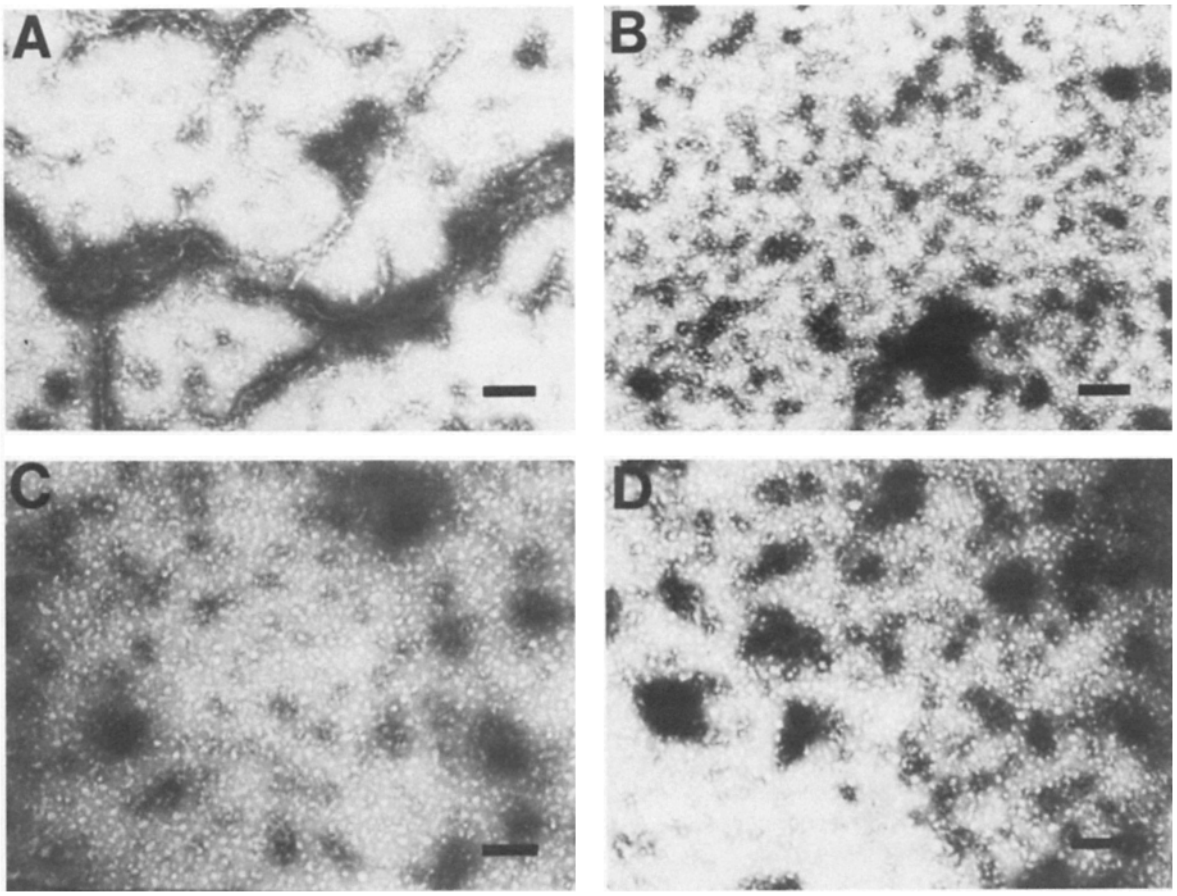

Figure 10. Effect of phosphorylation on reconstituted vimentin filaments. The figure shows electron micrographs of the following negatively stained reconstituted vimentin filaments. Untreated (*A*), phosphorylated with 3 μg catalytic subunit of cAMP-dependent kinase for 40 min (*B*), phosphorylated with 15 μg peak I kinase preparation for 60 min (*C*), and phosphorylated with 2 μg peak II kinase preparation for 60 min (*D*). Bars, 200 nm.

primarily due to a change in kinase activity and not to changes in serine site availability.

Fractionation of cytosol from nonmitotic and mitotic L-cells on DEAE-Sephacel and phosphocellulose columns indicates that there are at least two detectable protein kinase activities that will phosphorylate vimentin in vitro. Based on the order of elution from phosphocellulose, one of these activities, peak I, appears to be present in both mitotic and nonmitotic preparations. The second vimentin kinase activity, peak II, is detectable only in preparations from mitotically selected cells. On the basis of substrate specificity, and lack of sensitivity to characteristic inhibitors, both peak I and peak II protein kinase activities appear to be distinct from cAMP-dependent kinases and casein kinases.

Phosphopeptide mapping experiments demonstrate that the partially purified cytosol peak I and peak II kinase activities phosphorylate vimentin in the same region of the molecule and at sites that are similar to those that are phosphorylated in intact cells. The observation that peak I kinase phosphorylates vimentin at multiple sites while peak II phosphorylates only a single peptide is a further indication that these are distinct protein kinase species. Moreover, the evidence that peak II kinase phosphorylates vimentin in vitro at one of the two peptides that are more highly phosphorylated during mitosis is consistent with a similar role for this activity in intact cells. The inability of the semipurified vimentin kinase activities to phosphorylate all the vimentin peptides that are phosphorylated in vivo could indicate the existence of additional protein kinase activity that is not recovered in the fractionation and assay conditions used in these experiments.

The apparent absence of a significant cAMP-dependent kinase phosphorylation of vimentin in L-cells remains a puzzling finding. Vimentin is a good in vitro substrate for cAMP-dependent kinase (30, 31, 43), and there is good evidence that this enzyme is principally responsible for filament phosphorylation in skeletal muscle (41, 42). We have examined the enzymes that phosphorylate vimentin in hamster BHK-21 and human HeLa cells. Although the amount of apparent cAMP-dependent kinase vimentin phosphorylation was variable, preparations from these cell lines also contained cAMP-independent kinase activities with chromatographically similar properties to the kinase activities detected in L-cells. These results are consistent with a significant role for cAMP-independent kinases in intermediate filament phosphorylation and indicate that the kinase activity detected in mitotically selected cells may represent a conserved mechanism.

Previous reports have shown that the phosphate associated with vimentin turns over rapidly (8, 15, 17, 37). While most attention has been given to the characterization of kinase

activity, essentially nothing is known about the nature of vimentin dephosphorylation. The Ca^{2+}-stimulated phosphatase activity detected in cytosols prepared from mitotic cells presents the possibility that intracellular Ca^{2+} is important in the regulation of vimentin phosphorylation. While the present studies do not determine if the effect of Ca^{2+} on vimentin dephosphorylation is direct, it is tempting to speculate that a Ca^{2+}-activated phosphatase may be involved in the regulation of filament phosphorylation during mitosis. The rapid turnover of vimentin phosphate observed in mitotic cells (37) may be a reflection of both increased kinase and phosphatase activity.

The phosphorylation of intermediate filaments is explicitly complex. The present studies indicate that multiple kinases and phosphatases can phosphorylate and dephosphorylate vimentin at multiple sites within the NH_2-terminal region of the molecule. Although all protein kinase activities examined in these studies were capable of inducing in vitro filament disassembly, the relationship of this observation to filament phosphorylation in intact cells remains uncertain. Studies by Soellner et al. (48) have indicated that the small pool of tetrameric vimentin in cells is not more highly phosphorylated than filamentous vimentin. Although there appears to be considerable variation in the specific alteration in filament organization that occurs in different cell lines during mitosis (18, 54), Bravo et al. (6) have reported that there is no detectable highly phosphorylated form of soluble vimentin in mitotic HeLa cells. While phosphorylation-induced in vitro dissassembly may reflect an important mechanism, it seems likely that the effects of phosphorylation in cells may not be as simple.

Alterations in the phosphorylation of other proteins are prominent events during mitosis (56). In particular, changes in nuclear protein phosphorylation appear to be temporally related to changes in nuclear organization (21, 24, 27, 34, 45, 49). There is accumulating evidence that phosphorylation–dephosphorylation cycles may be essential regulatory steps in cell division (10, 11, 36, 38). Considerably less is known about the regulatory events which affect alterations in cytoplasmic organization during mitosis. Characterization of vimentin kinase–phosphatase activities will help determine if this represents a common regulatory mechanism which plays a role in both nuclear and cytoskeletal changes in mitotic cells.

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