

The relative roles of specific N- and C-terminal phosphorylation sites in the disassembly of intermediate filament in mitotic BHK-21 cells

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SUMMARY

Previously we identified p34^{cdc2} as one of two protein kinases mediating the hyperphosphorylation and disassembly of vimentin in mitotic BHK-21 cells. In this paper, we identify the second kinase as a 37 kDa protein. This p37 protein kinase phosphorylates vimentin on two adjacent residues (thr-457 and ser-458) which are located in the C-terminal non-alpha-helical domain. Contrary to the p34^{cdc2} mediated N-terminal phosphorylation (at ser-55) which can disassemble vimentin intermediate filaments (IF) *in vitro*, p37 protein kinase phosphorylates vimentin-IF without obviously affecting its structure *in vitro*. We have further examined the *in vivo* role(s) of vimentin phosphorylation in the disassembly of the IF network in mitotic BHK cells by transient transfection assays. In untransfected BHK cells, the interphase vimentin IF networks are disassembled into non-filamentous aggregates when cells enter mitosis.

Transfection of cells with vimentin cDNA lacking the p34^{cdc2} phosphorylation site (ser55:ala) effectively prevents mitotic cells from disassembling their IF. In contrast, apparently normal disassembly takes place in cells transfected with cDNA containing mutated p37 kinase phosphorylation sites (thr457:ala/ser458:ala). Transfection of cells with vimentin cDNAs lacking both the N- and C-terminal phosphorylation sites yields a phenotype indistinguishable from that obtained with the single N-terminal mutant. Taken together, our results demonstrate that the site-specific phosphorylation of the N-terminal domain, but not the C-terminal domain of vimentin plays an important role in determining the state of IF polymerization and supramolecular organization in mitotic cells.

Key words: Vimentin, Mitosis, Protein phosphorylation

INTRODUCTION

All intermediate filament (IF) proteins possess a tripartite structure with a conserved alpha-helical central rod domain flanked by two non-alpha-helical domains (Steinert and Roop, 1988). Although the central rod domain is sufficient for the formation of stable dimers and tetramers, deletion experiments illustrate the importance of end domains in the construction of higher order IF structures (Fuchs and Weber, 1994). In normal cells, the involvement of end domains in the process of IF structural dynamics is most likely mediated by protein phosphorylation and dephosphorylation events. Any perturbation of this balance, such as microinjection of protein kinase A (Lamb et al., 1989) or inhibition of protein phosphatase activities (Eriksson et al., 1992; Lee et al., 1992; Hirano and Hartshorne, 1993), results in hyperphosphorylation and either reorganization or disassembly of the IF network. A similar correlation between increased protein phosphorylation and changes in IF organization has also been observed in cells undergoing differentiation (Gard and Lazarides, 1982; Aletta et al., 1989; Baribault et al., 1989).

The most dramatic changes in IF structure, which are accompanied by increased levels of phosphorylation, are seen

when cells enter mitosis. This is a time when the restructuring of both cytoplasmic and nuclear IF networks takes place. More specifically, both vimentin (a type III IF protein) and nuclear lamins (type V IF proteins) are hyperphosphorylated by the mitosis-specific protein kinase p34^{cdc2}, and additional unidentified protein kinases (Chou et al., 1990; Peter et al., 1990; Ward and Kirschner, 1990; Luscher et al., 1991). Morphological studies have demonstrated that the organizational changes in vimentin IF vary from extensive disassembly in BHK and MDBK cells (Franke et al., 1984; Rosevear et al., 1990), to the formation of IF coils around the mitotic spindle in other cell lines (Aubin et al., 1980; Zieve et al., 1980; Jones et al., 1985). The mitotic disassembly of the nuclear lamins on the other hand is a universal phenomenon in vertebrate cells. Moreover the role of phosphorylation in this latter process appears to be critically important in that ablation of the two p34^{cdc2} mediated phosphorylation sites prevents nuclear lamina breakdown during mitosis in transiently transfected cells (Heald and McKeon, 1990).

In addition to p34^{cdc2} kinase, several well-characterized protein kinases such as cAMP-dependent protein kinase and protein kinase C are also capable of phosphorylating vimentin and desmin *in vitro* (Inagaki et al., 1987, 1988; Geisler et al.,

1989). All of these phosphorylation sites have been mapped to the N-terminal domain of these type III IF proteins (Ando et al., 1989; Geisler et al., 1989; Kitamura et al., 1989). Furthermore, *in vivo* phosphorylation of the C-terminal domain of vimentin and desmin has also been reported (Evans, 1988; Hirano and Hartshorne, 1993). However, the protein kinases responsible for the C-terminal phosphorylation and the physiological function of the phosphorylation of IF proteins *in vivo* have not been studied systematically. Here we report on the identification of a 37 kDa protein kinase that phosphorylates specifically the C-terminal domain of vimentin during mitosis. The physiological significance of the phosphorylation of vimentin at both the N- and C-terminal domains is examined *in vitro* by a disassembly assay employing negative stain electron microscopy and *in vivo* by transient transfection assays in mitotic BHK cells. Our results demonstrate that both the site and the extent of phosphorylation are crucial factors in mediating the structural changes of vimentin-IF in mitotic BHK cells *in vivo*.

MATERIALS AND METHODS

Cell culture and metabolic labeling

BHK cells were grown as previously described (Chou et al., 1989). For metabolic labeling, subconfluent cultures of BHK-21 cells were arrested in G₁ phase by incubation in isoleucine-free F-10 medium supplemented with 10% dialyzed calf serum for 38 hours (Tobey et al., 1988). Cells were then grown in standard culture medium for 4 hours and subsequently they were radio-labeled in phosphate-free Dulbecco's modified Eagle's (DME) medium buffered with 25 mM Hepes (pH 7.4), supplemented with 10% calf serum and 0.2 mCi/ml [³²P]phosphoric acid (ICN, Costa Mesa, CA) for 4 hours. Collection of mitotic cells, immunoprecipitation of vimentin from cell lysates, and two-dimensional phosphopeptide mapping were carried out as detailed previously (Chou et al., 1990).

Purification of p37 vimentin kinase

The two protein kinase activities which have been identified previously in mitotic BHK cell lysates were separated on an hydroxyapatite column. One of these two kinases has been shown to be a complex of p34^{cdc2}, 65 kDa and 110 kDa proteins (Chou et al., 1990). Fractions containing the other kinase (formerly described as vimentin kinase I, but now referred to as p37 protein kinase) were pooled and subjected to ammonium sulfate fractionation. The pellet which sedimented between 35-70% saturation was resuspended in 1 ml of 20 mM potassium phosphate (pH 7), 100 mM KCl, 1 mM EDTA, 1 mM DTT and 20 mM β-glycerolphosphate, and fractionated on a Sephacryl S-200 column (1.6 cm × 70 cm) previously equilibrated with the same buffer. The kinase containing fractions were pooled and then loaded onto a DEAE cellulose (1 ml) column equilibrated with the same Sephacryl S-200 column buffer described above. The flow-through fractions were collected and then fractionated on an S-Sepharose column (1 ml) and the proteins were eluted with a 100 to 500 mM KCl gradient in the same buffer used for the Sephacryl S-200 column. The fractions with peak kinase activities were pooled, dialyzed against 50% glycerol and frozen at -80°C. The specific vimentin kinase activity of mitotic cell lysates was typically around 0.6 nmol Pi/mg per minute, of which p37 protein kinase usually contributed about one third of the total vimentin kinase activities in the cell lysates. The final purified p37 protein kinase had a specific activity of 80 nmol Pi/mg per minute, which represented a 400-fold purification.

Antibodies used for western immunoblotting assays

Monoclonal anti-MAP kinase was purchased from Zymed (03-6600,

South San Francisco, CA). Polyclonal anti-mouse c-mos antibodies were generously provided by Drs G. Vande-Woude of the NIH (Yew et al., 1992) and R. Arlinghaus of the MD Anderson Hospital (Liu et al., 1990). A rabbit polyclonal antibody directed against the conserved PSTAIRE peptide found in all cyclin-dependent kinases was prepared in our own laboratory.

Identification of the sites of *in vitro* phosphorylation

Bacterially expressed human vimentin (0.45 mg, see below) was phosphorylated by purified p37 protein kinase (kinase:substrate(w/w)=1:20) for 60 minutes to a stoichiometry of 1.5 mol Pi/mol vimentin in a buffer of 10 mM Hepes (pH 7.2), 2 mM MgSO₄, 40 mM KCl and 0.1 mM [γ-³²P]ATP. The purification of the ³²P-labeled vimentin peptide utilized a procedure similar to the one that was employed to purify the p34^{cdc2}-phosphorylated vimentin peptides (Chou et al., 1991). Briefly, the ³²P-labeled vimentin was separated from other minor proteins by SDS-PAGE and was subsequently electro-eluted from gel slices. The protein was dialysed against 20 mM NH₄HCO₃ and lyophilized. The purified vimentin was proteolytically digested twice. The first enzymatic cleavage of vimentin was carried out with 5 μg of lysine-specific protease (Boehringer Mannheim, Indianapolis, IN) in 200 μl of 100 mM NH₄HCO₃ (pH 8.5) and 5 M urea for 4 hours at 37°C. Another 5 μg of this protease was added and incubated for an additional 4 hours. The proteolyzed sample was then fractionated by HPLC on a C18 reversed phase column with a linear gradient of 0-50% acetonitrile in 0.1% trifluoroacetic acid for 100 minutes. The majority (75%) of the ³²P activity was recovered in a peptide peak with a retention time of 40 minutes (data not shown). This fraction was vacuum-dried and subjected to a second proteolytic digestion with 1 μg of trypsin (Sigma, St Louis, MO) in 100 μl of 50 mM NH₄HCO₃ (pH 7.8) at 37°C for 5 hours. The cleaved peptides were again fractionated on a C18 column with a linear gradient of 0-40% acetonitrile in 0.1% trifluoroacetic acid for 80 minutes. The results are shown in Fig. 3. The single [³²P]peptide was shown to migrate to the spot 1 position in the 2-dimensional tryptic map shown in Fig. 2 (data not shown). The amino acid sequencing and phosphoamino acid analysis of this peptide were carried out exactly as described elsewhere (Chou et al., 1991).

Bacterial expression of vimentin

Human vimentin cDNA was cloned into the *NcoI-XbaI* sites of pET-7 (pETvim). Apart from an additional 3 amino acids (methionine, glycine and serine) at the N terminus, this subcloning strategy yielded the complete coding sequence of human vimentin (Ferrari et al., 1986). The pET vector system initiates protein synthesis of a cloned gene under the influence of T7 RNA polymerase after induction by isopropyl β-D-thiogalactopyranoside (Studier et al., 1990). *Escherichia coli* BL21 bacteria bearing the chloramphenicol resistant plasmid pLysE to prevent basal activity of T7 RNA polymerase were subsequently transformed with pETvim. The bacteria were then grown, the vimentin-rich inclusion bodies were isolated and the vimentin was purified using the same method developed for nuclear lamins (Moir et al., 1991). Vimentin was further purified by two rounds of assembly-disassembly as previously described (Zackroff and Goldman, 1979). The purity of vimentin was confirmed by the presence of a single band of 55 kDa on SDS gels (not shown). Vimentin was stored in aliquots at -80°C in unpolymerized form in 2 mM phosphate buffer (pH 7.2).

Site-directed mutagenesis

The human vimentin cDNA clone, flanked by *XbaI* sites in pET 7 was subcloned into the unique *XbaI* site of the vector pTZ 18U and mutagenesis was performed (Kunkel et al., 1991) using the Bio-Rad mutagenesis kit (Bio-Rad Richmond, Ca). Two single mutant constructs were obtained by substituting alanine residues separately for both the N-terminal (ser-55) and the C-terminal phosphorylation sites (thr-

457/ser-458) using the primers: S55:A---CGCCTCGGCCCGGG-CGG; and T456:A/S457:A---TCAACGAAGCTGCTCAGCATC.

All mutagenesis was confirmed by double stranded DNA sequencing. Double mutant constructs (with both the N- and C-terminal phosphorylation sites ablated) were subsequently prepared from the single mutant constructs by taking advantage of a unique *ClaI* site positioned between the two sites within the vimentin cDNA, and by swapping fragments between the two single mutant constructs. Constructs with the relevant sites altered were subcloned into the *BamHI* site of the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA) which bears the CMV promoter (Boshart et al., 1985). Our cloning strategy into pcDNA3 allowed us to delete the three additional residues at the N-terminal end of vimentin in the pET based bacterial expression system (mentioned above). A myc tag cassette was added at the C terminus of vimentin using a *SphI* site engineered at the end of the vimentin coding sequence. The myc tag has 15 amino acids listed in the single letter amino acid code as follows: GMQEQK-LISEEDLNV.

Transfection and light microscopy

BHK cells, plated onto poly-L-lysine coated coverslips in 35 mm culture dishes, were incubated in a transfection mixture composed of 2 μ g DNA and 6 μ l lipofectAMINE (Gibco-BRL, Gaithersburg, MD) in 1 ml of serum free DME medium. The transfection mixture was then replaced by standard culture medium after 5 hours and the cells on coverslips were incubated for 65 hours at 37°C. Whenever it was necessary to enrich for mitotically arrested cells, 0.4 μ g/ml nocodazole was added to the medium for the final 5 hours of incubation. The cells were fixed and processed for indirect immunofluorescence using standard techniques (Vikstrom et al., 1992).

Cells were observed by conventional epifluorescence on a Zeiss Axiophot or by confocal optics on a Zeiss Laser Scan microscope using $\times 40$, $\times 63$, and $\times 100$ oil immersion high NA objective lenses. Transfected cells were identified by the presence of myc-tagged vimentin using the mouse monoclonal primary antibody 9E10 specific for the myc tag (American Type Culture Collection, Bethesda, MD). Mitotic cells were identified by their condensed chromosomes stained with the DNA binding dye, Hoechst 33342 (Sigma, St Louis, MO). The secondary antibody used was fluorescein-conjugated goat anti-mouse IgG (Jackson Immunoresearch Labs, Inc., West Grove, PA).

RESULTS

Identification of a 37 kDa protein as a vimentin kinase

We have shown that the mitotic protein kinase, p34^{cdc2}, is one of two active vimentin kinases detected in mitotic BHK cell lysates. The other vimentin kinase, designated here as p37 protein kinase (see supporting data below), differs from p34^{cdc2} by its lack of H1 histone kinase activity and its phosphorylation of vimentin at different sites as determined by comparative two-dimensional phosphopeptide mapping (Chou et al., 1989, 1990).

We have been able to achieve a 400-fold enrichment of this p37 protein kinase through four chromatographic steps. The purified kinase has a specific activity of 80 nmol Pi/mg per minute (see Materials and Methods). Fractions containing this purified kinase preparation were not homogeneous when examined on silver-stained SDS-gels. However, when the relative kinase activities and the protein compositions in different fractions of the final chromatographic step (S-Sepharose column) were compared, the protein kinase activities were closely correlated with a 37 kDa protein (Fig. 1A and

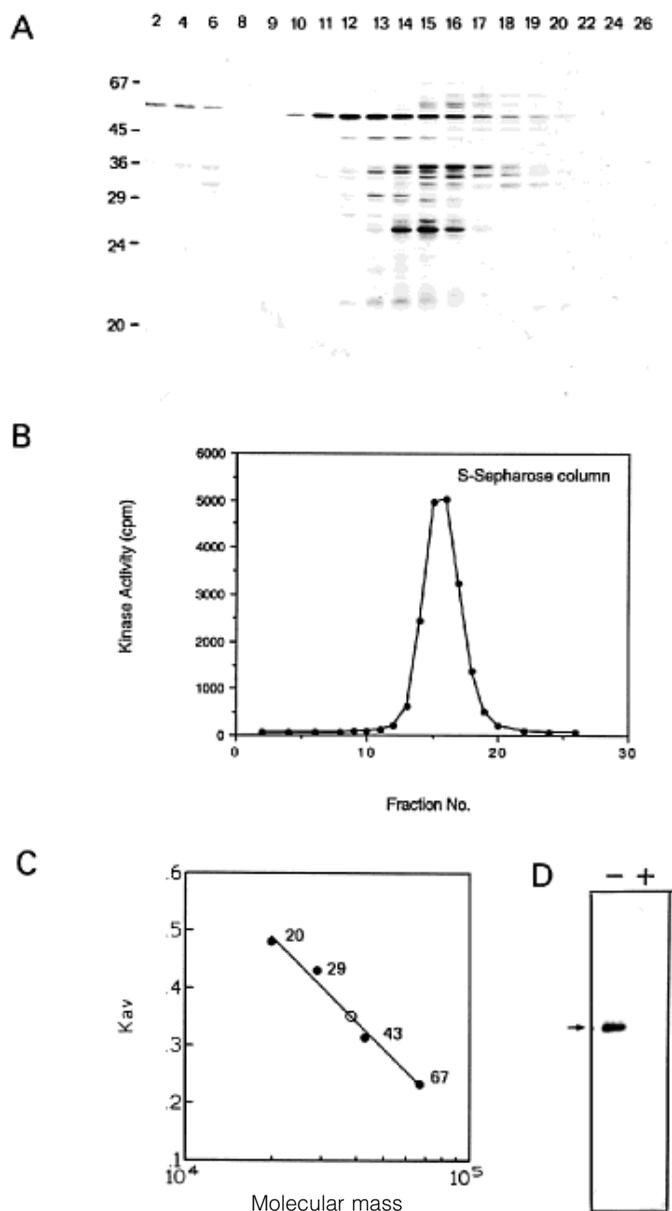


Fig. 1. Identification of a 37 kDa protein as vimentin kinase. (A) Analysis of S-Sepharose column fractions by SDS-PAGE using the silver-staining method (Wray et al., 1981). Fraction numbers are at the top of each lane and the positions of molecular mass markers are indicated at the left. The 37 kDa protein is most abundant in fractions 15 and 16. (B) Vimentin kinase activities in corresponding fractions of the S-Sepharose column. (C) Vimentin kinase has an apparent molecular mass of 37 kDa (open circle) on a Sephacryl S-200 column. Molecular mass markers used were: trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), egg ovalbumin (43 kDa), and bovine serum albumin (67 kDa). (D) Photoaffinity labeling of p37 kinase with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. Aliquots of purified kinase (as seen in column fraction 16 in A) were UV-irradiated at 4°C for 6 minutes as described (Potter and Haley, 1983) in 35 μ l of 10 mM Hepes (pH 7.2), 2 mM MgSO_4 , 50 mM KCl, 34 μ M 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and in the absence (-) or presence (+) of 5 mM MgATP. The samples were then separated on SDS-gels and autoradiographed. The position of the 37 kDa protein is indicated with an arrow.

B). Consistent with this correlation, fractionation of this kinase on a molecular sieve column (Sephacryl S-200) with proteins of known molecular mass also suggested that the kinase had an apparent molecular mass of 37 kDa (Fig. 1C). Additional evidence that the 37 kDa protein was a protein kinase came from its identification as an ATP-binding protein. The fractions containing peak kinase activities were incubated with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, an ATP analogue that can be activated and covalently crosslinked to the bound protein by 254 nm UV irradiation (Potter and Haley, 1983). As shown in Fig. 1D, the 37 kDa protein was the only band labelled with ^{32}P under these conditions. When UV irradiation was carried out in the presence of excess ATP, no ^{32}P incorporation was seen. Taken together, the results strongly indicate that the 37 kDa protein is a bona fide vimentin kinase.

We have also attempted to determine whether the p37 protein kinase is one of the known cell cycle-dependent protein kinases with similar molecular masses: these include mitogen activated kinase (42 kDa, Boulton et al., 1990), c-mos kinase (37 kDa, Liu et al., 1990; Yew et al., 1992), and cyclin-dependent kinases (33 kDa, Meyerson et al., 1992). Our approaches to date have involved the use of antibodies directed against these protein kinases (see Materials and Methods). In each case the antibodies tested showed no cross reactivity on western blots (data not shown).

Mitotic phosphorylation of vimentin is mediated by two protein kinases

Previously, we had determined that p34^{cdc2} phosphorylates vimentin at one of two major mitosis-specific sites (Chou et al., 1990). Therefore, we investigated whether the p37 protein kinase might be responsible for the phosphorylation of the other major site. Two-dimensional phosphopeptide maps of vimentin phosphorylated in vitro by purified p37 protein kinase were compared with similar maps derived from mitotic or interphase cells labeled with ^{32}P in vivo. As shown in Fig. 2A, two prominent phosphopeptides are present in the mitotic map (spots 1 and 2). Spot 2 is known to be phosphorylated by p34^{cdc2} (Chou et al., 1990), and the other major site (spot 1) comigrates with the spot phosphorylated in vitro by purified p37 protein kinase (Fig. 2C and D). Neither peptide 1 nor peptide 2 comigrates with any spots phosphorylated during interphase (Fig. 2B,E and F). Although two minor phosphopeptides (spots 3 and 4) are shared by both mitotic and interphase samples (Fig. 2A,B and E), the kinases responsible for their phosphorylation are different. Peptides 3 and 4 are phosphorylated only slightly by p34^{cdc2} at ser-41 and ser-65 (Chou et al., 1991), while the same two peptides are phosphorylated at ser-38 and ser-65 during interphase probably by A kinase and C kinase (Ando et al., 1989). In summary, our results show that p37 vimentin kinase activity can only be detected during cell division, and together with p34^{cdc2}, accounts for the major mitosis-specific vimentin phosphorylation sites.

p37 kinase phosphorylates vimentin on its C-terminal tail domain

To determine the amino acid residue(s) phosphorylated by p37 protein kinase, we employed a procedure similar to the one used for localizing the p34^{cdc2}-mediated vimentin phosphorylation sites (Chou et al., 1991). Briefly, purified human vimentin was phosphorylated in vitro with purified p37 protein

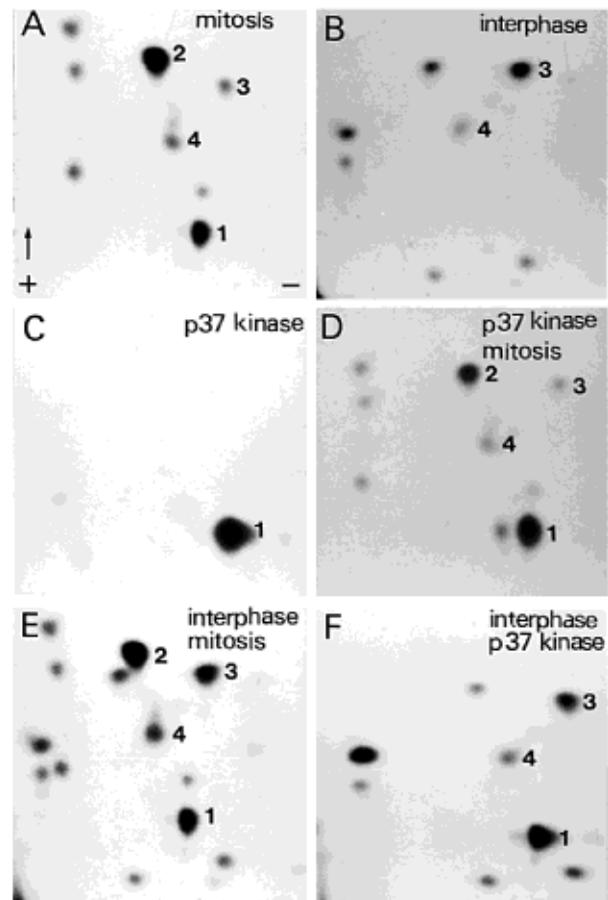


Fig. 2. Phosphorylation of vimentin by p37 protein kinase at mitosis-specific sites. Autoradiograph of tryptic ^{32}P -peptide maps of vimentin phosphorylated in vivo during mitosis (A), interphase (B), and in vitro by purified p37 protein kinase (C). The uniqueness of spots 1 and 2, which are phosphorylated only during mitosis, was confirmed by the comigration of paired samples from interphase, mitosis and p37 kinase-phosphorylated vimentin (D,E,F). Directions of electrophoresis (+, -) and ascending chromatography (arrow) are indicated in (A).

kinase using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The ^{32}P -labeled vimentin was run on SDS-gels, and then electrophoretically eluted from gel slices (see Materials and Methods). The eluted vimentin was digested with lysine-specific protease and the resulting peptide fragments were separated by reversed phase HPLC. Over 75% of the ^{32}P activity eluted at a retention time of 30 minutes (data not shown). Sequential Edman degradations of the sample in this fraction revealed a dominant peptide corresponding to the human vimentin sequence from thr-445 to glu-465 (Ferrari et al., 1986).

To further purify this ^{32}P -peptide, the sample was subjected to tryptic cleavage and again fractionated by reversed phase HPLC. All of the ^{32}P activity was recovered in a single peptide peak with a retention time of 42 minutes (Fig. 3A and B). About 400 pmols of this peptide were recovered and its sequence corresponded to the last tryptic peptide of vimentin (residues 450-465, Fig. 3B). Since it is known that phosphorylated serine and threonine residues have a stronger tendency, compared to their unphosphorylated counterparts, to be converted to dithiothreitol

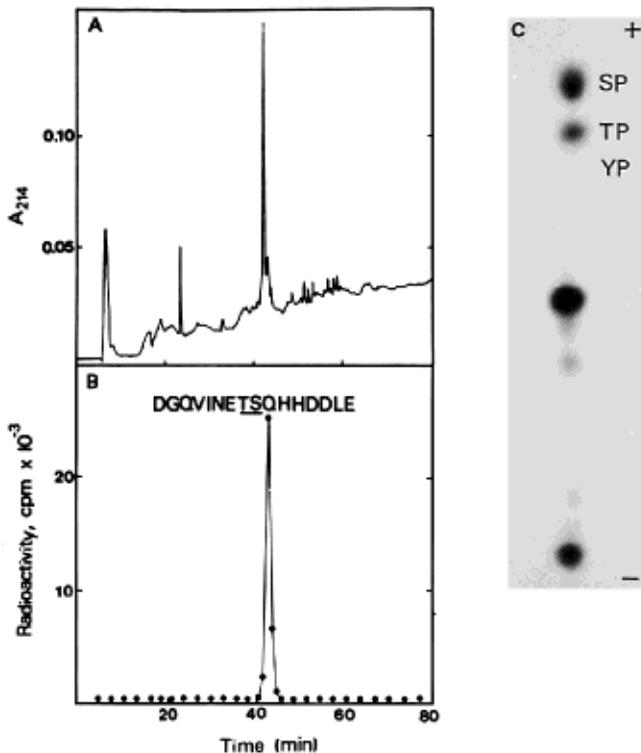


Fig. 3. Identification of p37 kinase phosphorylation sites in the C-terminal domain. Vimentin phosphorylated by p37 kinase was subjected to two cycles of proteolysis and reversed phase HPLC as described in Materials and Methods. The peptide profile obtained from the second HPLC purification step is shown in A. The majority of ^{32}P activity is eluted at a retention time of 42 minutes as seen in B, which coincides with the dominant UV-absorbing band seen in A. The amino acid sequence of this ^{32}P -containing peptide was determined and is shown in single-letter code. The designated phosphorylation sites (thr-457, ser-458) are underlined. (C) The result of phosphoamino acid analysis of this peptide. The positions of serine-phosphate (SP), threonine-phosphate (TP) and tyrosine-phosphate (YP) are indicated.

adducts (Meyer et al., 1986), the relatively low yield of phenylthiohydantoin-serine at Edman degradation cycle 9 suggested that this residue was phosphorylated. Furthermore, phosphoamino acid analysis confirmed that the majority of the ^{32}P was associated with serine, and a lesser amount with threonine (Fig. 3C). As there is only one serine and one threonine in this peptide, we conclude that ser-458 is the preferred phosphorylation site of p37 kinase. Thr-457 is also phosphorylated, but to a much lesser extent under the *in vitro* conditions used. These two residues are conserved across species lines in all vimentins that have been sequenced to date.

Phosphorylation at the N-, but not the C-terminal domain disassembles vimentin-IF *in vitro*

Numerous studies have shown that phosphorylation of the N-terminal domain disassembles type III IF *in vitro* (Inagaki et al., 1987, 1988; Chou et al., 1989, 1990). However, there are no studies aimed at determining the effects of phosphorylation in the C-terminal domain. Therefore the effects of phosphorylation at the N and C termini on vimentin IF disassembly *in vitro* were compared. IF formed from vimentin were phosphorylated by either p34^{cdc2} or p37 kinase and negatively stained samples were then examined by electron microscopy. Consistent with our previous observation, phosphorylation by p34^{cdc2} induced IF disassembly. However, there was no obvious change in IF structure when vimentin-IF were phosphorylated in the presence of p37 kinase (Fig. 4).

Ablation of the N- and C-terminal phosphorylation sites

In order to begin to study the *in vivo* function of both the N- and C-terminal phosphorylation sites, we employed transient transfection assays. Vimentin and mutant vimentin cDNAs in pET vectors were subcloned into a eukaryotic expression vector under the control of the CMV promoter (Boshart et al., 1985). To facilitate the distinction between host and exogenous proteins, a fifteen amino acid myc tag was placed at the end of the vimentin coding region so that the exogenous vimentin could be readily recognized by a monoclonal antibody specific

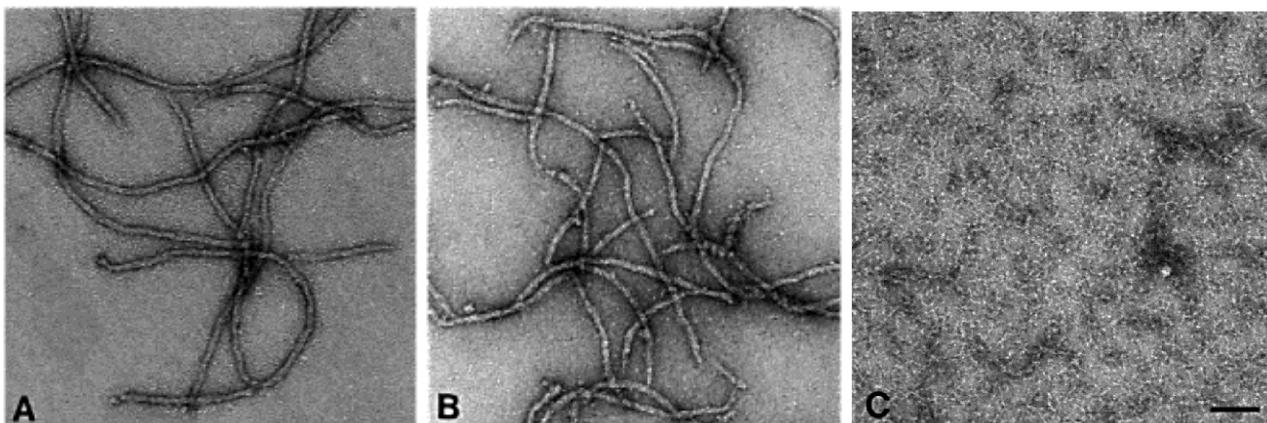


Fig. 4. Phosphorylation at the N- but not the C-terminal domain disassembles vimentin-IF *in vitro*. (A) Unphosphorylated wild-type vimentin IF. (B) p37 kinase phosphorylated wild-type vimentin IF (1.06 mol Pi/mol protein). Note that the filaments appear to be similar to those seen in (A). (C) p34^{cdc2} phosphorylated vimentin IF (1.62 mol Pi/mol protein). Note the lack of obvious 10 nm diameter IF. Only vimentin IF disassembly products are present. Vimentin phosphorylation and disassembly were carried out in conditions as described (Chou et al., 1990). Bar, 85 nm.

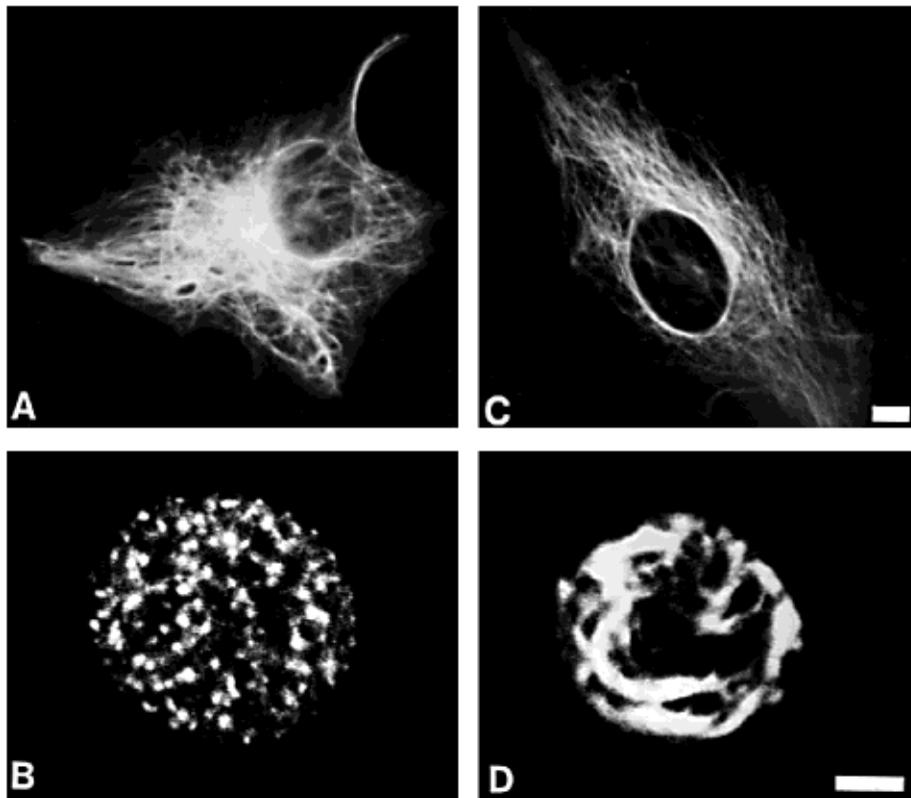


Fig. 5. Ablation of the N-terminal phosphorylation prevents IF disassembly during mitosis in transfected BHK cells. Immunofluorescence observations of IF networks in interphase and mitotic BHK cells observed at 65 hours post transfection. (A) BHK cell transfected with myc tagged wild-type vimentin. This cell was fixed and stained with anti-myc for immunofluorescence. Note the normal appearance of the vimentin network. (B) Transfected and mitotically arrested BHK cell using the same myc tagged wild-type vimentin as in (A). There is a conversion of a continuous filamentous network pattern into a punctate pattern in the majority of cells (see E) indicating disassembly of the IF network in mitosis (Rosevear et al, 1990). (C) BHK cells transfected with the double mutant construct (S55:A/T457:A/S458:A) showing a normal appearing interphase IF network. (D) Mitotic BHK cells transfected with the same double mutant construct as in (C). Note the filamentous pattern in contrast to the punctate pattern seen in (B). (A and C) Images taken with conventional epifluorescence optics; (B and D) confocal images resulting from the stacking of 10 sections, taken at 0.5 μm focal increments. The majority of cells transfected with the mutant construct S55:A were identical to the ones seen in C and D (not shown), while

E	Untransfected	Wild type	S55:A	S55:A/T457:A/S458:A	T457:A/S458:A
Punctate	209 (81.3%)	106 (65%)	46 (22.8%)	54 (18.1%)	208 (63.4%)
Filamentous	48	57	155	244	120
Total (n)	257	163	201	298	328

the mutant (T457:A/S458:A) appeared indistinguishable from the wild-type transfectants seen in (A) and (B) (not shown). (E) A summary of the data obtained for the two mitotic phenotypes seen for each type of transfectant. Bar, 5 μm .

for this tag. This strategy has been successfully used for the mutational analysis of IF assembly *in vivo* (Wong and Cleveland, 1990; Dent et al., 1992). When wild-type vimentin cDNA was expressed by transient transfection and the cells were observed at 65 hours after transfection, the myc sequence did not appear to interfere with the formation of the endogenous vimentin IF network in BHK-21 cells (Fig. 5A). Our protocol routinely yielded a transfection efficiency of 20-30%, which allowed us to obtain sufficient numbers of cells for the analyses described below.

We have shown that the vimentin IF network is changed from a filamentous to a spotty or punctate (non-filamentous) pattern in prometaphase/metaphase BHK cells. These spots were shown to consist of aggregates of 2-5 nm diameter vimentin containing protofibrils by electron microscopy (Rosevear et al., 1990). Therefore, immunofluorescence provides a convenient assay for testing the role of vimentin phosphorylation on IF structure *in vivo*. If phosphorylation indeed participates in the process of disassembly, one would predict that overexpression of non-phosphorylatable protein in these cells could inhibit a proportion of mitotic vimentin IF from disassembling.

We examined the IF networks of mitotically arrested BHK

cells at 65 hours post-transfection. The IF pattern in transfected cells in mitosis was scored either as consisting of spots or filamentous structures, and the numbers of cells in each category were tabulated as shown (see Fig. 5E). The majority (over 80%) of the non-transfected mitotic cells clearly demonstrated a punctate vimentin pattern (Fig. 5B). Cells transfected with the double mutant cDNA (S55:A/T457:A/S458:A) showed a significant decrease in the percentage of mitotic cells with this pattern of disassembled IF (approximately 18%). Most of these latter transfected mitotic cells displayed thick filamentous vimentin-rich arrays (Fig. 5D). To further examine the individual contributions of N- and C-terminal phosphorylation sites, the cells were transfected with the cDNA constructs lacking either the C- or N-terminal phosphorylation sites. While the N-terminal mutant (S55:A) yielded a phenotypic distribution comparable to the double mutants, the C-terminal mutant displayed morphological features very similar to the wild-type vimentin construct (e.g. the majority appeared as in Fig. 5B; also see Fig. 5E). Interestingly, transfection with wild-type human vimentin cDNA also exhibited a slight reduction in the percentage of cells with a punctate vimentin pattern (see Fig. 5E).

In order to make certain that the thick filamentous structures

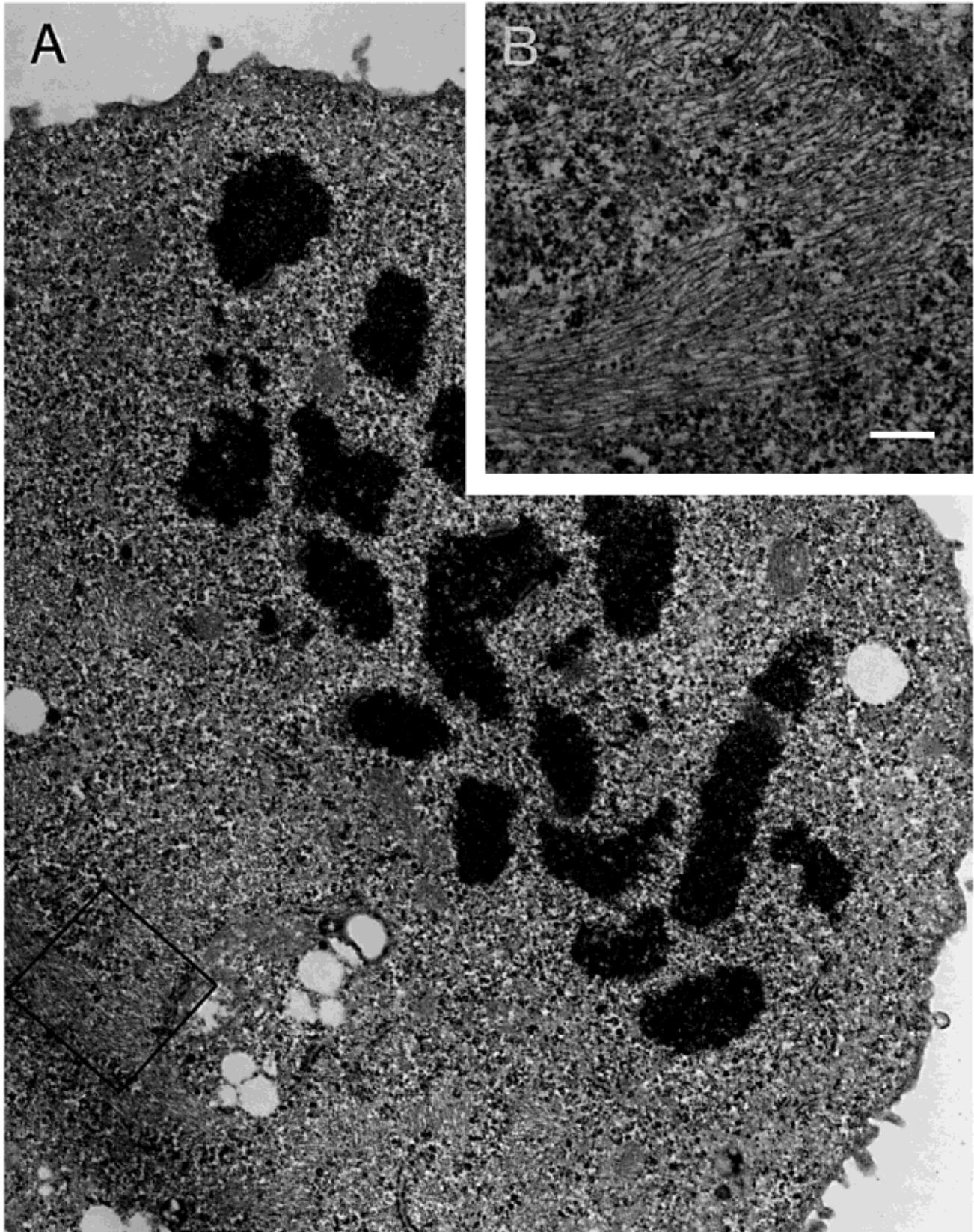


Fig. 6. Electron micrograph of a thin section of a mitotic cell transfected with mutant cDNA (S55:A). (A) Low magnification showing condensed chromosomes and numerous 10 nm filament bundles throughout the cytoplasm. The area marked by the square is shown at higher magnification (B). Bar, 250 nm.

(see Fig. 5D) consisted of 10 nm diameter IF and not other types of protein aggregates, we examined either N-terminal or double mutant transfectants by thin section electron microscopy. Mitotic cells transfected with either one of these mutant cDNAs could be easily distinguished from untransfected cells by the presence of many large bundles of 10 nm IF (Fig. 6). We have never been able to observe a similar structure in untransfected mitotic cells. As we have documented in detail before, almost all of the detectable vimentin IF proteins are present in aggregates of short 2-5 nm diameter protofilaments in mitotic BHK cells (Rosevear et al., 1990). Taken together these results suggest that blocking the N-terminal phosphorylation site alone is sufficient to significantly inhibit IF disassembly, while blocking the C-terminal phosphorylation site does not appear to play a significant role in this respect.

DISCUSSION

There is little doubt that phosphorylation is an important post-translational regulatory factor in controlling the state of assembly of cytoskeletal IF proteins (Skalli et al., 1992). However, most studies to date have revealed very little information about either the cell type specific kinases or the specific sites of IF subunit phosphorylation involved in regulating IF assembly states *in vivo*. In general, *in vivo* studies have been hampered by the fact that in most cells, the vast majority of cytoskeletal IF proteins appear to be polymerized throughout most of the cell cycle. In order to establish a more direct correlation between specific kinases, their sites of phosphorylation and their function in regulating IF protein assembly, we have taken advantage of both the hyperphosphorylation of vimentin and the unusually dramatic transition from assembled to disassembled IF in synchronized mitotic BHK cells (Rosevear et al., 1990). Previously, we determined that two protein kinases are responsible for this mitosis-specific hyperphosphorylation. One of these kinases is p34^{cdc2} which phosphorylates ser-55 in the N terminus of vimentin and coincidentally drives the disassembly of IF *in vitro* (Chou et al., 1990). In the present study, we have further purified and characterized the second kinase, p37 kinase, and mapped its phosphorylation sites to thr-457 and ser-458 in the C-terminal domain of vimentin. Using site-directed mutagenesis and transient transfection, we have been able to test more directly the role of phosphorylation in regulating the disassembly of IF in mitotic BHK cells.

N-terminal phosphorylation is essential for IF disassembly in mitotic BHK cells

The results tabulated in Fig. 5 suggest that both the site and the amount of phosphate on vimentin determine its ability to undergo disassembly *in vivo*. With respect to the sites of phosphorylation, there are dramatic differences between the N- and C-terminal domains, even though they are phosphorylated to similar extents during mitosis (Fig. 2). The ability of the N-terminal mutated vimentin (S55:A) to significantly decrease (from approximately 65% to 23%) the fraction of transfected mitotic cells with disassembled IF networks, strongly suggests that phosphorylation by p34^{cdc2} is essential for IF disassembly *in vivo*. On the other hand, the majority of cells transfected

with the C-terminal mutant (T457:A/S458:A) continue to disassemble their IF in mitosis. Therefore it is not surprising to find that the double mutant (S55:A/T457:A/S458:A) also prevents IF disassembly to approximately the same extent as the single N-terminal mutant. These data suggest that the C-terminal phosphorylation sites do not participate in IF disassembly, nor do they appear to facilitate or potentiate the phosphorylation of the N-terminal site. It should also be noted that when keratins are ectopically expressed in 3T3 and SW13 cells, ablation of one of the major interphase phosphorylation sites, enhances the resistance of keratin-IF to okadaic acid-induced disassembly during interphase and mitosis (Ku and Omery, 1994).

Interestingly, transfection with wild-type vimentin also reduces the number of mitotic cells with disassembled IF (from approximately 81% to 65%). This observation suggests that expression of additional vimentin in transfected cells decreases the kinase/substrate ratio, which conceivably could lower the amount of phosphate incorporated into vimentin polymer. This could explain the decrease in the extent of IF disassembly in individual cells. Using this scenario, alterations in IF structure would be dependent on both the stoichiometry and sites of phosphorylation. This is consistent with a recent report demonstrating that the various extents of mitotic vimentin IF disassembly seen in different cell types appear to be correlated with the amount of p34^{cdc2} kinase activity (Tsujiyama et al., 1994). In the light of this dose-dependent effect, a unique feature of BHK IF is the presence of desmin (Quinlan and Franke, 1982), which possesses two p34^{cdc2} sites in its N terminus (Kasubata et al., 1993). This additional phosphorylation site might render mitotic BHK IF especially sensitive to phosphorylation-mediated disassembly. Our results demonstrate that phosphorylation-mediated IF disassembly in mitosis is site-specific and dose dependent (also see preliminary report of Carpenter et al., 1992). These results can also help to reconcile the differences in the extent of IF disassembly in different cell types during mitosis (Aubin et al., 1980; Jones et al., 1985).

In addition to possible differences in p34^{cdc2}:vimentin ratios, the maintenance of polymerized vimentin IF in some cell types during mitosis also suggests the possibility that phosphorylation of vimentin by p34^{cdc2} is essential, but may not be sufficient for the complete disassembly of IF during mitosis. In this respect, phosphorylation might work synergistically with other factors to ultimately disrupt IF structure. Several such protein factors, such as α -crystallins, have been shown to affect IF structure (Dent et al., 1992; Nicholl and Quinlan, 1994). We therefore speculate that the dynamic and complex behavior of vimentin IF in different cells, might reflect the interplay of such putative regulatory factors in addition to phosphorylation.

Potential roles for phosphorylation in the C-terminal domain

In vitro, tailless vimentin forms IF which have a tendency to aggregate along their lengths (Kaufmann et al., 1985; Coulombe et al., 1990; Bader et al., 1991; Eckelt et al., 1992). Desmin-IF assembled in the presence of a peptide, corresponding to a subdomain of the tail region, appear to be more loosely packed than normal IF (Birkenberger and Ip, 1990). These observations support the involvement of the C terminus in the lateral packing of vimentin IF. Point mutation analyses have also identified an essential element for IF formation in the

conserved β -turn motif in the C-terminal domain of vimentin (Kouklis et al., 1993a; McCormick et al., 1993). The proximity of the C-terminal phosphorylation sites (thr-457, ser-458) to this conserved β -turn (at gly-451) suggests the intriguing possibility that the phosphorylated residues characterized in this study may be involved in regulating the supramolecular organization of IF in vivo. In light of this, it is somewhat disappointing that we have not yet detected a phenotype with our C-terminal mutant. However, it is quite possible that subtle alterations in IF structure due to C-terminal phosphorylation would not be detected by conventional microscopic methods.

The C-terminal domain of vimentin, as well as other type III IF proteins, have also been implicated in interactions with nuclear lamin B in vitro (Georgatos and Blobel, 1987; Maison et al., 1993). Consistent with this observation is the recent finding that microinjection of vimentin antibodies delays exit from mitosis by interfering with the formation of daughter cell nuclei (Kouklis et al., 1993b). A role for the C-terminal domain of vimentin in regulating nuclear shape has also been proposed (Sarria et al., 1994). In addition, it appears that phosphorylation of the C-terminal domain could be involved in regulating a variety of other functions including the organization of IF networks (Gotow et al., 1994; Tu et al., 1995), as well as interactions between IF and microtubules (Hisanaga et al., 1991), and between IF and actin filaments (Cary et al., 1994).

In summary, our interest in the molecular basis of IF dynamics has led us to study the correlation between mitotic IF hyperphosphorylation and disassembly in BHK cells. We have shown that the hyperphosphorylation of vimentin in mitosis represents the combined action of two protein kinases, p34^{cdc2} and p37 kinase. Although a large number of protein kinases are capable of phosphorylating vimentin in vitro, p34^{cdc2} and p37 kinase constitute the first two kinases identified to date which specifically phosphorylate this type III IF protein in vivo. The identification of the major mitotic vimentin phosphorylation sites has also permitted us to test the causal relationship between vimentin phosphorylation and disassembly in mitotic BHK cells by using transient transfection assays. While p34^{cdc2} plays an essential role in the disassembly of IF in mitotic BHK cells, the function of the p37 kinase remains unknown. However, the identification for the first time of an endogenous kinase that phosphorylates specific sites in the C-terminal domain provides us with the unique opportunity to look into other alternative functions for vimentin phosphorylation, such as its role in regulating the interaction between type III IF proteins and other cellular components. This is an especially pertinent issue in light of the recent observation that vimentin-null mice lack a detectable phenotype (Colucci-Guyon et al., 1994). Undoubtedly, a greater understanding of all of the factors involved in the posttranslational control of vimentin IF disassembly and dynamics will shed light on their specific functions in cells in which they are normally expressed. In addition, the lack of a detectable phenotype for vimentin tail phosphorylation suggests the possibility that there may be other targets for p37 kinase. In this regard, p37 kinase may be a new cell cycle-dependent protein kinase whose activity is part of the protein phosphorylation cascade involved in activating and maintaining the mitotic process of dividing cells. The search for other substrates for this kinase, and how this kinase is regulated, will certainly aid in the elucidation of the greater physiological role that p37 kinase plays during mitosis.

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