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Keratin 8 Phosphorylation *in Vitro* by cAMP-Dependent Protein Kinase Occurs within the Amino- and Carboxyl-Terminal End Domains

Shoji Ando,[1] Toshiya Tokui,[2] Takeo Yano, and Masaki Inagaki

Biophysics Unit and Laboratory of Biochemistry, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan

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We reported earlier that phosphorylation *in vitro* of keratin filaments reconstituted from rat type I keratin 18 and type II keratin 8 by cAMP-dependent protein kinase induces disassembly of the keratin filament structure. Keratin 8 rather than keratin 18 was the major target of the kinase. We have now identified the sites on rat keratin 8 for cAMP-dependent protein kinase. Sequential analysis of the purified phosphopeptides, together with the known primary sequence, revealed that four major sites, Ser-12, Ser-23, Ser-36, and Ser-50, and three minor sites, Ser-8, Ser-33, Ser-42, are located in the amino-terminal head domain, while three minor sites, Ser-416, Ser-423 and Ser-425 locate in the carboxyl-terminal tail domain.

Keratins are the most complex and diversified molecules within the intermediate filament protein family and are expressed differentially in various epithelial cells and tissues (1). Keratins are divided into two distinct groups; type I keratins (*i.e.* keratins 9–19) have a relatively small molecular mass (40–57 kDa) and are relatively acidic (pI = 4.5–5.5), whereas type II keratins (*i.e.* keratins 1–8) are larger (52–67 kDa) and slightly basic (pI = 5.5–7.5). Formation of keratin filaments requires at least the two types of keratin proteins.

Although mechanisms regulating intracellular assembly/disassembly of keratin filaments have remained obscure, a temporal relationship between changes in keratin filament organizations and alterations in phosphorylation of their subunit proteins was noted. In particular, keratin filaments are significantly reorganized in cells during mitosis, and there is an increase in filament phosphorylation (2). Increase in phosphorylation of keratin has also been observed in interphase cells of some epithelial cell lines incubated with phorbol ester (3) or dibutyryl-cAMP (4), and in response to depolarization-induced Ca^{2+} influx (5).

We reported earlier that rat liver keratin 8 (type II) and keratin 18 (type I) serve as excellent *in vitro* substrates for cAMP-dependent protein kinase, protein kinase C, and calmodulin-dependent protein kinase, and that phosphorylation induces disassembly of the keratin filaments (6). Kinetic analysis suggested that the protein kinases preferentially phosphorylate keratin 8, compared to keratin 18 (6).

We have now identified sites on keratin 8 for cAMP-dependent protein kinase. While keratins, as well as other intermediate filament proteins, are composed of the amino-terminal head domain, the central α-helical rod domain and the carboxyl-terminal tail domain (1), seven serine sites were located in the head domain, and three in the tail domain. The significance of domain specific phosphorylation in dynamic keratin filaments is discussed.

MATERIALS AND METHODS

Purification of proteins. The catalytic subunit of cAMP-dependent protein kinase was prepared from bovine heart by the

[1] To whom correspondence should be addressed at present address: Chemistry Laboratory, Saga Medical School, Nabeshima, Saga 849, Japan. Fax: 81-952-30-3393.

[2] Present address: Department of Thoracic and Cardiovascular Surgery, Mie University School of Medicine, Tsu 514, Japan.

Abbreviations: HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate.

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method of Beavo *et al.* (7). Purified keratin 8 (type II keratin, molecular mass 55 kDa) and keratin 18 (type I keratin, molecular mass 47 kDa) were obtained by extraction of crude keratin filament preparations from rat liver, as described (6).

Phosphorylation of keratin 8. Keratin 8 (0.175 mg/ml) was phosphorylated by incubation with 3 μg/ml of the catalytic subunit of cAMP-dependent protein kinase, 0.1 mM [γ-^{32}P]ATP, 0.7 mM $MgCl_2$, 7 mM imidazole/HCl, pH 7.0, at 25°C, for 60 min. The extent of phosphorylation was assayed using SDS-polyacrylamide gel electrophoresis, as described (6).

Phosphopeptides from radioactive keratin 8. Phosphorylated keratin 8 (1 mg) was digested with trypsin, and the resulting phosphopeptides were separated by reversed-phase HPLC, as described in the legend to Fig. 1A. The radioactive fractions were further purified by anion exchange HPLC on a TSK gel DEAE-5PW (0.75 × 7.5 cm) column and then by reversed-phase HPLC on a YMC-Pack ODS-AP (0.46 × 25 cm) column, as described (8).

Sequence analysis. Amino acid sequences were determined using an Applied Biosystems 470A gas-phase sequencer equipped with a 120A on-line PTH amino acid analyzer, using the 03R PTH program. To determine the location of phosphoserine, phosphopeptides were treated with ethanethiol in an alkaline condition, as described (8, 9), and then applied on the sequencer.

Phosphoamino acid analysis. Phosphorylated keratin or each phosphopeptide was subjected to acid hydrolysis in 6 M 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 (6).

RESULTS AND DISCUSSION

To identify the sites phosphorylated by cAMP-dependent protein kinase, keratin 8 (1 mg) was incubated with [γ-^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase, which incorporated approximately 4 mol of phosphate/mol of keratin 8. The radioactive keratin was completely digested with trypsin, then the materials were subjected to HPLC on a reversed-phase column. As shown in Fig. 1A, the HPLC procedure separated six major radioactive peaks, I to VI, comprising 91% of the incorporated ^{32}P label. Each radioactive peak was further purified by anion exchange HPLC (Fig. 1B). While the phosphopeptides from the peaks I, II, and VI appeared as one major peak, peak III, IV or V appeared as plural peaks, namely IIIa and IIIb, IVa and IVb, or Va, Vb and Vc, respectively. Each of these peptides was re-chromatographed on reversed-phase HPLC, and appeared as a single and symmetric peak (data not shown). The phosphopeptides analyzed for gas-phase Edman degradation, as described under "Materials and Methods" and amino acid sequences are listed in Table 1. By comparison with the reported sequence of rat type II keratin 8 (10), the phosphopeptides were found to derive from the amino- and carboxyl-terminal end domains (Table 1).

Phosphoamino acid analysis of keratin 8 phosphorylated by cAMP-dependent protein kinase revealed the presence of only phosphoserine, as reported (6). Since peptide I contained one serine residue, it was assumed that radioactive phosphate was located on Ser-8. Other peptides, however, contained more than two serine residues and the exact phosphorylation sites were determined, as follows: Each peptide was treated with ethanethiol in an alkaline condition to convert specifically the phosphoserine residues to S-ethylcysteine, and positions of S-ethylcysteine were identified by gas-phase sequencing, as described (8, 9). The high release of S-ethylcysteine was observed at the second cycle for peptide II, the third cycle for IIIa, the fifth cycle for peptide IVa, the second and the fifth cycle for peptide IVb, the third cycle for peptide Va, the third and twelfth cycle for peptide Vb, the tenth and twelfth cycle for peptide Vc, and the second cycle for peptide VI (Fig. 1C). A small but significant amount of S-ethylcysteine was also observed at the twelfth cycle for peptide Va, the tenth cycle for peptide Vb, and the third cycle for peptide Vc (Fig. 1C). Therefore, Ser-12, Ser-33, Ser-36, Ser-42, Ser-50, Ser-416, Ser-423 and Ser-425 were phosphorylated. On the other hand, S-ethylcysteine was not observed within peptide IIIb (data not shown). If phosphoserine possesses a free amino or carboxyl terminus, transformation into S-ethylcysteine would not occur (9), thus, Ser-23 may be a phosphoserine. During gas-phase sequencing, normal serine residues were identified as phenylthiohydantoin-serine (PTH-Ser) and the dithiothreitol (DTT) adduct of PHT-Ser. In contrast, phosphoserine provides exclusively the DTT adduct of PTH-Ser (8, 9). Under the conditions we used, only the DTT adduct of PTH-Ser was observed at the first cycle whereas both PTH-Ser and its DTT adduct were observed at the fourth cycle for peptide IIIb (data not

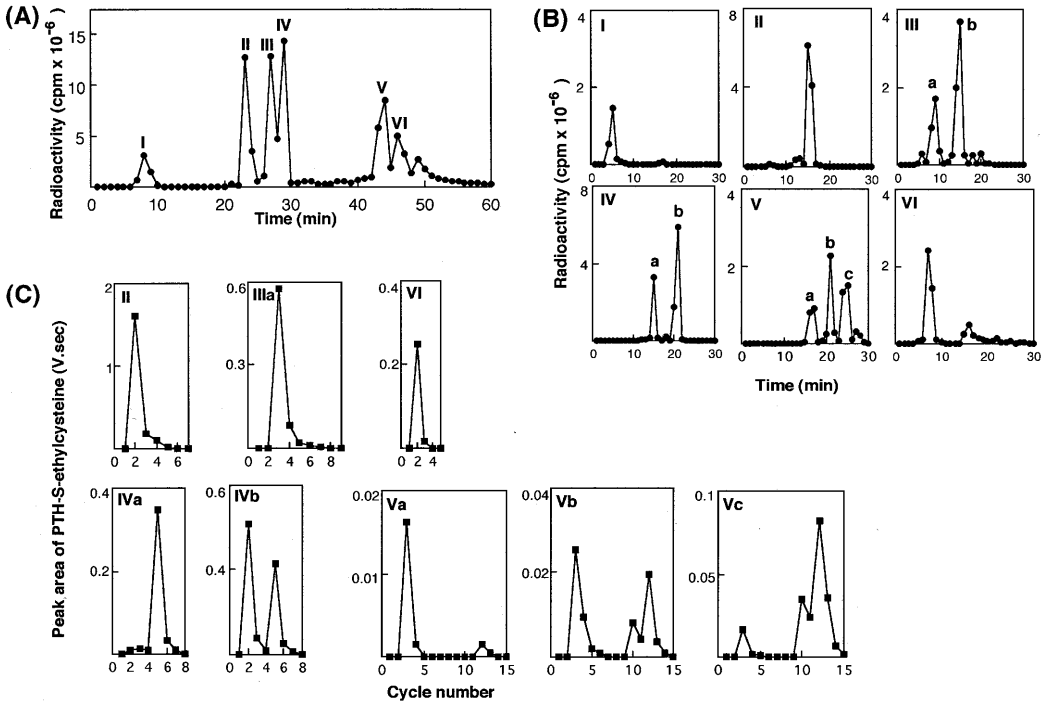

FIG. 1. Purification of phosphopeptide fragments derived from keratin 8 and identification of phosphorylation sites for cAMP-dependent protein kinase. (A) Reversed-phase HPLC of phosphopeptide fragments of keratin 8 phosphorylated by cAMP-dependent protein kinase. After treatment of the phosphorylated keratin 8 with trypsin, the resulting material was applied on a YMC-Pack ODS-AP (0.46 × 25 cm) HPLC column and was eluted with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid over 50 min at a flow rate of 0.8 ml/min. Elution of the fragments was monitored according to the radioactivity of each fraction. (B) Anion exchange HPLC of phosphopeptide fragments. Radioactive fractions were applied on a TSK gel DEAE-5PW (0.75 × 7.5 cm) column 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. (C) Detection of S-ethylcysteine in sequential Edman degradation of ethanethiol-treated phosphopeptides. Peptides (0.1–0.3 nmol) were treated with ethanethiol and subjected to gas-phase sequence analysis, as described under Materials and Methods. The peak area of S-ethylcysteine in the PTH-chromatogram of each degradation step is shown. For peptide VI having the long sequence, S-ethylcysteine was detected only at the second cycle.

shown), thereby indicating that Ser-23 but not Ser-26 is phosphorylated. Location of the phosphorylation sites on rat keratin 8 for cAMP-dependent protein kinase is summarized in Fig. 2.

cAMP-dependent protein kinase reacts normally with serine and threonine residues close to the carboxyl-terminal side of arginine or lysine (11). The sites identified in this study, except for Ser-36, Ser-423 and Ser-425 conform to the substrate recognition sequences. Phosphorylation of Ser-36, Ser-423 and Ser-425 might be dependent on the three-dimensional structures around the sites, as observed for phosphorylation at Ser-356 on rat liver fructose-1,6-bisphosphatase by cAMP-dependent protein kinase (12).

Of the ten sites identified in this study, Ser-8, Ser-12, Ser-23, Ser-33, Ser-36, Ser-42 and Ser-50 are located in the head domain, and Ser-416, Ser-423 and Ser-425 are located in the tail domain (Fig. 2). Approximately 76% of the total phosphates located in the head domain, and 15% were in the tail domain (Table 1). The head domains of intermediate filament proteins may be important for assembly into the filament structures (1, 13). Data on the *in vitro* phosphorylation provided evidence indicating that several protein kinases, including cAMP-dependent protein kinase readily phosphorylate the head domain of vimentin, desmin, glial fibrillary acidic protein or neurofilament-L, and induce disassembly of the filaments (8, 13–19 and references therein). The head domain of

TABLE 1
Amino Acid Sequences and Phosphorylation Sites of Phosphopeptides for cAMP-Dependent Protein Kinase

Isolated peptide	Sequence[a]	Relative amount[c] of phosphate (%)
I	SYK (residues 8–10)	4
II	MSTSGPR (residues 11–17)	20
IIIa	VGSSSSSFR (residues 40–48)	6
IIIb	SFTSGPGAR (residues 23–31)	12
IVa	ISSSSFSR (residues 32–39)	7
IVb	ISSSSFSR (residues 32–39)	15
Va	TTSGYAGGLSSSYGGLTSPGFSYG MSSFQPGFGSVGGS[b] (residues 414–451)	4
Vb	TTSGYAGGLSSSYGGLTSPGFSYG MSSFQPGFGSVGGS[b] (residues 414–451)	6
Vc	TTSGYAGGLSSSYGGLTSPGFSYG MSSFQPGFGSVGGS[b] (residues 414–451)	5
VI	GSLGGFGGAGVGGITAVTVNQSLL NP[b] (residues 49–74)	12

[a] Amino acid sequences are represented by the single-letter code. The phosphorylated serine residues are determined as described in "Materials and Methods", and are underlined. The numbers in parentheses indicate the residue numbers determined from cDNA of rat keratin 8 (10). We postulate that the first methionine residue is removed during processing. [b] Partial sequences detected in peptides V and VI are shown. [c] Determined from radioactivity in the reversed-phase and anion exchange HPLC analyses.

keratin 8 shares unique characteristics (*e.g.* highly basic and Gly/Ser-rich) with those of other intermediate filament proteins. The incorporation of phosphates along the head domain of keratin 8 is probably a major factor linked to the *in vitro* disassembly of keratin 8/keratin 18 filaments. The significance of phosphorylation in the tail domain of keratin 8 remains unknown. Hatzfeld and Weber reported that tail domains of type I and II keratins are not essential for filament formation (20). However, the tail domain might have a role in stabilization of the keratin filaments (13), and the possibility that phosphorylation of the tail domain of keratin 8 affects the stability of filament structures would need to be excluded.

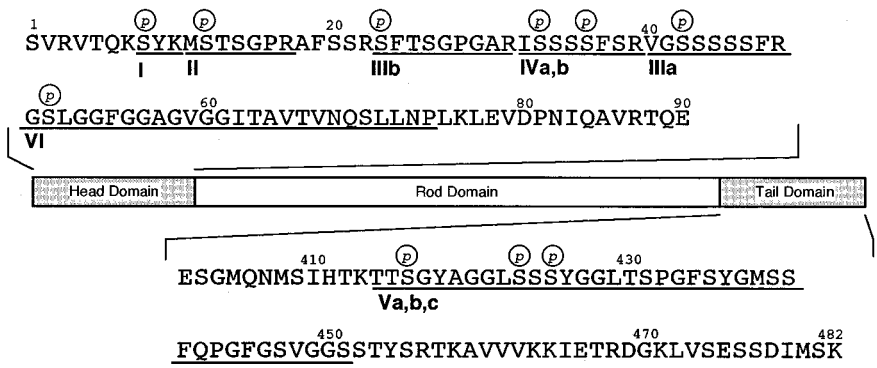

FIG. 2. Location of phosphoserine in rat keratin 8 phosphorylated by cAMP-dependent protein kinase. Amino acid sequences in the amino-terminal head and carboxyl-terminal tail domains of rat keratin 8 (10) are shown in the single-letter code. Amino acids detected in the sequence analysis of the isolated phosphopeptides are underlined. Phosphorylation sites are shown by a P within a circle.

There are reports on phosphorylation sites in keratins in cells or tissues. Steinert found that phosphorylation of human keratin 1 (type II) in the terminally differentiating epidermis occurs in both the head and tail domains (21). Ku and Omary found Ser-52 (in the head domain) to be the major phosphorylation site in human keratin 18 expressed in insect or mammalian cells (22). However, functional significance of location of the phosphorylation sites as well as the involved protein kinases remains to be elucidated. It is known that site-specific phosphorylation along the head domain of vimentin or glial fibrillary acidic protein is involved in organizational changes of their filaments during mitosis of cells (23–26). Our present results provide a clue to elucidation of the role of phosphorylation in the regulation of keratin filaments in cells.

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