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Domain- and Sequence-Specific Phosphorylation of Vimentin Induces Disassembly of the Filament Structure†

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ABSTRACT: We reported that stoichiometric phosphorylation by either cAMP-dependent protein kinase or protein kinase C induces disassembly of vimentin filaments [Inagaki, M., Nishi, Y., Nishizawa, K., Matsuyama, M., & Sato, C. (1987) *Nature 328*, 649-652; Inagaki, M., Gonda, Y., Matsuyama, M., Nishizawa, K., Nishi, Y., & Sato, C. (1988) *J. Biol. Chem. 263*, 5970-5978]. In the present work, we attempted to identify the sites of vimentin phosphorylated by each protein kinase. Sequential analysis of the purified phosphopeptides, together with the known primary sequence, revealed that Ser-8, Ser-9, Ser-20, Ser-25, Ser-33, and Ser-41 were specifically phosphorylated by protein kinase C, whereas Ser-46 was phosphorylated preferentially by cAMP-dependent protein kinase. Both kinases reacted with Ser-6, Ser-24, Ser-38, Ser-50, and Ser-65. Specific phosphorylation sites for protein kinase C are mostly located close to the amino-terminal side of arginine while those for cAMP-dependent protein kinase are located close to the carboxyl-terminal side of arginine. The phosphorylation sites exclusively occur in the amino-terminal non-α-helical head domain, particularly at the β-turn region. These results provide clues to the molecular mechanisms of phosphorylation-dependent disassembly of vimentin filaments.

The cytoskeletons of most animal cells are composed of three major fiber systems that can be distinguished by electron microscopy: microfilaments (6 nm in diameter), microtubules (24 nm in diameter), and intermediate filaments (10 nm in diameter). Although intermediate filaments appear to play a significant role in maintenance of the organization of cytoplasmic space (Ishikawa et al., 1968; Lazarides, 1980), the cytoplasmic regulatory mechanisms that govern the assembly–disassembly of these structures are not well characterized. Until recently, most information on the regulatory mechanisms of filament assembly–disassembly has come from the other two classes of cytoskeletal filaments, microfilaments and microtubules, which together with batteries of associated proteins have been extensively characterized (Mohri, 1976;

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Olmsted & Borisy, 1973; Pollard & Weihing, 1974; Clarke & Spudich, 1977). We recently demonstrated the role of phosphorylation of vimentin and desmin in vitro (Inagaki et al., 1987, 1988); vimentin and desmin are excellent in vitro substrates for protein kinase C and cAMP-dependent protein kinase, but not of several other kinases. Vimentin and desmin phosphorylated by each protein kinase do not polymerize, and the polymerized filaments tend to depolymerize after phosphorylation.

Information regarding the structural features of intermediate filaments and subunit proteins has accumulated. Intermediate filaments share similar biochemical properties, an α-type X-ray diffraction pattern, and a common electron microscopic appearance (Geisler & Weber, 1986). The amino acid sequence data and results from limited proteolytic digestion led to a definition of the three-domain structures of the intermediate filament proteins (Geisler & Weber, 1986; Geisler et al., 1982). The filament wall is primarily based on interaction patterns of double-stranded coiled-coils provided by the central α-helical rod domains of the proteins. The proteolytic derivatives indicate that the amino-terminal domain but not the carboxyl-terminal domain has a direct effect on filament stability and polymerization (Traub & Vorgias, 1983; Kaufmann et al., 1985).

As a first step toward defining the molecular mechanism of phosphorylation-dependent disassembly of intermediate filaments, we identified sites of vimentin phosphorylated by either cAMP-dependent protein kinase or protein kinase C. These studies were deemed necessary in order to assign the phosphate positions along the three-domain structure of vimentin and to define the site specificities of cAMP-dependent protein kinase and protein kinase C. Most recently, it was reported that phosphorylations of desmin (Geisler & Weber, 1988) and vimentin (Evans, 1988a) by cAMP-dependent protein kinase are restricted to the non-α-helical head domain. In the present experiments, we obtained further evidence on the sites of phosphorylation in vimentin, which depolymerizes filaments, as follows. (1) Not only cAMP-dependent protein kinase but also protein kinase C phosphorylated serine residues within the amino-terminal head domain of vimentin. Five common sites of phosphorylation were identified. (2) Each protein kinase differed essentially in site recognition. (3) Most of the phosphorylation sites are likely to be located in the β-turn structures. Identification of the phosphorylation sites has important implications for understanding the domain structure of intermediate filament proteins and the molecular mechanisms of phosphorylation-dependent disassembly of intermediate filaments.

Experimental Procedures

Purification of Vimentin. Purified vimentin was obtained by extraction of the crude intermediate filament preparation from Furth's murine mastocytoma cells with 8 M urea and subsequent chromatography on DEAE-cellulose and CM-cellulose columns in the presence of urea, as described (Inagaki et al., 1987, 1988).

Preparation of Protein Kinases. The catalytic subunit of cAMP-dependent protein kinase was prepared from bovine heart by the method of Beavo et al. (1974). Protein kinase C was prepared from rat brain by the method of Inagaki et al. (1985).

Phosphorylation of Vimentin. Vimentin (0.4 mg/mL) was phosphorylated by incubation with 5 μg/mL protein kinase C, 0.1 mM [γ-^{32}P]ATP, 0.3 mM $MgCl_2$, 50 μg/mL phosphatidylserine, 2.5 μg/mL diacylglycerol, and 25 mM Tris-HCl, pH 7.0, and 25 °C for 4 h or by incubation with 5 μg/mL catalytic subunit of cAMP-dependent protein kinase, 0.1 mM [γ-^{32}P]ATP, 0.3 mM $MgCl_2$, and 25 mM Tris-HCl, pH 7.0, at 25 °C for 4 h.

Isolation of Phosphorylated Vimentin. Two milliliters each of the phosphorylation mixture containing vimentin (0.4 mg/mL) was applied to a Zorbax C8 (0.46 × 20 cm) column attached to a Waters high-performance liquid chromatography (HPLC)[1] system consisting of Model 510 pumps, a Model 490 detector, and an automated gradient controller. The phosphorylated vimentin was eluted at around 39 min, using a linear gradient of 5–90% acetonitrile in 0.1% trifluoroacetic acid over 50 min at a flow rate of 0.8 mL/min. Elution was monitored by UV at 230 nm using a Chromatocorder 11 (System Instruments Corp.) or by radioactivity of each fraction (0.8 mL), using a Beckman scintillation counter LS 5801. Fractions containing the radioactive phosphorylated vimentin were pooled and lyophilyzed.

Fragmentation of Phosphorylated Vimentin. The phosphorylated vimentin isolated by reverse-phase HPLC was dissolved in 50 mM Tris-HCl (pH 7.4) at the concentration of 0.2 mg/mL and was treated with L-1-(tosylamino)-2-phenylethyl chloromethyl ketone treated trypsin [Sigma, 1/50 (w/w) of vimentin] at 37 °C for 2 h. The sample was further treated with lysyl endopeptidase [Wako, 1/100 (w/w) of vimentin] at 30 °C for 6 h. An aliquot (1.5–2 mL) of the reaction mixture was applied to a Zorbax C8 (0.46 × 20 cm) column and was eluted with a linear gradient of 5–50% acetonitrile in 0.1% trifluoroacetic acid over 50 min followed by a further linear gradient of 50–80% acetonitrile in 0.1% trifluoroacetic acid over 20 min at a flow rate of 0.8 mL/min. Elution of the fragments was monitored by UV at 230 nm and by the radioactivity of each fraction (0.8 mL). Each radioactive fraction was separately lyophilized and stored at 4 °C.

Purification of Fragments. The radioactive fractions were applied to an anion-exchange column of TSK gel QAE-2SW (0.46 × 25 cm) and were eluted with a linear gradient of 0–0.5 M NaCl in 20 mM Tris-HCl (pH 7.5) over 40 min at a flow rate of 0.8 mL/min. Each radioactive fraction obtained was then applied to a Zorbax C8 (0.46 × 20 cm) column and was eluted with the same linear gradient used in the first fractionation of the phosphopeptides, at the same flow rate. Radioactive fractions obtained were lyophilized and stored at 4 °C.

Modification of Phosphoserine Residues. The purified radioactive fragments (1–7 nmol) were treated with 100 μL of solution consisting of 9.9 μL of ethanethiol, 33.1 μL of water, 33.1 μL of dimethyl sulfoxide, 13.2 μL of ethanol, and 10.7 μL of 5 N NaOH, at 50 °C for 1 h, as described by Meyer et al. (1986). Prior to sequence analysis, the reaction mixtures were cooled and stored at −20 °C, after adding 10 μL of acetic acid.

Sequence Analysis. An aliquot of either the purified fragments dissolved in 0.1% trifluoroacetic acid or the chemically modified fragments in the reaction mixture was analyzed with an ABI gas-phase sequencer, Model 470 A, using the manufacturer's program. The PTH-amino acids were analyzed on an ABI PTH-C18 (0.46 × 22 cm) cartridge column attached to an HPLC system consisting of Spectra-Physics 8700 pump, an ISCO V^4 absorbance detector, and a Spectra-Physics 4270 integrator.

Phosphoamino Acid Analysis. Vimentin was phosphorylated by each protein kinase as mentioned above. The ra-

[1] Abbreviations: HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin; DTT, dithiothreitol; A-kinase, cAMP-dependent protein kinase.

Table I: Amino Acid Sequences and Phosphorylation Sites of Phosphopeptides for cAMP-Dependent Protein Kinase

peptide	amino acid sequence[a]	rel amount[b] of phosphate in peptide (% of total)
1a	Ser-*Ser*-Ala-Val-Arg (residues 64–68)	18
2a	Ser-Val-*Ser*-Ser-Ser-Ser-Tyr-Arg (residues 4–11)	14
4a	Met-Phe-Gly-Gly-Ser-Gly-Thr-Ser-Ser-Arg-Pro-*Ser*-Ser-Asn-Arg (residues 13–27)	11
5a	*Ser*-Leu-Tyr-Ser-Ser-Ser-Pro-Gly-Gly-Ala-Tyr-Val-Thr-Arg (residues 50–63)	17
6a	Thr-Tyr-*Ser*-Leu-Gly-Ser-Ala-Leu-Arg-Pro-*Ser*-Thr-Ser-Arg (residues 36–49)	40

[a] Phosphorylation sites are shown italicized. Residue numbers in parentheses are determined from the amino-terminal end of murine vimentin (Fischer et al., 1986). [b] Determined from radioactivity in the HPLC analysis as shown in Figure 1.

Table II: Amino Acid Sequences and Phosphorylation Sites of Phosphopeptides for Protein Kinase C

peptide	amino acid sequence[a]	rel amount[b] of phosphate in peptide (% of total)
1c	Ser-*Ser*-Ala-Val-Arg (residues 64–68)	23
2c	Ser-Val-*Ser*-Ser-*Ser*-*Ser*-Tyr-Arg (residues 4–11)	14
3c	Ser-Tyr-Val-Thr-Thr-*Ser*-Thr-Arg (residues 28–35)	13
4c	Met-Phe-Gly-Gly-Ser-Gly-Thr-*Ser*-Ser-Arg-Pro-*Ser*-*Ser*-Asn-Arg (residues 13–27)	16
5c	*Ser*-Leu-Tyr-Ser-Ser-Ser-Pro-Gly-Gly-Ala-Tyr-Val-Thr-Arg (residues 50–63)	20
6c	Thr-Tyr-*Ser*-Leu-Gly-*Ser*-Ala-Leu-Arg-Pro-Ser-Thr-Ser-Arg (residues 36–49)	14

[a] Phosphorylation sites are shown italicized. Residue numbers in parentheses are determined from the amino-terminal end of murine vimentin (Fischer et al., 1986). [b] Determined from radioactivity in the HPLC analysis as shown in Figure 1.

dioactive vimentin was isolated by SDS–polyacrylamide gel electrophoresis, eluted from gel, and subjected to acid hydrolysis in 6 N HCl for 1.5 h at 110 °C. The phosphoamino acids were resolved by electrophoresis at pH 3.5 on a cellulose thin-layer plate, as described (Hunter & Sefton, 1980).

Prediction of Secondary Structure. The secondary structure of vimentin was predicted, using DNASIS (Hitachi Software Co.).

RESULTS

Isolation of Phosphopeptides Derived from Phosphorylated Vimentin. To identify the sites phosphorylated by cAMP-dependent protein kinase and by protein kinase C, vimentin (4 mg) was incubated with [γ-^{32}P]ATP and either cAMP-dependent protein kinase (50 μg) or protein kinase C (50 μg) in a 10-mL mixture for 4 h, under conditions similar to those reported elsewhere (Inagaki et al., 1987, 1988). cAMP-dependent protein kinase and protein kinase C incorporated 3.8 mol and 4.6 mol of phosphate into 1 mol of vimentin, respectively. The materials were directly subjected to HPLC equipped with a reverse-phase column, as described under Experimental Procedures. The radioactive vimentin (1.8–2.0 mg) was eluted as a sharp peak between 67% and 72% acetonitrile.

The radioactive vimentin was freeze-dried, dissolved in 9 mL of 50 mM Tris-HCl, pH 7.4, and digested with trypsin [40 μg, treated with L-1-(tosylamino)-2-phenylethyl chloromethyl ketone] for 2 h at 37 °C. The sample was further treated with lysyl endopeptidase (20 μg) for 6 h at 30 °C.

The materials were then subjected to HPLC equipped with a reverse-phase column, as described under Experimental Procedures. The radioactive fragments were eluted by application of a linear gradient of acetonitrile. As shown in Figure 1, the HPLC procedure separated several phosphopeptides for samples phosphorylated by cAMP-dependent protein kinase and by protein kinase C. The radioactive phosphopeptides that are indicated by the same number in this figure were eluted at the same retention time. Each phosphopeptide was lyophilized, taken up with a small volume of 20 mM Tris-HCl, pH 7.5, and chromatographed on an anion-exchange HPLC column, as described under Experimental Procedures. Finally, each phosphopeptide was rechromatographed on the reverse-phase column of HPLC. Each phosphopeptide appeared as a single and symmetric peak, and the amount of the phosphopeptide purified in this way was in the range of 2–14 nmol.

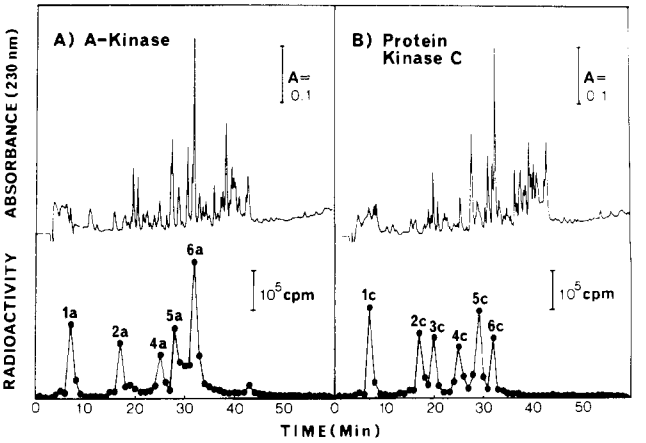

FIGURE 1: Reverse-phase HPLC of phosphopeptide fragments derived enzymatically from phosphorylated vimentin. Vimentin phosphorylated by cAMP-dependent protein kinase (A) or protein kinase C (B) was digested successively with trypsin and lysyl endopeptidase, as described under Experimental Procedures. An aliquot of each reaction mixture was applied to a Zorbax C8 (0.46 × 20 cm) column and eluted under the conditions described under Experimental Procedures. Elution was monitored by UV at 230 nm, and the radioactivity of each fraction (0.8 mL) was determined, as described under Experimental Procedures.

Amino Acid Sequences of Phosphopeptides. Each purified radioactive phosphopeptide was analyzed for gas-phase Edman degradation, as described under Experimental Procedures. The amino acid sequences of these phosphopeptides were as given in Tables I and II. The peptides with the same number in Figure 1 showed the same sequence. Phosphopeptides 4 and 6 appeared to be products of incomplete digestion. This incomplete digestion may be due to the primary sequence (Arg-22-Pro-23; Arg-44-Pro-45) (Li et al., 1955; Ambler & Brown, 1967; Fischer et al., 1986).

The sequence of all the radioactive phosphopeptides is compatible with the reported amino-terminal sequence of murine vimentin (Fischer et al., 1986). These results suggest that the amino-terminal non-α-helical head domain (containing 72 amino acid residues) is the common target domain for cAMP-dependent protein kinase and for protein kinase C. Any phosphopeptides derived from the rod domain (containing 345 amino acid residues) or from the tail domain (containing 48

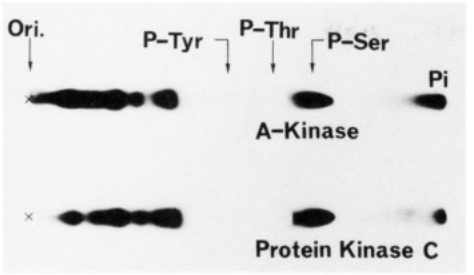

FIGURE 2: Phosphoamino acid analysis of vimentin phosphorylated by cAMP-dependent protein kinase or protein kinase C. Vimentin phosphorylated by cAMP-dependent protein kinase or protein kinase C was subjected to phosphoamino acid analysis as described under Experimental Procedures. The positions of the Origin (Ori.), phosphoserne (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr), and inorganic phosphate (Pi) are indicated.

amino acid residues) (Quax et al., 1983) were not identified.

Assignment of Phosphorylation Sites. Phosphoamino acid analysis of vimentin phosphorylated by either cAMP-dependent protein kinase or protein kinase C showed the presence of only phosphoserine (Figure 2). Since all the phosphopeptides contained more than two serine residues, as shown in Table I and II, the exact phosphorylation sites had to be defined. For this purpose, each fragment was treated with ethanethiol in an alkaline condition to convert specifically the phosphoserine residues to *S*-ethylcysteine. Normal serine residues were not affected by this treatment (Meyer et al., 1986). The positions of *S*-ethylcysteine residues within peptides were identified by gas-phase sequencing (Meyer et al., 1986). Figure 3 shows the sequence analysis of peptides 1a, 2a, 4a, and 6a treated with ethanethiol prior to Edman degradation. The high release of *S*-ethylcysteine was observed at the second cycle for peptide 1a, the third cycle for peptide 2a, the twelfth cycle for peptide 4a, and the third and the eleventh cycles for peptide 6a, indicating that the phosphate was located on Ser-65, Ser-6, Ser-24, Ser-38, and Ser-46, respectively. Figure 4 also shows the sequence analysis of peptides 1c, 2c, 3c, 4c, and 6c treated with ethanethiol prior to Edman degradation. The high release of *S*-ethylcysteine was observed at the second cycle for peptide 1c, the fifth and sixth cycles for peptide 2c, the sixth cycle for peptide 3c, the eighth cycle for peptide 4c, and the third cycle for peptide 6c, indicating that the phosphate was located on Ser-65, Ser-8, Ser-9, Ser-33, Ser-20, and Ser-38, respectively. The small but significant amount of *S*-ethylcysteine was also observed at the third cycle for peptide 2c, the twelfth and thirteenth cycles for peptide 4c, and the sixth cycle for peptide 6c, indicating that the phosphate was also located on Ser-6, Ser-24, Ser-25, and Ser-41, respectively.

Although peptides 5a and 5c were identified as the radioactive phosphopeptides, *S*-ethylcysteine was not observed within both the peptides (data not shown). It has been reported that if phosphoserine possesses a free amino or carboxyl terminus, no transformation into *S*-ethylcysteine will occur (Meyer et al., 1987). Since peptides 5a and 5c have serine residues (Ser-50) at the amino-terminal position, the possibility arises that Ser-50 is a phosphoserine. Thus, another procedure was also employed to determine the location of phosphoserine by using phosphopeptides untreated with ethanethiol. During gas-phase sequencing, normal serine residues are identified as phenylthiohydantoin-serine (PTH-serine) and the dithiothreitol (DTT) adduct of PTH-serine. In contrast, phosphoserine provides exclusively the DTT adduct of PTH-serine (Meyer et al., 1986). The phosphopeptides 1a, 2a, and 5a, which have a serine residue at the amino-terminal position, were analyzed

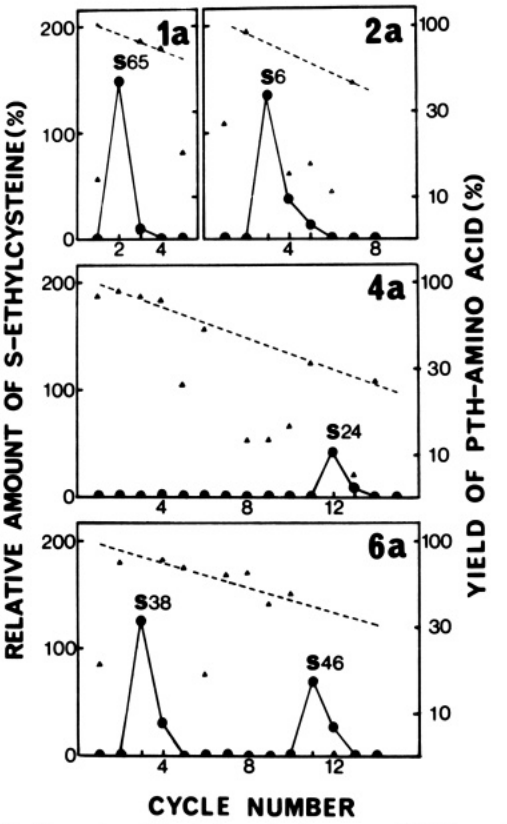

FIGURE 3: Detection of *S*-ethylcysteine in sequential Edman degradation of ethanethiol-treated phosphopeptides. Phosphopeptides (0.5–0.8 nmol) by cAMP-dependent protein kinase were treated with ethanethiol in alkaline conditions and subjected to gas-phase sequence analysis, as described under Experimental Procedures. The relative amount (●) of *S*-ethylcysteine was determined from the peak area as the percentage ratio to the internal standard (100 pmol). The serine residue (S) detected as *S*-ethylcysteine is shown with the residue number in vimentin (Fischer et al., 1986). The repetitive yield of PTH-amino acid is shown by a triangle.

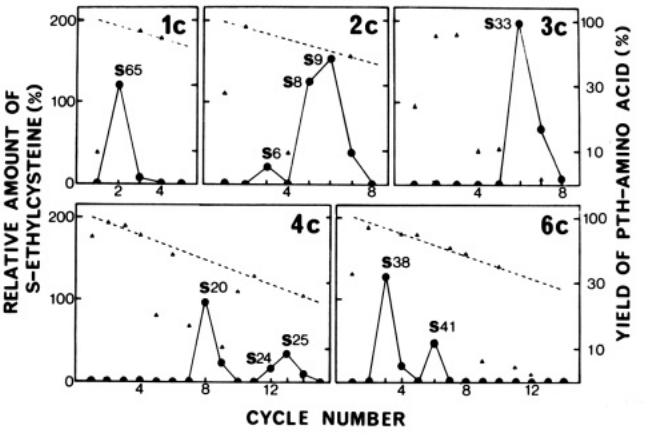

FIGURE 4: Detection of *S*-ethylcysteine in sequential Edman degradation of ethanethiol-treated phosphopeptides. Phosphopeptides (0.5–1.2 nmol) by protein kinase C were treated with ethanethiol in alkaline conditions and subjected to gas-phase sequence analysis, as described under Experimental Procedures. The relative amount (●) of *S*-ethylcysteine was determined from the peak area as the percentage ratio to the internal standard (100 pmol). The serine residue (S) detected as *S*-ethylcysteine is shown with the residue number in murine vimentin (Fischer et al., 1986). The repetitive yield of PTH-amino acid is shown by a triangle.

by the method described above (Figure 5). Under the conditions employed, both PTH-serine and the DTT adduct of PTH-serine were observed at the first cycle for peptide 1a and peptide 2a. On the other hand, only the DTT adduct of PTH-serine was observed at the first cycle for peptide 5a,

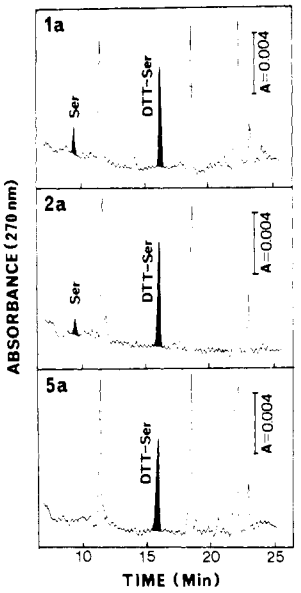

FIGURE 5: Sequence analysis of phosphopeptides containing amino-terminal serine. Phosphopeptides 1a, 2a, and 5a (0.3–0.5 nmol) were subjected to gas-phase sequence analysis without pretreatment with ethanethiol, as described under Experimental Procedures. PTH chromatograms of the first cycle of Edman degradation are shown.

indicating that the phosphate was located on Ser-50 (Figure 5). Phosphopeptides 1c, 2c, 3c, and 5c were also analyzed by the same method. Both PTH-serine and the DTT adduct of PTH-serine were observed at the first cycle for peptides 1c, 2c, and 3c, while only the DTT adduct of PTH-serine was observed at the first cycle for peptide 5c, indicating that the phosphate was located on Ser-50 (data not shown). The location of phosphoserine in the head domain of vimentin phosphorylated by cAMP-dependent protein kinase and protein kinase C is summarized in Tables I and II.

The secondary structural prediction (Chou & Fasman, 1978) for the head-domain sequence of vimentin showed that most of the identified phosphorylation sites were likely to be located in the β-turn structures (Figure 6).

Discussion

We reported that vimentin can serve as a substrate for phosphorylation by cAMP-dependent protein kinase or by protein kinase C (Inagaki et al., 1987). Subsequently, each phosphorylation was shown to induce the disassembly of vimentin filaments (Inagaki et al., 1987, 1988). In the present study, we identified phosphorylation sites along the vimentin molecule, as follows. cAMP-dependent protein kinase phosphorylated Ser-6, Ser-24, Ser-38, Ser-46, Ser-50, and Ser-65, and protein kinase C did Ser-6, Ser-8, Ser-9, Ser-20, Ser-24, Ser-25, Ser-33, Ser-38, Ser-41, Ser-50, and Ser-65. These serine residues were located at sites close to arginine residues. Phosphorylation sites by both the enzymes are present in the amino-terminal non-α-helical head domain, particularly at the β-turn region.

The location of the phosphorylation sites along the head domain agrees with earlier aspects of vimentin filament structure (Geisler & Weber, 1986). The head domain is basic because of a wealth of arginine residues. This highly basic domain has a direct effect on filament stability and polymerization; specific removal of the head domain by Ca^{2+}-activated proteinase leaves a protein unable to polymerize (Traub & Vorgias, 1983), and a vimentin fragment of 12K most likely covering the head domain acts as an inhibitor in vimentin assembly (Traub & Vorgias, 1983). Anionic charges of the phosphoserine residues not only reduce the total basicity of

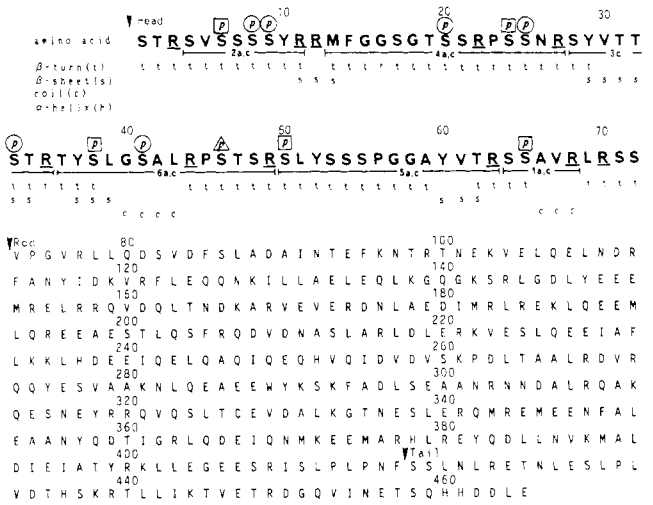

FIGURE 6: Location of phosphoserine in vimentin phosphorylated by cAMP-dependent protein kinase and protein kinase C. The isolated phosphopeptides underlined are aligned according to the sequence of vimentin (Quax et al., 1983; Fischer et al., 1986). Amino acids are represented by the single-letter code. Phosphorylation sites by cAMP-dependent protein kinase and protein kinase C are shown by a P within a triangle or circle, respectively. The common phosphorylation sites are shown with a P in a square. Involvement of each residue in α-helix (h), β-sheet (s), β-turn (t), and coli (c) structures is predicted for the head domain sequence by using the method of Chou and Fasman (1978).

the head domain but also may mask the bivalent cationic charges of the adjacent arginine residues which are important for local conformation, or for interaction with other vimentin domains to form the filament structure. Furthermore the evidence that sites phosphorylated by these two types of protein kinases are located at the β-turn region supports that these two protein kinases readily phosphorylate not only soluble vimentin but also the vimentin filaments.

To assess the substrate specificity of cAMP-dependent protein kinase or protein kinase C, vimentin appears to be an ideal substrate, since this protein is phosphorylated at multiple sites by each protein kinase. The primary structure of the vicinity of the aminoacyl residue to be phosphorylated is one important determinant factor for substrate recognition, and cAMP-dependent protein kinase reacts normally with the serine and threonine residues located close to the carboxyl-terminal side of lysine or arginine (Kemp et al., 1977; Feramisco et al., 1980). For vimentin, we have identified Ser-6, Ser-24, Ser-38, Ser-46, Ser-50, and Ser-65 as the sites to be phosphorylated by cAMP-dependent protein kinase. All these serine residues are located close to the carboxyl-terminal side of arginine. On the other hand, protein kinase C has indistinct and different types of site recognition for vimentin, as compared with cAMP-dependent protein kinase. The present analysis has identified Ser-6, Ser-8, Ser-9, Ser-20, Ser-24, Ser-25, Ser-33, Ser-38, Ser-41, Ser-50, and Ser-65 as sites to be phosphorylated by protein kinase C. Among them, Ser-8, Ser-9, Ser-20, Ser-24, Ser-25, Ser-33, Ser-41, and Ser-65 are located close to the amino-terminal side of arginine. However, Ser-6, Ser-38, and Ser-50, which are phosphorylated by both protein kinase C and cAMP-dependent protein kinase, are located close to the carboxyl- but not the amino-terminal side of arginine. Using myelin basic protein as a model substrate, it was proposed that contrary to cAMP-dependent protein kinase, protein kinase C preferentially reacts with the serine and threonine residues that are located close to the amino-terminal side of lysine or arginine (Kishimoto et al., 1985). On the other hand, the sequences around the known phos-

phorylation sites by protein kinase C in histone H1 (Nishizuka et al., 1978), histone H2B (Nishizuka et al., 1978), glycogen synthase (Ahmad et al., 1984), and acetylcholine receptor (Safran et al., 1987) indicate that their phosphorylation sites are located close to the carboxyl- but not the amino-terminal side of lysine or arginine. For vimentin, we have shown that the sites phosphorylated specifically by protein kinase C are always located close to the amino-terminal side of arginine, but not to the sites phosphorylated by both the protein kinases. Further exploration and/or new approaches are needed to clarify the principle of the site recognition of protein kinase C.

What, if any, is the physiological significance of the domain- and/or site-specific phosphorylation of vimentin by cAMP-dependent protein kinase and protein kinase C? It will be important to demonstrate whether this type of phosphorylation occurs in vivo. A temporal relationship between changes in vimentin and desmin filament organizations and alterations in phosphorylations of their subunit proteins has been demonstrated (Gard & Lazarides, 1982; Spruill et al., 1983). In particular, vimentin and desmin filaments are significantly reorganized in cells during mitosis (Blose & Bushnell, 1982; Franke et al., 1982; Zieve et al., 1980), as accompanied by an increase in filament phosphorylations (Evans & Fink, 1982; Celis et al., 1983; Fey et al., 1983; Westwood et al., 1985). Recently, Evans (1988b) has demonstrated that increased phosphorylations of vimentin and desmin observed during cell division occur within the amino-terminal domains. Experiments similar to that conducted by Evans (1988b) would be of help to discuss a potential role of the domain- and/or site-specific phosphorylation of vimentin and desmin, in intact cells. Although the physiological importance of the experiments reported here remains to be established, they reinforce the importance of identifying the domain and/or sites of phosphorylation, and they would verify the possibility that phosphorylation is directly related to changes in vimentin and desmin filament organizations.

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