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Phosphorylation *in vitro* of vimentin by protein kinases A and C is restricted to the head domain

Identification of the phosphoserine sites and their influence on filament formation

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The *in vitro* phosphorylation of vimentin, the intermediate filament protein of mesenchymal cells, by kinases A and C is serine-specific and involves only the N-terminal head domain. In oligomeric protofilament units each kinase recognizes five sites, which have been identified by sequence analysis. Kinase C introduces 1.5 mol phosphate/mol vimentin, while kinase A treatment results in 4 mol phosphate/mol. Kinase-A-treated oligomers do not polymerize in standard assays whereas kinase C treatment has no inhibitory effect. Filaments exposed to kinase A remain stable and incorporate only 1.7 mol phosphate/mol vimentin. These phosphates are essentially restricted to two of the five kinase A sites found in protofilament units. Thus the head domain, previously related to *in vitro* assembly competence and filament stability, changes in accessibility between the oligomeric and polymeric state. We discuss the possibility that *in vivo* phosphorylation of vimentin filaments by kinase A may not necessarily be accompanied by an extensive depolymerization. It could instead involve a dynamic change of the filament surfaces, which could alter the interaction of the filaments with other cellular structures.

Vimentin, the intermediate filament protein of mesenchymal cells, is an *in vivo* substrate for protein kinases [1 – 8]. Several studies have shown that vimentin phosphorylation is enhanced during cell division [3, 9 – 11]. Early work on chick embryo myotubes indicated that vimentin and desmin are subject to phosphorylation by kinase A [2, 5] and in other *in vivo* studies phosphorylation of vimentin by kinase C was reported [6]. Recently Inagaki et al. [12] studied the *in vitro* phosphorylation of murine vimentin by kinases A and C. They reported a direct depolymerization of vimentin filaments upon phosphorylation with kinase A and raised the possibility that this type of modification could lead to an *in vivo* depolymerization of filaments.

To begin to understand the properties of phosphorylated desmin and vimentin, we recently characterized the *in vitro* modification of desmin by kinase A [13]. We described three kinase A sites on the sequence level and showed that phosphorylation of desmin, the myogenic intermediate filament protein, is restricted to the N-terminal head domain, which has been implicated in polymerization competence and filament stability [14 – 16]. In agreement with this view, kinase-A-modified oligomers of desmin proved incompetent in standard polymerization tests [13, 17]. In addition, a recent report by Evans [18] has shown that mitosis-specific phosphorylation of vimentin is also restricted to the head domain. Thus a more detailed analysis of the *in vitro* phosphorylation of vimentin is necessary. Here we present the protein chemical identification of the phosphate sites identified in vimentin treated by either kinase A or kinase C.

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MATERIALS AND METHODS

Materials

Porcine vimentin, purified from eye lens by standard procedures [19], was used without a further dephosphorylation step. It was dialyzed at room temperature at a concentration of 0.6 mg/ml from 8 M urea into a protofilament buffer: 25 mM imidazole/HCl pH 7.0, 30 mM NaCl [12]. For phosphorylation with kinase A, 10 ml of the vimentin solution was supplemented with $MgCl_2$, dithiothreitol and [γ-^{32}P]ATP (80 Ci/mol) to final concentrations of 0.3 mM, 0.5 mM, and 0.1 mM respectively. The reaction was started by the addition of 60 µg purified catalytic subunit of beef heart kinase A (kindly provided by Dr H. D. Söling). In other preparations a commercial enzyme (Sigma) was used. After 3 h at 20°C the reaction was stopped by addition of EDTA to a final concentration of 25 mM. For reaction with kinase C, 3 mg vimentin protofilaments at 0.6 mg/ml was supplemented with $MgCl_2$, $CaCl_2$, phosphatidylserine and [γ-^{32}P]ATP (80 Ci/mol) to final concentrations of 0.3 mM, 0.6 mM, 50 µg/ml and 0.1 mM respectively. Then 23 µg kinase C from rat brain (kindly donated by Dr H. D. Söling) was added. After 3 h at 20°C, the reaction was stopped by addition of EGTA to 25 mM.

Vimentin filaments were obtained by dialyzing vimentin at 4°C from 8 M urea into 10 mM Tris/HCl, pH 7.5, 170 mM NaCl and 1 mM 2-mercaptoethanol. The filament solution was then supplemented with $MgCl_2$ to 0.3 mM, and the filaments were subjected to phosphorylation by kinase A (1% by mass) at room temperature for 4 h. Throughout this time, aliquots were withdrawn for identification of phosphorylated residues and simultaneously filament integrity was monitored by electron microscopy after staining with 1% uranyl acetate.

Phosphorylated proteins were precipitated by the addition of a sixfold volume of cold ethanol, harvested by centrifugation and again washed with ethanol. For complete removal of unreacted [^{32}P]ATP, phosphorylated protein was dissolved in 8 M urea and gel filtered through a column of Sephadex G-50 equilibrated with buffer (10 mM Tris-HCl pH 7.5, 1 mM 2-mercaptoethanol) containing 8 M urea. In other experiments small amounts of phosphorylated vimentin were purified by applying the reaction mixture directly to a C_4 HPLC column. Protein was eluted by an acetonitrile gradient in 0.1% trifluoroacetic acid.

The extent of phosphorylation of the intact protein, its large fragments, and its peptides (see below) was calculated from quantitative amino acid analyses and aliquots directly counted in Hydroluma (Baker). In some cases bands were cut from polyacrylamide gels after electrophoresis in the presence of SDS and directly counted in Hydroluma. Autoradiograms on Fuji RX X-ray film were used for documentation.

Domain assignment of phosphorylation sites

Vimentin was cleaved at its sole cysteine site with 2-nitro-5-thiocyanobenzoic acid as before [21]. Limited proteolysis of vimentin (1 mg/ml 20 mM ammonium bicarbonate) with thrombin (Sigma, 10 U/mg vimentin) was performed at 37°C for different times. It released the peptides from the amino-terminal head domain leaving the remainder of the molecule intact. Reaction products were separated by gel filtration and by gel electrophoresis. Radioactivity was monitored as above.

Identification of phosphorylation sites

Phosphorylated vimentin (5 mg from a kinase A and 2.5 mg from a kinase C phosphorylation experiment) at 1 mg/ml was dialyzed from 8 M urea into 20 mM ammonium bicarbonate and digested with thrombin (10 U/mg protein) at 37°C for 20 h. Digests were applied to a Sephadex G-100 column (350 ml) equilibrated in ammonium bicarbonate. Fractions eluting close to the salt volume contained most of the radioactivity. They were pooled and lyophilized. The peptide material was redissolved in ammonium bicarbonate and further digested with trypsin (treated with tosylphenylalanylchloromethane, Worthington) at a relative concentration of 1% (by mass, 5 h at 37°C). Digests were lyophilized, redissolved in pH-6.5 electrophoresis buffer (pyridine/acetic acid) and applied to Whatman 3MM paper (kinase-A-treated vimentin) or Whatman No. 1 paper (kinase-C-treated vimentin). After electrophoresis at pH 6.5 descending chromatography (*n*-butanol/acetic acid/pyridine/water) was used at right angles. Peptides were detected with fluorescamine (0.0002% in acetone) and radioactive peptides were identified by autoradiography. Aliquots of the eluted peptides were counted in Hydroluma (Baker) to determine the specific radioactivity in conjunction with quantitative amino acid analysis. All radioactive peptides encountered and some of the unlabelled peptides were sequenced on a gas-phase sequenator (model 470A, Appplied Biosystems) equipped with an on-line phenylthiohydantoin (Pth) analyser. The 03 RPTH program of the manufacturer was used.

Standard phosphoamino acid analysis showed exclusive serine phosphorylation for both protein kinases. Phosphoserine residues were identified by the following procedures. During gas-phase sequencing, normal serine residues are identified as Pth-Ser and its dithiothreitol adduct. In the case of phosphoserine usually only the dithiothreitol adduct is encountered (procedure 1; see [13, 20]). In procedure 2 the phosphorylated peptides were modified with ethanethiol and the phosphoserines detected as the phenylthiohydantoin of *S*-ethylcysteine [20]. In one peptide, which displayed an amino-terminal phosphoserine (see Results), positive identification was not possible by procedures 1 and 2. Here procedure 3 was used. It is based on the manual Edman degradation. Before each step an aliquot was removed for pH-3.5 electrophoresis followed by autoradiography. The appearance of labelled inorganic phosphate was used to identify the position of the serine phosphate [13].

Polymerization experiments

Polymerization was monitored by standard assays [14]. Vimentin protofilaments, phosphorylated by kinase A or kinase C, were purified by HPLC and lyophilized (see above). The protein was dissolved in 8 M urea buffer at 250 µg/ml and dialyzed against 50 mM Tris/HCl, 170 mM NaCl and 1 mM dithioerythritol at pH 7 to promote polymerization. Samples for electron microscopy were prepared by staining with 1% uranyl acetate.

RESULTS

Phosphorylation sites locate to the head domain

Porcine vimentin was phosphorylated in the protofilamentous form by kinases A and C using [^{32}P]ATP. To locate the phosphate sites, the labelled derivatives were chemically cleaved at the sole cysteine of vimentin using 2-nitro-5-thiocyanobenzoic acid [21] and analyzed by SDS/PAGE followed by autoradiography. As shown in Fig. 1 only the large N-terminal fragment C1 is heavily labelled. Direct scintillation counting of the excised bands showed that 99% and 97% of the label introduced by kinase A and C, respectively, can be assigned to the fragment C1.

To locate the phosphate sites more precisely, we used proteolysis with thrombin. In pilot experiments using unphosphorylated vimentin we found that thrombin acts exclusively on the head domain with the most carboxy-terminal cleavage point situated at arginine 70 (data not shown). Thus the molecule is converted into a very large fragment plus several small peptides, which together cover the head domain. The same limited proteolytic cleavage pattern was earlier observed with desmin [14]. Thrombic digests of vimentin phosphorylated by kinase A and C were subjected to gel filtration on Sephadex G-100 (Figs 1 and 2). The first-eluting peak (Fig. 2) contained the large thrombic core (Fig. 1), while the small peptides were recovered in the second peak. Radioactivity measurements showed that the second peak accounted for 95% of the label incorporated into vimentin by both kinase A and C (Fig. 2). Thus the amino-terminal head domain is the preferred target when vimentin is phosphorylated in protofilamentous form by either kinase A or C. This assignment was further strengthened by direct sequence analysis. The thrombic core isolated from vimentin, which had been phosphorylated by kinase A, starts with leucine 78 of the known sequence [22]. As the thrombic core of normal vimentin begins with serine 71 (see above), phosphorylation at serines 71 and 72 (see below) seems to influence the thrombic cleavage pattern. When intact vimentin filaments were phosphorylated by kinase A, thrombic digestion showed that the phosphate sites were again located in the head domain.

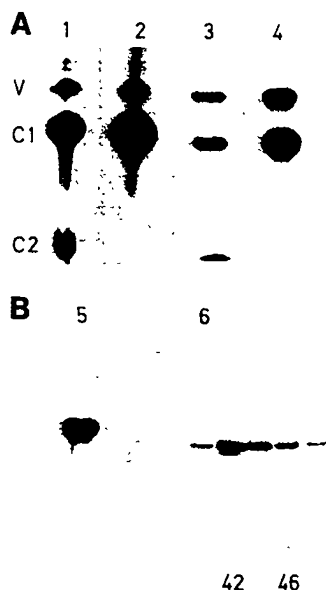

Fig. 1. *Identification of phosphorylated vimentin domains.* (A) The gel patterns (slots 1, 3) and corresponding autoradiograms (slots 2, 4) of phosphorylated vimentin after cleavage at the single cysteine. Phosphorylation by kinase C (slots 1, 2) and kinase A (slots 3, 4) was performed on vimentin in protofilamentous form. Note the strong ^{32}P incorporation in the large N-terminal fragment C1 and the virtual absence of ^{32}P in the C-terminal fragment C2. Direct counting in Hydroluma provided a ratio of 1600:50 for C1 and C2 from the kinase-C-labelled vimentin (V). Corresponding values for the kinase-A-labelled protein were 25600:300. (B) Slots 6, the dye-stained gel pattern of the fractions 40–48 from the gel filtration given in Fig. 2. In this experiment kinase-C-labelled vimentin was analyzed. The same pattern was observed with kinase-A-treated vimentin. Slot 5 shows intact vimentin, which has a higher molecular mass than the thrombic core (slots in 6). Note that the core contains only small amounts of ^{32}P (compare Fig. 2). Dots at the right side mark start and front of the gel

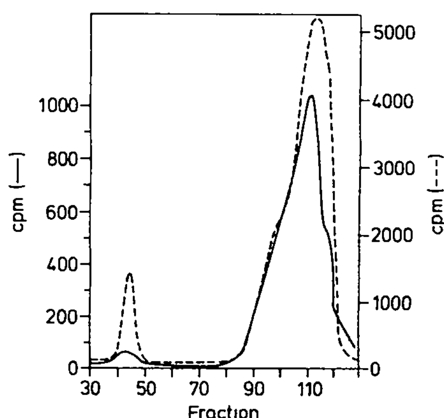

Fig. 2. *Isolation of radioactive peptides by gel chromatography.* Thrombin digests of vimentin protofilaments phosphorylated by kinase A (----) and kinase C (——) were subjected to gel filtration on Sephadex G-100. The core portions, eluting in fractions 38–50 (compare Fig. 1 B) are well separated from the small peptides originating from the head domains. These elute close to the salt volume and contain most of the radioactivity. The column (350 ml) was run in 20 mM ammonium bicarbonate and the protein sample was applied in a volume of 700 µl. Fraction size was 3 ml. The radio-activity values given correspond to the 5-µl aliquots which were used for counting

Identification of the serine phosphate sites in the head domain

Kinase A incorporated under our experimental conditions 4 mol phosphate/mol vimentin, when the protein substrate was used in the protofilamentous form. Phosphoamino acid analysis showed exclusively serine phosphorylation. Preparative fingerprinting of the kinase-A-treated vimentin protofilaments provided seven labelled peptides (Fig. 3). Their amino acid sequences accounted for five distinct serine phosphorylation sites in the head domain (Tables 1 and 2). Two of the sites, i.e. serine 38 and serine 72, were quantitatively modified. In the other three positions phosphorylation was non-stoichiometric i.e. 27% at serine 24, 53% at serine 65 and 76% at serine 71. All other serines remained unmodified. When intact vimentin filaments rather than protofilaments were used, kinase A introduced only 1.7 mol phosphate/mol protein during the reaction time of 4 h. These phosphates arise from only three serine phosphate sites (Tables 1 and 2). They were also encountered among the five sites identified with vimentin protofilaments. Phosphorylation of serines 38 and 72 was 70% and 63% respectively, still high but clearly not stoichiometric. Serines 24 and 65 were unmodified, while serie 71 showed only marginal phosphorylation (4%). The relative distribution of the phosphates was very similar even at the earliest time points of the reaction. After 15 min only 0.1 mol phosphate/mol were incorporated into the vimentin filaments. The analysis of the radioactive peptides showed that the ratio of the phosphorylated residues 38 and 72 was 1.2:1 and the phosphorylated residue 71 war hardly detectable. Although some of the sequence around the serine residues phosphorylated by kinase A could possibly also allow for phosphorylation by calcium/calmodulin-dependent protein kinase, we note that Inagaki et al. [12] reported that this kinase does not act *in vitro* on vimentin.

Phosphorylation of vimentin protofilaments by kinase C leads to 1.5 mol phosphate/mol protein. Characterization of seven of the eight radioactive peptides (Fig. 3) by protein chemical data identified five distinct phosphorylation sites (Tables 1 and 3). Serines 9 and 50 are the preferred sites of kinase C, but their modification involves about 50% only of the maximal phosphorylation. Serines 25, 33 and 65 are even less extensively modified. The eighth peptide (Fig. 3, peptide c8) was isolated from the acidic side of the fingerprint. Several attempts to sequence the peptide failed. Since it carried about 20% of the total radioactivity incorporated by kinase C, there could be an as yet unidentified further kinase C site. We can however not exclude another possibility. Spot c8 could consist of a mixture of the peptides already identified together with some of the phospholipid introduced in the phosphorylation reaction and not removed in the following preparation steps.

Sequences surrounding the phosphorylation sites

The three kinase A sites of desmin show the sequence RXS [13] and this feature is also seen in three of the five sites of vimentin i.e. serines 24, 65 and 72 (Fig. 4). In the two other kinase A sites of vimentin the motif is RXXS with the serine separated by two residues from the next N-terminally located arginine (Fig. 4). Of the five kinase C sites in vimentin, three sites (serines 9, 25 and 33) reveal a similar situation as in other proteins [23]. Here the phosphorylated serine is separated by one residue from a following lysine or arginine residue. In contrast, the low occupation site 65 shows a separation by two residues from arginine 68 and by one residue from arginine 63 (Fig. 4). Curiously, the highly phosphorylatable serine 50 is

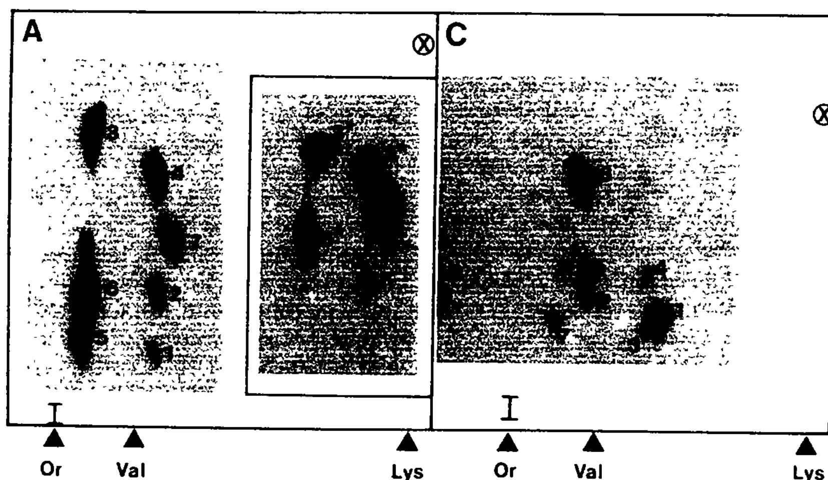

Fig. 3. *Preparative fingerprinting of labelled peptides.* Thrombic peptides from the head domain were isolated (Fig. 2) and redigested with trypsin. Digests were electrophoresed in the horizontal direction at pH 6.5 and then subjected to descending chromatography (*n*-butanol/ acetic acid/pyridine/water) in the vertical direction. The positions of the origin (Or) and of the neutral and basic marker amino acids valine (Val) and lysine (Lys) are indicated. The encircled X marks the position of the dye xylene xyanole FF used as marker during chromatography. The larger parts of the acidic sides (left) of the fingerprints are not shown because they did not contain any peptide material. Only the autoradiograms of the corresponding fingerprints are displayed. (A) The peptides obtained after kinase A phosphorylation of protofilaments; (C) Corresponding results obtained after kinase C phosphorylation. The insert in A shows the peptides obtained when intact filaments were phosphorylated by kinase A. In A and its insert, identical peptides are marked by the same numbers with the stars reserved for the peptides in the insert. The numbering system corresponds to the order of the peptides along the sequence of the head domain (see Fig. 4). The total radioactivity (cpm) contained in the spots was 508000 (a1), 980000 (a2), 3680000 (a3), 1800000 (a4), 2910000 (a5), 8368000 (a6), 1350000 (a7), 465000 (a3*), 490000 (a4*), 110000 (a6*), 790000 (a7*), and 286000 (c1), 81000 (c2), 92000 (c3), 53000 (c4), 63000 (c5), 307000 (c6), 71000 (c7), 248000 (c8)

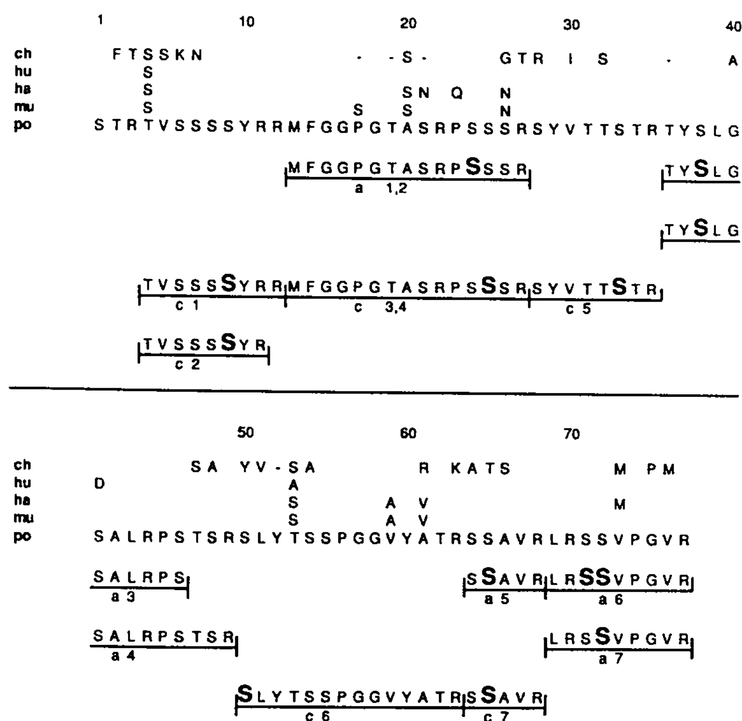

Fig. 4. *Alignment of the identified phospho-peptides (Table 2) with the sequence of the head domain.* The complete line gives the sequence for residues 1–77 of porcine vimentin (po). Where the vimentin sequences of chicken (ch), mouse (mu), hamster (ha) and human (hu) vimentin differ from the porcine sequence this is indicated by the corresponding amino acid replacement. The sequences are found in the following references: po [22], ch [28], mu [29], ha [30] and hu [31]. The porcine peptides containing phosphoserine due to kinase A treatment (a1–7) or kinase C treatment (c1–7) are shown below the full line. They were identified and characterized in Fig. 3 and Table 1. Phosphorylated serine residues are indicted by bold-letter type in the sequence given for the corresponding peptides. For relative yields of the peptides see Table 1. Note two corrections at positions 52 and 60 from our previously published sequence of the porcine vimentin head domain [22], which was deduced without automated sequencing

directly adjacent to arginine 49 and the next C-terminally located basic residue appears only with arginine 63.

Electron microscopy of phosphorylated vimentin derivatives

Vimentin protofilaments phosphorylated with kinase A (4 mol phosphate/mol vimentin) failed to form filaments when dialyzed from 8 M urea into polymerization buffer (Fig. 5A). In contrast, kinase-C-treated protofilaments containing 1.5 ml phosphate/mol polymerized into normal filaments, when the same experiment was performend (Fig. 5B). Intact vimentin filaments treated with kinase A contained 1.7 mol phosphate/mol vimentin after 4 h. This phosphorylation did not affect filament integrity. No depolymerization could be detected during the phosphorylation reaction (compare Fig. 5D and C).

DISCUSSION

We have shown that *in vitro* phosphorylation of porcine vimentin by kinases A and C is restricted to the N-terminal head domain, a serine-rich, non-helical structure, which precedes the coiled-coil region [24]. Vimentin protofilament units incorporated up to 4 mol phosphate/53 kDa with kinase A, while kinase C yielded 1.5 mol. Interestingly phosphorylation of intact filaments by kinase A leads to only 1.7 mol phosphate/mol. Using a direct protein-chemical approach, we have identified five serine phosphate sites each for kinase A and C and mapped these sites along the known sequence of the head domain (Fig. 4 and Tables 2 and 3). With the exception of serine 65, the two kinases show non-overlapping sites. These results confirm and extend the work of Evans [18, 25]. He deduced from cleavage patterns that *in vivo* phosphorylation of vimentin in BHK cells, as well as the *in vitro* phosphorylation by kinase A, involved only the head domain.

Our results on the polymer-forming ability of phosphorylated vimentins agree in general with the findings of Inagaki et al. [12], although some differences clearly exist. Using their conditions we find vimentin protofilaments to be a relatively poor substrate for kinase C. Only 40 – 50% modification occurs at serines 50 and 9, while the other three sites, serines 25, 33 and 60, are even less effectively recognized (Table 3). Thus, as previously reported, the resulting protofilament preparation shows normal assembly properties. Under quite different experimental conditions, which we have not yet used, Inagaki et al. [17] recently found that vimentin assembly can also be inhibited by increased kinase C phosphorylation. Kinase-A-dependent phosphorylation has an inhibitory effect on vimentin assembly [12, 17]. While we could show this effect with phosphorylated protofilaments, we did not detect a direct disassembly of filaments upon phosphorylation. The reason for this discrepancy is not known. It could however arise from slightly different experimental conditions or from the different protein concentrations used. Alternatively since the different vimentin preparations are from different species, i.e. pig versus mouse, this could also have some influence.

The strong reduction in kinase A phosphorylation observed for intact filaments versus protofilaments (1.7 mol versus 4 mol) seems to be related to a differential accessability of the different sites (Table 2). Serines 24 and 65 seem not to be accessible in intact filaments and the phosphorylation of serine 71 drops to background levels. In contrast the stoichiometric modification of serines 38 and 72 observed for protofilaments is only moderately affected in filaments as the two sites still show 60 – 70% phosphorylation. These results have some interesting implications for filament structure. Current models of intermediate filaments agree that the structure relies primarily on an interaction between the coiled coils with the terminal domains protruding from the filament wall. Defined

Table 1. *Identification of phosphorylated serine residues*
Peptides a1 – a7 were isolated from kinase-A-treated protofilaments, peptides a3*, a4*, a6* and a7* from kinase-A-treated intact filaments and peptides c1 – c7 from kinase-C-treated protofilaments (see Fig. 3). Their position along the sequence of the head domain (Fig. 4) was obtained by automated sequencing. The three procedures (1, 2 and 3) for the identification of the phosphorylated serine residues are described in detail in Materials and Methods. The yield of the labelled peptides was calculated by relating the quantity of the peptide as isolated from the fingerprint (Fig. 3) to the quantity of unlabelled peptides also isolated from the same finterprint. The peptide comprising residues 50 – 63 was taken as 100% standard for kinase A peptides as this region lacks a serine phosphate. The dipeptide comprising residues 69 – 70 was taken as standard for kinase-C-generated peptides. The peptides a1, a2, c3 and c4 were identical except for the oxidation state of the amino-terminal methionine. Where the position of the phosphorylated serine residue is given in parentheses, a separate site determination was not done. Peptide a3 and a3* end with serine 46 and therefore arose from an unusual cleavage by either thrombin or trypsin

Peptide number	Residues in head domain	Phosphorylated residue	Relative yield	Method of identification
			%	
a1	13 – 27	Ser 24	9	1
a2	13 – 27	(Ser 24)	18	–
a3	36 – 46	Ser 38	67	1
a4	36 – 49	Ser 38	33	1
a5	64 – 68	Ser 65	53	1
a6	69 – 77	Ser 71, 72	76	1
a7	69 – 77	Ser 72	24	1
a3*	36 – 46	Ser 38	34	1
a4*	36 – 49	Ser 38	36	1
a6*	69 – 77	Ser 71, 72	4	1
a7*	69 – 77	Ser 72	59	1
c1	4 – 12	Ser 9	40	1, 2
c2	4 – 11	Ser 9	11	1
c3	13 – 27	Ser 25	13	1
c4	13 – 27	(Ser 25)	8	–
c5	28 – 35	Ser 33	9	1, 2
c6	50 – 63	Ser 50	43	3
c7	64 – 68	Ser 65	10	2, 3

Table 2. *Relative phosphorylation levels of individual serine residues by kinase A*
The phosphorylation levels were calculated from results given in Table 1

Residue number	Phosphorylation in protofilaments	Phosphorylation in filaments
	%	
Ser 24	27	0
Ser 38	100	70
Ser 65	53	0
Ser 71	76	4
Ser 72	100	63

Table 3. *Relative phosphorylation levels of individual serine residues by kinase C*
The phosphorylation levels were calculated using the data from Table 1 by combining the appropriate peptides

Residue number	Phosphorylation in protofilaments
	%
Ser 9	51
Ser 25	21
Ser 33	9
Ser 50	43
Ser 65	10

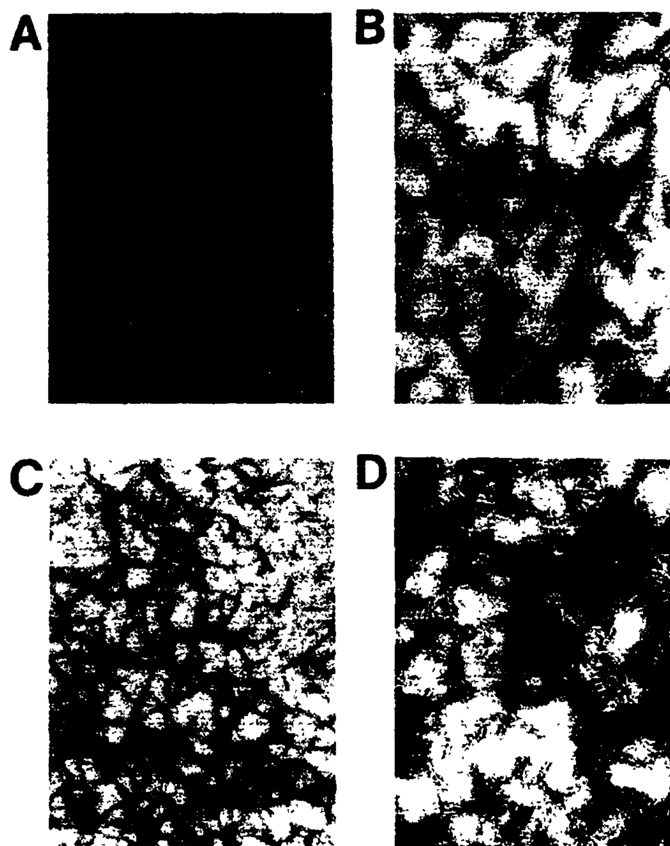

Fig. 5. *Electron microscopic observations on filament assembly of phosphorylated vimentins.* Vimentin was phosphorylated in protofilament form and repurified by HPLC. Kinase-A-treated vimentin containing 4 mol phosphate/mol protein did not form filaments, when dialyzed from 8 M urea into normal assembly buffer (A). Kinase-C-treated vimentin containing 1.5 mol phosphate/mol protein showed normal assembly under the same conditions (B). Preformed vimentin filaments treated with kinase A (1% by mass) for 4 h at room temperature showed 1.7 mol phosphate/mol protein. During the course of this phosphorylation filaments appeared normal (D) when compared with control filaments (C). The bar in D is 0.4 μm. The same magnification is used for all micrographs. Staining was with 1% uranyl acetate

proteolytic derivatives of vimentin and desmin show, however, that the head domains play, at least *in vitro*, a direct role in polymerization and filament integrity [14–16]. The enhanced phosphorylation of vimentin protofilaments, together with their impaired polymerization, either point to a structural change of the head domain during polymerization or indicate that parts of the head domain become protected in the intact filament.

The work of Evans [18, 25] together with our results shows that specific serines of the head domain are the preferred phosphorylation sites for the *in vivo* and *in vitro* phosphorylation of vimentin. As this assignment also holds for the increased phosphorylation during mitosis [25], an interesting problem concerning filaments in the cell arises. Inagaki et al. [12, 17] assume that phosphorylation leads to depolymerization. We feel that the situation is more complex given the immunocytochemical results on various cells. In spite of increased phosphorylation, mitotic cells still contain filaments, although they may be rearranged, bundled or collapsed towards the nucleus [32, 33]. Interestingly, also during interphase, vimentin phosphorylation can increase without disassembly as recently shown for cells previously microinjected with kinase A [26]. Since such cells show induction of filament bundling we view phosphorylation as a means to modulate or to change the interaction patterns of the filaments with other cellular structures without a drastic depolymerization. Obvious affected targets could include the microtubules, the integrity of which influences the display of vimentin during mitosis and in certain drug-induced situations [32, 33]. The two serine residues accessible to kinase A under *in vitro* conditions (38 and 72, see Results) could therefore be targets of a dynamic modulation of the filament surface, which could influence cellular interaction sites without affecting filament stability. This view would also explain why phosphorylated vimentin isolated from extracted cells due to its insolubility can still cycle *in vitro* through depolymerization and re-polymerization [27]. We note, however, that neither our *in vitro* results nor the *in vivo* results of Lamb et al. [26] exclude the possibility that a kinase distinct from kinase A could induce some mitotic depolymerization of vimentin in certain cell types.

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