Protein Kinase C Phosphorylation of Desmin at Four Serine Residues within the Non-α-helical Head Domain*

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We reported that phosphorylation by either cAMP-dependent protein kinase or protein kinase C (Ca⁡⁺⁺/phospholipid-dependent enzyme) in vitro induces disassembly of the desmin filaments (Inagaki, M., Gonda, Y., Matsuyama, M., Nishizawa, K., Nishi, Y., and Sato, C. (1988) J. Biol. Chem. 263, 5970–5978). For this subunit protein, Ser-29, Ser-38, and Ser-56 within the non-α-helical head domain were shown to be the sites of phosphorylation for cAMP-dependent protein kinase (Geisler, N., and Weber, K. (1988) EMBO J. 7, 15–20). In the present work, we identified the sites of desmin phosphorylated in vitro by other protein kinase which affects the filament structure. The protein kinase C-phosphorylated desmin was hydrolyzed by trypsin, and the phosphorylated peptides were isolated by reverse-phase chromatography. Sequential analysis of the purified phosphopeptides, together with the known primary sequence, revealed that Ser-12, Ser-29, Ser-38, and Ser-56 were phosphorylated by protein kinase C. All four sites are located within the non-α-helical head domain of desmin. Ser-12, Ser-29, and Ser-56 specifically phosphorylated by protein kinase C, have arginine residues at the carboxyl-terminal side (Arg-14, Arg-42, and Arg-59, respectively). Ser-29 phosphorylated by both protein kinase C and cAMP-dependent protein kinase has arginine residues at the amino and carboxyl termini (Arg-27 and Arg-33). These findings support the view that the head domain-specific phosphorylation strongly influences desmin filament structure; however, each protein kinase differed with regard to site recognition on this domain.

Phosphorylation, a major modification common to all intermediate filament proteins has been examined in various in vivo systems (1–3). Intermediate filaments undergo dynamic changes during mitosis, cell locomotion, and in pathologic processes (4–6). A temporal relationship between changes in intermediate filament organizations and alterations in phosphorylation of their subunit proteins has been demonstrated (7–14). In particular, intermediate filaments are significantly reorganized in cells during mitosis, following an increase in filament phosphorylation (9–14). These observations led to speculation that phosphorylation plays an important role in regulating organization of the intermediate filament component of the cytoskeleton (2, 3).

There are reports on in vivo phosphorylation sites and/or domains of the intermediate filament proteins. These proteins contain the amino-terminal head domain, the central α-helical rod domain and the carboxyl-terminal tail domain (3, 15). In vertebrate and invertebrate neurofilaments, most if not all of the phosphorylation of serine residues occurs in the extended tail domain (16–19). These phosphoserine sites mainly contain the sequence Lys-Ser(P)-Pro (18, 19). Keratin intermediate filament proteins are significantly phosphorylated on both serine and threonine residues in vivo, and the bulk of this phosphorylation occurs on the amino- and carboxyl-terminal domains (20–23). Steinert (24) most recently reported that the major sites of phosphorylation of the keratin 1 chain mainly involve sequences containing Arg-X-Ser(P) within the amino- and carboxyl-terminal domains. Analysis of desmin and vimentin from nonmitotic and mitotic cells has shown that the increased phosphorylation of desmin and vimentin observed during cell division occurs exclusively within the amino-terminal head domains (25). However, the precise effects of phosphorylation on intermediate filaments in intact cells remained to be determined.

We reported the role of phosphorylation of desmin and vimentin, in vitro, to be as follows (26, 27): vimentin and desmin are excellent in vitro substrates for protein kinase C (Ca⁡⁺⁺/phospholipid-dependent enzyme) and cAMP-dependent protein kinase, but not of several other kinases. Desmin and vimentin phosphorylated by each protein kinase do not polymerize. The filaments that do polymerize tend to depolymerize after phosphorylation. Moreover dephosphorylation by phosphoprotein phosphatase leads to a reassembly of soluble desmin into filaments.

Geisler and Weber (28) reported that the sites of desmin phosphorylated by cAMP-dependent protein kinase in vitro are restricted to the non-α-helical head domain. We have now obtained findings that not only cAMP-dependent protein kinase but also protein kinase C phosphorylates serine residues within the amino-terminal head domain of desmin in different site recognition. Together with the reported data (26–28), the present results provide clues to the molecular mechanisms of phosphorylation-dependent disassembly of desmin filament, and the functional role of in vivo phosphorylation of desmin filaments is better understood.

EXPERIMENTAL PROCEDURES

Purification of Proteins—Purified desmin was obtained by extraction of the crude intermediate filament preparation from chicken.
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gizzard with 8 m urea and the subsequent chromatography on DEAE-cellulose and CM-cellulose columns in the presence of urea, as described (27). Protein kinase C was prepared from rat brain by the method of Inagaki et al. (29).

Phosphorylation of Desmin—Desmin (0.25 mg/ml) was phosphorylated by incubation with 5 μg/ml protein kinase C, 0.1 mM [γ-32P]ATP, 0.2 mM MgCl₂, 50 μg/ml phosphatidylserine, 2.5 μg/ml diazylglycerol, 25 mM Tris-HCl, pH 7.0 at 25 °C for 4 h.

Isolation of Phosphorylated Desmin—Two ml each of the reverse-phase mixture containing desmin (0.25 mg/ml) was applied to a Zorbax C8 (0.46 × 20 cm) column attached to a Waters HPLC system consisting of model 510 pumps, a model 490 detector, and an automatic gradient controller. The phosphorylated desmin was eluted at around 35 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., Dover, MA) or by radioactivity of each fraction (0.8 ml), using a Beckman scintillation counter LS 5801. This separation was repeated eight times. Fractions containing the radioactive phosphorylated desmin were pooled and lyophilized.

Fragmentation of Phosphorylated Desmin—The phosphorylated desmin isolated by reverse-phase HPLC was dissolved in 50 mM Tris-HCl (pH 7.5) at a concentration of 0.25 mg/ml and was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma, 1:50 (w/w) of desmin) at 37 °C for 4 h. Fresh trypsin was again added, and the mixture was incubated for an additional 4 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 5% acetonitrile, 0.1% trifluoroacetic acid, and subsequently with linear 5–50% acetonitrile gradient in 0.1% trifluoroacetic acid. The flow rate was 0.8 ml/min, 0.8-ml fractions. Each radioactive fraction was separately 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. (30). 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 sequenator, model 470, using the manufacturer’s program. The PTH-derivatives were analyzed on an ABI PTH-C18 (0.46 × 22 cm) cartridge column attached to an HPLC system consisting of Spectra-Physics 8700 pump, ISC0 Vλ absorbance detector and Spectra-Physics 4270 integrator.

Phosphoamino Acid Analysis—The radioactive fragments were 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 (31).

RESULTS AND DISCUSSION

Isolation of Phosphopeptides Derived from Protein Kinase C-phosphorylated Desmin—To identify the sites phosphorylated by protein kinase C, desmin (4 mg) was phosphorylated with [γ-32P]ATP by protein kinase C to approximately 3.0 mol of phosphate/mol of desmin (see “Experimental Procedures”). The material was directly subjected to HPLC equipped with a reverse-phase column, as described under “Experimental Procedures,” and the radioactive desmin (2.4 mg) was eluted as a sharp peak between 61 and 67% acetonitrile. The radioactive desmin was then completely digested with trypsin (see “Experimental Procedures”). The phosphopeptides were isolated by HPLC equipped with a reverse-phase column, as described under “Experimental Procedures.” As shown in Fig. 1, the HPLC procedure separated several phosphopeptides and each was further purified by anion exchange and reverse-phase HPLC columns, as described under “Experimental Procedures.” Each phosphopeptide appeared as a single and symmetric peak, and the amount of the phosphopeptide purified in this manner was in the range of 3–10 nmol.

Phosphorylation Sites Locate to the Head Domain—Each purified radioactive phosphopeptide was analyzed for gas-phase Edman degradation, as described under “Experimental Procedures.” The amino acid sequences of these phosphopeptides are listed in Table I. By comparison with the reported sequence of chicken gizzard desmin (32), peptides 1, 2, 3, and 4 were located at desmin residues 34–42, 10–14, 28–33, and 49–59, respectively. Thus, all the phosphorylation sites of desmin for protein kinase C apparently locate at the amino-terminal head domain (residues 1–69) (15, 33).

The domain location of the protein kinase C phosphorylation sites agrees with certain aspects of intermediate filament structure (3, 15). Although the filament wall seems to arise from interaction of the double-stranded coiled-coils provided by the rod domain, the non-α-helical terminal domains may well provide a stabilizing factor. Use of defined proteolytic derivatives of desmin has led to increasing attention directed to the head domain (33). Moreover, Geisler and Weber (28) reported that cAMP-dependent desmin phosphorylation occurs exclusively in the amino-terminal head domain. Thus, our present observations add support to the view that the

1 The abbreviations used are: HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin.

FIG. 1. Reverse-phase HPLC of phosphopeptide fragments derived enzymatically from phosphorylated desmin. Desmin phosphorylated by protein kinase C was digested successively with trypsin 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 radioactivity of each fraction (0.8 ml) was determined as described under “Experimental Procedures.”
Table I  

| Amino acid sequence* | Isolated peptide | Amount of phosphateb (mol/mol desmin) |
|----------------------|------------------|-------------------------------------|
|                      |                  | Protein kinase C | Protein kinase A |
| Preferable sites for | -Gly-Ser-Arg-     |                       |                  |
| Protein Kinase C      | Gly-Ser-Gly-Ser(Ser(P))-Val-Thr-Ser-Arg-Val- | 1 | 1.0 |
|                      | -Ser-Ser-Ser-Gln-Arg-Val-Ser(Ser(P))-Tyr-Arg-Arg-Thr-Phe- | 2 | 0.8 |
|                      | Arg-Thr-Ser-Ala-Val-Pro-Thr-Leu-Ser(P)-Thr-Phe-Arg-Thr- | 4 | 0.4 |
| Preferable sites for | -Gly-Ser-Arg-     |                       |                  |
| Protein Kinase A      | Gly-Ser(P)-Gly-Ser-Val-Thr-Ser-Arg-Val- | 1' | 0.9 |
|                      | Arg-Thr-Ser(P)-Ala-Val-Pro-Thr-Leu-Ser(P)-Thr-Phe-Arg-Thr- | 4' | 1.0 |
| Common site for both | -Ser-Pro-Val-Phe-Pro-Arg-  |                       |                  |
| Kinases              | Ala-Ser(P)-Phe-Gly-Ser-Arg-Gly- | 3' | 0.8 |

*Residue numbers above the sequences were determined from the amino-terminal end of chicken gizzard desmin (32). The rectangles show the amino acid sequences of the isolated phosphopeptides. Solid and broken lines indicate the phosphorylated serine and arginine residues, respectively.

**The amount of phosphate of each peptide listed was quantitated by HPLC analysis. Protein kinase A, cAMP-dependent protein kinase.

The sites of phosphorylation for cAMP-dependent protein kinase (28) were located at the same tryptic peptide obtained in this study.

Identification of Phosphorylation Sites in the Head Domain—Phosphoamino acid analysis of each phosphopeptide revealed the presence of only phosphoserine (Fig. 2). Since all the phosphopeptides contained more than 2 serine residues, as shown in Table I, the exact phosphorylation sites had to be defined. For this purpose, each fragment was treated with ethanethiol in alkaline condition to specifically convert the phosphoserine residues to S-ethylcysteine. Normal serine residues were not affected by this treatment (30). The positions of S-ethylcysteine residues within the peptides were identified.
by gas-phase sequencing (30). Fig. 3 shows the sequence analysis of each phosphopeptide treated with ethanethiol prior to Edman degradation. A high release of S-ethylcysteine was observed at the fifth cycle for peptide 1, the third cycle for peptide 2, the second cycle for peptide 3, and the eighth cycle for peptide 4, thereby indicating that the phosphate was located on Ser-38, Ser-12, Ser-29, and Ser-56, respectively.

**Primary Structure of the Vicinity of the Phosphorylation Sites**—The primary structure of the vicinity of the amino acid residue to be phosphorylated is one important factor for determining substrate recognition, and cAMP-dependent protein kinase reacts normally with serine and threonine residues located at the carboxyl-terminal side close to lysine or arginine (34, 35). In the case of desmin, Geisel and Weber (28) identified Ser-29, Ser-35, and Ser-50 as the sites to be phosphorylated by protein kinase C. However, sequences around known phosphorylation sites, that is, protein kinase C in histone H1 (39), histone H2B (39), glycogen synthase (40), and acetylcholine receptor (41) indicate that the phosphorylation sites are located at the carboxyl- but not amino-terminal side close to lysine or arginine. Moreover, studies by House et al. (42) who used as substrates various types of synthetic peptides suggest that protein kinase C can recognize primary specificity determinants on either the amino- or carboxyl-terminal side of the phosphorylatable residue. Some of the protein kinase C phosphorylation sites of desmin do not have an appropriate protein kinase C substrate site, usually arginine. This basic residue is separated from the phosphorylation site by one to two neutral amino acids (37, 42, 43). In Ser-29 and Ser-38, the carboxyl-terminal arginine is separated from the phosphorylation sites by 3 neutral amino acids.

**The Secondary Structure of Phosphorylation Sites and Their Vicinity**—Prediction of the secondary structure (44) for the head domain sequence of desmin shows that most of the phosphorylation sites of both kinases are likely to be located in the β-turn structures (Fig. 4). This would support the observation (27) that these two protein kinases readily phosphorylate not only soluble desmin but also the desmin filament, since the β-turn structures generally locate at the solvent-accessible protein surface (45).

Desmin is phosphorylated in vitro by protein kinase C which in turn leads to desmin filament disassembly in vitro (27). There are 4 serine residues phosphorylated and only one is also phosphorylated by cAMP-dependent protein kinase. However, in these in vitro experiments, phosphorylation of desmin by either protein kinase C or cAMP-dependent protein kinase was performed under conditions of low salt and low MgCl2 (26–28). The concentrations of both salt and MgCl2 affect the rate of desmin phosphorylation by these protein kinases. In particular, increasing the MgCl2 concentrations (>3 mM) dramatically decreased the rate of desmin phosphorylation by the protein kinases through formation of the abnormal desmin filament, as was the case with vimentin (26, 27). For desmin, precise locations of the phosphoamino acids within the head domain in vivo have not been determined. Studies are ongoing to examine possible physiological significance of our findings.

Acknowledgments—We are grateful to Dr. Y. Nishizuka of our institute for kind encouragement and to Drs. S. Nakasu and M. Ohara for comments and discussions. We also thank Drs. K. Nishizawa and Y. Ukai for technical assistance and S. Tokumasu for secretarial services.

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