Vol. 142, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1987 Pages 155-161

PHOSPHORYLATION BY PROTEIN KINASE C OF A SYNTHETIC HEPTAPEPTIDE BEARING A LYSINE RESIDUE ON THE C TERMINAL SIDE OF SERINE

Hiroki Kondo [1], Yoshimi Baba[1], Katsunori Takaki[1], Kiyoshi Kondo[2], and Hiroyuki Kagamiyama[2]

[1]Laboratory of Bioorganic Chemistry, Department of Industrial Chemistry, Nagasaki University, Nagasaki 852, Japan
[2]Department of Medical Chemistry, Osaka Medical College, Takatsuki, Osaka 569, Japan

Received November 4, 1986

A peptide, Ala-Ser-Gly-Ser-Phe-Lys-Leu, which corresponds to Ala103 ∿ Leu109 of H1 histone, was synthesized and tested as substrate for protein kinase C. The serine residue at position 4 was phosphorylated specifically. Another peptide lacking the lysine at position 6 was not phosphorylated by the same enzyme, indicating the importance of that basic residue as the recognition site for protein kinase C.

It has become increasingly clear that Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) plays a vital role in the transduction of extracellular signals and stimuli (1-4). Many proteins have been found to be phosphorylated by this enzyme, of which the epidermal growth factor receptor and myelin basic protein are the sole examples whose phosphorylation sites have been determined (5-7). The information derived therefrom has shown that at least one basic amino acid residue is always present in the vicinity of the phosphorylated serine or threonine residue. However, it remains to be clarified whether the N-terminal or the C-terminal basic residue is the primary determinant of substrate activity. This question may be best

Abbreviations: kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; PS, phosphatidylserine; AEAP, 2-aminoethyl-3-aminopropyl; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography.

0006-291X/87 $1.50

explored using synthetic peptides. Earlier reports have indicated that peptides such as kemptide carrying basic amino acids on the N-terminal side of the serine residue can serve as substrates for protein kinase C (8,9), while others have claimed that the enzyme phosphorylates peptides having basic residues on the C-terminal side of serine (10). It is, thus, evident that the substrate specificity of protein kinase C is still debatable.

Hl histone is a protein often employed as a substrate for protein kinase C (11-13). This protein is phosphorylated by protein kinase C as well as by cAMP-dependent protein kinase at multiple but mutually distinct sites. It has also been suggested that one of the sites modified by protein kinase C is located in the middle of the molecule (personal communication from Dr. Y. Nishizuka). One possible site is Ser-106 (numbering based on the calf thymus histone). To confirm this and further to gain insight into the factors dictating the substrate activity of peptides and proteins we prepared a heptapeptide representing residues 103-109 in the sequence of the histone: Ala-Ser-Gly-Ser-Phe-Lys-Leu (peptide-1). Since it was felt that the penultimate lysine might be important in peptide-1, another peptide lacking this residue was also prepared for comparison: Ala-Ser-Gly-Ser-Phe-Leu (peptide-2). It was found that protein kinase C phosphorylates peptide-1 but not peptide-2.

MATERIALS AND METHODS

Materials. The following materials were obtained from the specified sources. DEAE cellulose and P81 paper, Whatman. SP-Sephadex, Sephadex G-10, Phenyl Sepharose, and AH Sepharose, Pharmacia. Biogel P-150, BioRad. PS, diolein and type IIIS histone (Hl rich), Sigma. Carrier-free [γ-^{32}P]inorganic phosphate, Japan Atomic Energy Research Institute. [γ-^{32}P]ATP was either prepared by the method of Glynn and Chappell (14) or purchased from ICN. Homogeneous catalytic subunit of bovine heart cAMP-dependent protein kinase was prepared by J. Kinoshita in our laboratory.

Peptide Synthesis. Peptide-1 and -2 were prepared by the solid phase method (15). Experimental procedures were essentially the same as those described previously (16). Crude

peptides were purified by SP-Sephadex and Sephadex G-10 chromatography. The purity and identity of the peptides were assessed by high voltage paper electrophoresis at pH 3.6 and amino acid analysis.

Enzyme Purification and Assay. Protein kinase C was purified from porcine spleen according essentially to the method of Humble et al. (17) and from rat brain by the method of Kitano et al. (18,19). Most of the experiments were carried out with the former preparation, but the results presented in Table 1 were obtained with the latter preparation. The assay mixture (200 µl) consisted of the following components: 20mM Tris·HCl, pH 7.5, 1mM 2-mercaptoethanol, 5mM $MgCl_2$, 0.5mM $CaCl_2$, 10 µg/ml PS, 1 µg/ml diolein, 200 µg/ml type IIIS histone, 20 µM [γ-^{32}P]ATP (100-300 cpm/pmol) and enzyme. Assay was usually carried out at 30°C for 5 min. Phosphorylated histone was collected on P81 paper.

Peptide Phosphorylation. The composition of the assay mixture was identical to that described above except for substitution of peptide for histone. The phosphorylated peptide was separated from other assay components by high-voltage paper electrophoresis in pyridine-acetic acid-water (1:10:89) buffer, pH 3.6, on Toyo Roshi filter paper No. 51A. The dried paper was exposed to Fuji X-ray film for 2-3 days to locate the radioactive spots, the areas representing the phosphorylated peptide were cut out and subjected to scintillation counting.

Peptide Sequencing. The phosphorylated peptide was sequenced by automated Edman degradation on an LKB 4030 solid phase sequencer equipped with an autoconverter (LKB 4025). The sample was coupled to 2-aminoethyl-3-aminopropyl glass through the amino groups (20). The PTH derivatives of amino acids were identified by HPLC on an LS 410 C_{18} column (Toyo Soda) by isocratic elution according to the method of Zimmerman et al. (21). The PTH derivatives were also monitored for radioactivity.

Miscellaneous. Protein was quantitated by the method of Lowry et al. (22) with bovine serum albumin as the standard. Radioactivity associated with filter paper was determined with 5 ml of toluene-based scintillation cocktail on an Aloka liquid scintillation counter LSC-703. Amino acid analysis was carried out on a Jeol JLC-6AH amino acid analyzer. High-voltage paper electrophoresis was run using a Toyo HPE-406 apparatus.

RESULTS AND DISCUSSION

Phosphorylation of synthetic peptides by protein kinase C was carried out under the conditions described in Materials and Methods. The progress of the reaction was followed by high-voltage paper electrophoresis at pH 3.6 (23). Incubation of peptide-**1** with the enzyme and [γ-^{32}P]ATP generated a radioactive product with a mobility of 0.47 toward the cathode relative to

Table 1. Effects of Ca^{2+} and lipids on the phosphorylation of peptide-1 by protein kinase C

Condition[a]	^{32}P Incorporated[b], cpm
Complete	518 ± 28
-Ca^{2+}	272 ± 22
-PS, diolein	254 ± 79
-Ca^{2+}, PS, diolein	57 ± 23

[a] The complete system contained 0.10 mM $CaCl_2$, 10 µg/ml PS and 1 µg/ml diolein as activators.
[b] Mean ± SD of duplicate runs. The background radioactivity (19 cpm) was subtracted.

that of peptide-1. This phosphorylation of peptide-1 was diminished markedly by the omission of Ca^{2+} and/or phospholipids (Table 1). In fact, peptide phosphorylation in the complete system was 9 times greater than that obtained in the absence of Ca^{2+}, PS and diolein. Rates of peptide phosphorylation in the complete system were determined under initial rate conditions. The data were analyzed using the Henri-Michaelis-Menten equation. The K_m for peptide-1 was determined to be 100 µM, while the V_{max} was 19 nmol/min · mg. Sigma type IIIS histone was also phosphorylated analogously. The kinetic parameters for this substrate were: K_m 1.0 µM and V_{max} 3 nmol/min · mg.

As peptide-1 possesses two serine residues, it was necessary to determine which of them was phosphorylated. Thus the peptide was phosphorylated up to ∿0.1 mol per mol of peptide. The phosphorylated peptide was separated by high-voltage paper electrophoresis as described above, extracted from the paper with water, and then subjected to Edman degradation (Fig. 1). The PTH derivatives of Ser (0.34 nmol), Gly (1.09 nmol), and Phe (1.02 nmol) were identified at Steps 2, 3 and 5, respectively. Ala1 and Lys6 were not identified because they were attached to the AEAP-glass, and the seventh residue was unable to remain on the

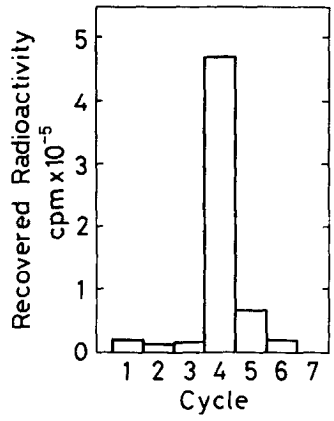

Fig. 1. Determination of the phosphorylation site by Edman degradation. Peptide-1 was phosphorylated under the conditions specified in Materials and Methods. Approximately 5 nmol of phosphorylated peptide-1 was subjected to the analysis. About 1.5 nmol of the sample was estimated to be linked to the AEAP glass.

glass after Step 6. Although a considerable amount of radioactivity was released at every step, including Steps 1 and 6, due presumably to partial dephosphorylation in the course of Edman degradation, Fig. 1 demonstrates unambiguously that the serine at position 4 was preferentially phosphorylated. Peptide-2, which lacks the penultimate lysine, was not phosphorylated to any detectable extent under identical conditions, indicating that this amino acid is essential for the peptide to be phosphorylated by protein kinase C. In other words, this lysine serves as a recognition site in peptide-1 for the kinase. Neither peptide-1 nor peptide-2 was phosphorylated by cAMP-dependent protein kinase, as expected (24).

These data suggest that Ser106 of H1 histone has the potential to be modified by protein kinase C, though final proof of this possibility awaits identification of the phosphorylation site on the histone molecule. It also became clear that protein kinase C is capable of phosphorylating a peptide bearing a basic lysine residue on the C-terminal side of serine. This finding is consistent with the results reported recently by Ferrari et al.

(10). Meanwhile, it has been known that peptides such as kemptide carrying basic residues on the N-terminal side of serine can serve as substrates for this kinase (8,9). From these results we conclude tentatively that protein kinase C is able to phosphorylate a site bearing basic amino acids on either the C-terminal or the N-terminal side. In this sense protein kinase C may be regarded as being more versatile than cAMP-dependent protein kinase which exhibits preference for serines having arginine or lysine residues on the N-terminal side (24).

Three further points should be discussed. Firstly, a single lysine residue, when placed at an appropriate position, is sufficient for a peptide to become a substrate for protein kinase C. This is probably the minimal requirement for the substrate activity of peptides and proteins. Secondly, of the two serine residues in peptide-1, the one closer to the lysine is preferentially phosphorylated by protein kinase C. In other words, the enzyme appears to show rather strict recognition of the serine to be modified. Finally, the fact that peptide-1 is a good substrate for protein kinase C and that it is not phosphorylated by cAMP-dependent protein kinase makes this compound useful as a substrate for the selective assay of protein kinase C activity in a complex mixture of protein kinases.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Y. Nishizuka and his collaborators for kindly informing of their unpublished results as well as for helpful discussions. They also thank Ms. J. Kinoshita for her expert technical assistance.

REFERENCES

1. Nishizuka, Y. (1984) Nature 308, 693-698.
2. Takai, T., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695.
3. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341-13348.
4. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851

5. Hunter, T., Ling, N., and Cooper, J.A. (1984) Nature 311, 480-483.
6. Turner, R.S., Kemp, B.E., Su, H., and Kuo, J.F. (1985) J. Biol. Chem. 260, 11503-11507.
7. Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y., and Nishizuka, Y. (1985) J. Biol. Chem. 260, 12492-12499.
8. Magino, P.E., de la Houssaye, B.A., and Masaracchia, R.A. (1983) Biochem. Biophys. Res. Commun. 116, 675-681.
9. O'Brian, C.A., Lawrence, D.S., Kaiser, E.T., and Weinstein, I.B. (1984) Biochem. Biophys. Res. Commun. 124, 296-302.
10. Ferrari, S., Marchiori, F., Borin, G., and Pinna, L. (1985) FEBS Lett. 184, 72-77.
11. Iwawa, Y., Takai, Y., Kikkawa, U., and Nishizuka, Y. (1980) Biochem. Biophys. Res. Commun. 96, 180-187.
12. Turner, R.S., Chou, C-H.J., Kibler, R.F., and Kuo, J.F. (1982) J. Neurochem. 39, 1397-1404.
13. Glynn, B., Colliton, J., McDermott, J., and Witters, L.A. (1985) Biochem. J. 231, 489-492.
14. Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147-149.
15. Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149-2154.
16. Kondo, H., Uno, S., Komizo, Y., and Sunamoto, J. (1984) Int. J. Peptide Protein Res. 23, 559-564.
17. Humble, E., Heldin, P., Forsberg, P., and Engstrom, L. (1984) J. Biochem. 95, 1435-1443.
18. Kitano, T., Go, M., Kikkawa, U., and Nishizuka, Y. (1986) Methods Enzymol. in press.
19. Kondo, H., Kinoshita, J., Matsuba, T., and Sunamoto, J. (1986) FEBS Lett. 201, 94-96.
20. Laursen, R.A. (1977) Methods Enzymol. 47, 277-288.
21. Zimmerman, C.L., Apella, E., and Pisano, J.J. (1979) Anal. Biochem. 77, 567-573.
22. Lowry, O.H., Resebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
23. Zetterqvist, O., Ragnarsson, U., Humble, E., Berglund, L., and Engstrom, L. (1976) Biochem. Biophys. Res. Commun. 70, 696-703.
24. Glass, D.B. and Krebs, E.G. (1980) Annu. Rev. Pharmacol. Toxicol. 20, 363-388.