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SELECTIVE ASSAY OF PROTEIN KINASE C WITH A SPECIFIC PEPTIDE SUBSTRATE

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INDEXING WORDS

protein kinase C; peptide substrate; selective assay

SYNOPSIS

Protein kinase C is a family of multifunctional protein serine/threonine kinase and generally accepted to be involved in a wide variety of cellular signal transduction. Biochemical and immunochemical studies as well as sequence analysis of its cDNA clones have revealed the existence of multiple subspecies of this enzyme with obvious tissue-specific expression. Enzymatic properties of type I, II, and III protein kinase C subspecies, which are encoded by γ-, βI- and βII, and α-cDNA, respectively, are well characterized. Many proteins and peptides are reported as phosphate acceptors of these protein kinase C subspecies. In this study, it is shown that a synthetic peptide, Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu, which corresponds to amino acid

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Abbreviations used are: protein kinase A, cAMP-dependent protein kinase; EGTA, [ethylenebis (oxyethylenenitrilo)] tetraacetic acid.

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residues 4-14 of bovine myelin basic protein, is the most specific and convenient substrate for selective assay of protein kinase C among various phosphate acceptor proteins and peptides. This peptide is phosphorylated at Ser-8, but not Ser-11 by protein kinase C subspecies in a manner dependent on Ca^{2+}, phosphatidylserine, and diacylglycerol. This peptide is not phosphorylated by other protein serine/threonine kinases such as cyclic AMP-dependent protein kinase. Thus, it is possible to assay protein kinase C activity in the crude tissue extracts selectively using this peptide as a phosphate acceptor.

INTRODUCTION

Protein kinase C is known to be activated by 1,2-diacylglycerol generated by receptor-mediated hydrolysis of inositol phospholipids and is regarded as a receptive protein for tumor-promoting phorbol esters.[12] Protein kinase C catalyzes the phosphorylation of seryl- and threonyl-residues in many proteins and recent analysis has indicated that protein kinase C exists as a family of multiple subspecies which shows subtly different enzymatic properties and distinct expression among various mammalian tissues and cells.[13] Among protein kinase C subspecies, type I, II, and III, which are encoded by γ-, βI- and βII, and α-cDNA, respectively, are shown to have similar substrate specificity and H1 histone is routinely used to assay the enzyme activity of these subspecies.[9] This substrate, however, is not an ideal phosphate acceptor protein to provide an accurate measure of protein kinase C, because basic proteins sometimes influence the kinetic properties of protein kinase C.[1] In addition, especially in crude tissue fractions, many other protein kinases can phosphorylate H1 histone, and basic proteins such as H1 histone are known to enhance phosphorylation of some endogenous proteins catalyzed by casein kinases.[22] On the other hand, it is reported that a pseudosubstrate peptide, which corresponds to the pseudosubstrate sequence of protein kinase C itself and a potent inhibitory peptide for this enzyme, does not inhibit protein kinase C activity when the enzyme activity is assayed using H1 histone as substrate.[5]

To assay protein kinase C selectively a number of peptide substrates have been prepared.[3,6,10,14,17,20,21] Nonetheless, cAMP-dependent protein kinase (protein kinase A), Ca^{2+}/calmodulin-dependent protein kinase II, and phosphorylase kinase phosphorylate seryl- or threonyl-residues in most of these peptide substrates non-selectively and, in some cases, *Km* values for the peptide substrates are relatively high. A previous report has shown that Ser-8 of myelin basic protein is one of the major phosphorylation sites in this protein by protein kinase C, whereas phosphorylation of this serine residue by protein kinase A was very slow.[8] In the present study, a synthetic peptide corresponding to amino acid residues 4-14 of myelin basic protein, Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu, is synthesized and shown as a specific and sensitive substrate for the assay for type I, II, and III protein kinase C. Using this peptide as substrate, it is possible to measure the protein kinase C activity in crude tissue preparations accurately with low background.

MATERIALS AND METHODS

Materials

Phosphatidylserine (bovine brain) and 1,2-diolein were obtained from Serdary Research Laboratories. [γ-32P]ATP was purchased from Amersham. DEAE-cellulose (DE-52) and P-81 ion exchange paper were obtained from Whatman. Sephadex G-15 was from Pharmacia. Calf thymus H1 histone and bovine brain myelin basic protein were prepared by the methods of Oliver *et al.*[15] and Oshiro and Eylar [16], respectively.

Peptide synthesis

Peptides used in this study were as follows.

MBP(4-14)	Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu
MBP(4-12)	Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys
MBP(4-10)	Gln-Lys-Arg-Pro-Ser-Gln-Arg
EGFR(650-658)	Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu
S6(232-239)	Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala

Pseudosubstrate Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val

These peptides were synthesized using the Merrifield procedure[4] on an Applied Biosystems Model 430A peptide synthesizer using PAM resins conjugating 0.5 mmol of appropriate t-Boc (t-butoxycarbonyl) amino acid and 1 mmol of t-Boc amino acids for each cycle. The resins and t-Boc amino acids are supplied by Applied Biosystems. Peptide-resins thus obtained were employed for the standard cleavage procedure with trifluoromethan sulfonic acid according to the manufacture's instruction. The crude synthetic peptides were first dissolved in 1 ml of 2 M acetic acid and applied on gel filtration on a Sephadex G-15 column (0.7 × 50 cm) equilibrated with the same solution. The fractions which contain the peptide are pooled and lyophilized. After lyophylization, these desalted peptides were dissolved in 2 ml of 0.1% trifluoroacetic acid and divided into 0.2 ml-aliquots. Each aliquot was further purified by reverse-phase HPLC on an Aquapore Prep-10 column (C-8, 1 × 10 cm, Applied Biosystems) using an acetonitrile gradient in the presence of 0.1 % trifluoroacetic acid. Purified peptides were quantitated by amino acid analysis after acid hydrolysis using an Applied Biosytems Model 420A delivertizer equipped with an on-line 130A PTC-amino acid analyzer.

Enzyme purification

Protein kinase C, specific activity 1,000-1,500 units/mg protein, was purified from rat brain and separated into three fractions, types I, II, and III, by hydroxyapatite column chromatography.[19] The catalytic subunit of protein kinase A, specific activity 2,900 units/mg protein, was purified from rabbit skeletal muscle as described by Bechtel *et al.*[2] Protein concentration was determined by the method of Lowry *et al.*[11]

Enzyme assay

Phosphorylation of peptides by protein kinase C was carried out in a reaction mixture (0.05 ml) containing 20 mM Tris/HCl at pH 7.5, 5 mM magnesium acetate, 10 μM [γ-^{32}P]ATP (3-

6 × 10^5 cpm), phosphatidylserine (0.5 μg) plus 1,2-diolein (0.05 μg), 0.1 mM $CaCl_2$, a protein kinase C fraction, and various concentrations of each peptide. Before being added, phosphatidylserine and 1,2-diolein were dispersed in 20 mM Tris/HCl at pH 7.5 by sonication and employed as described previously.[17] After incubation in a plastic tube at 30°C, the reaction was stopped by spotting a 30-μl aliquot of the reaction mixture onto a P-81 ion exchange paper (2 × 2 cm), and immediately placed in 75 mM H_3PO_4. Then, P-81 paper was washed four times for 5 min with the same acid solution followed by counting the radioactivity of ^{32}P in 10 ml H_2O with Cerenkov radiation. Basal activity was measured in the presence of EGTA instead of $CaCl_2$, phosphatidylserine and 1,2-diolein. With the catalytic subunit of protein kinase A, reactions were performed under the same conditions except that $CaCl_2$, phosphatidylserine, and 1,2-diolein were omitted from the assay mixture. Where indicated, either H1 histone or myelin basic protein was used instead of synthetic peptides as phosphate acceptor for the protein kinase assay. One unit of protein kinase was defined as the amount of enzyme which incorporated 1 nmol of phosphate from ATP into H1 histone per 1 min under the respective standard conditions.

Amino acid sequence analysis

A sensitive gas-phase sequence analysis was performed with an Applied Biosystems Model 477A protein sequenator, equipped with an on-line Model 120A PTH-amino acid analyzer using the manufacture's standard program.

Preparation of crude extract

Rats are decapitated, and brain tissues were quickly removed and chilled on ice. All procedures described below were performed at 0-4°C. The tissues were homogenized in a Potter-Elvehjem Teflon-glass homogenizer with 6 volumes of 20 mM Tris/HCl at pH 7.5 containing 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mM leupeptin. The homogenates were centrifuged for 60 min at 100,000 x g. The supernatant was employed as crude extract.

RESULTS

Phosphorylation of MBP (4-14)

Fig. 1A shows the time-dependent phosphorylation of MBP(4-14) catalyzed by protein kinase C type II purified from rat brain. The time course of phosphorylation is linear at least for 10 min. Kinetic studies indicated that an apparent *Km* value for MBP(4-14) is 7 μM (Fig. 1B), and this value is similar to those for protein substrates such as H1 histone and myelin basic protein (3-5 μM),[8] and lower than those for other peptide substrates.[3,6,10,14,17,20,21] Thus, MBP(4-14) at a final concentration of 25 μM is employed for standard assay.

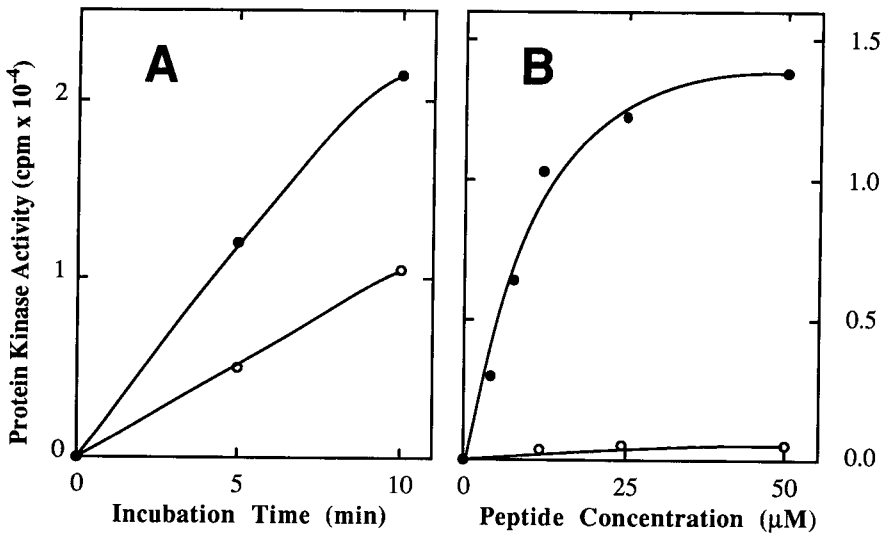

Fig. 1
Phosphorylation of MBP(4-14) by protein kinase C. *A*, Time course of MBP(4-14) phosphorylation. MBP(4-14) was phosphorylated by purified protein kinase C type II for various time indicated in the presence of phosphatidylserine, 1,2-diolein, and Ca^{2+}. (●), with 0.001 unit enzyme; (○), with 0.0005 unit enzyme. *B*, Dose-dependent phosphorylation of MBP(4-14). MBP(4-14) at various concentrations was phosphorylated by purified protein kinase C type II (0.001 unit) for 5 min. (●), in the presence of phosphatidylserine, 1,2-diolein, and Ca^{2+}; (○), in the presence of EGTA instead of phosphatidylserine, 1,2-diolein, and Ca^{2+}.

MBP(4-14) is phosphorylated by type I, II, and III enzymes, which requires the simultaneous addition of Ca^{2+}, diacylglycerol, and phosphatidylserine for full activation. Type I, II, and III enzymes showed subtly different requirements for these activators when assayed using H1 histone as a substrate.[19] Similar results are obtained when assayed using MBP(4-14). For example, MBP(4-14) was phosphorylated by protein kinase C type II in a manner dependent on Ca^{2+}, diacylglycerol, and phosphatidylserine, and this enzyme subspecies exhibited considerable activity for this peptide without added Ca^{2+} as described for H1 histone (Table 1). When assayed with P81 filter for this peptide substrate, the background radioactivity was much lower than that of trichloroacetic acid precipitation procedure. Blank incubation (lacking enzyme) of this assay normally showed negligible radioactivity (less than 150 cpm).

Table 1 Requirement of phosphatidylserine, 1,2-diolein, and Ca^{2+} for phosphorylation of MBP(4-14) by protein kinase C.

MBP(4-14) at a final concentration of 25 μM was phosphorylated by protein kinase C type II (0.003 unit) for 6 min under the standard conditions in the presence or absence of phosphatidylserine, 1,2-diolein, and Ca^{2+}.

Conditions			
Phosphatidyl-serine	1,2-diolein	Ca2+	Phosphorylation (pmol/min)
-	-	-	0.36
-	-	+	0.45
+	-	-	1.17
+	-	+	6.06
+	+	-	4.35
+	+	+	7.50

MBP(4-14) was fully phosphorylated after prolonged incubation, and approximately 0.9 mol of phosphate was incorporated into each mol of the peptide. Subsequent incorporation of radioactive phosphate is extremely slow. The phosphorylated MBP(4-14) is isolated with a reverse phase column on HPLC and subjected to sequential Edman degradation.

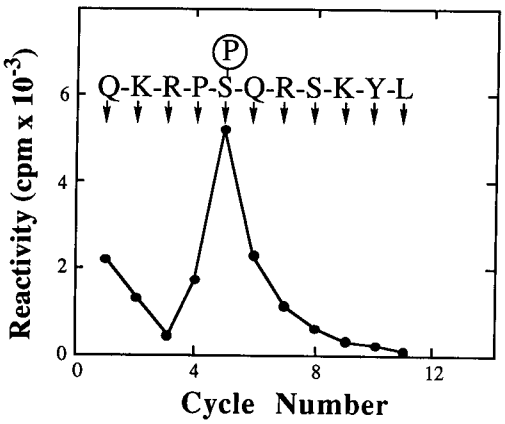

Fig. 2
Sequence analysis of phosphorylated MBP(4-14) by protein kinase C with sequential Edman degradation. MBP(4-14) (1.25 nmol) was phosphorylated by type II PKC (0.024 unit) under the standard condition. Same amount of MBP(4-14) was phosphorylated under the comparable conditions using nonradioactive ATP. Then, the reaction mixture was diluted with 0.2 ml of 0.1% trifluoroacetic acid. A 200-µl aliquot of the solution was injected on to a TSK-gel ODS-120T reverse-phase column (Toso, 5 µm silica, 120Å pore, 250 x 4.6 mm). MBP(4-14) was eluted using an acetonitrile gradient in the presence of 0.1% trifluoroacetic acid and freeze-dried. Purified MBP(4-14) phosphorylated with nonradioactive ATP was employed for the gas-phase sequence analysis. The ^{32}P-labeled MBP(4-14) thus purified was employed for Edman degradation sequence analysis. The deduced sequence is indicated in inset by using one-letter amino acid abbreviation. A circled P shows the seryl-residue phosphorylated by protein kinase C.

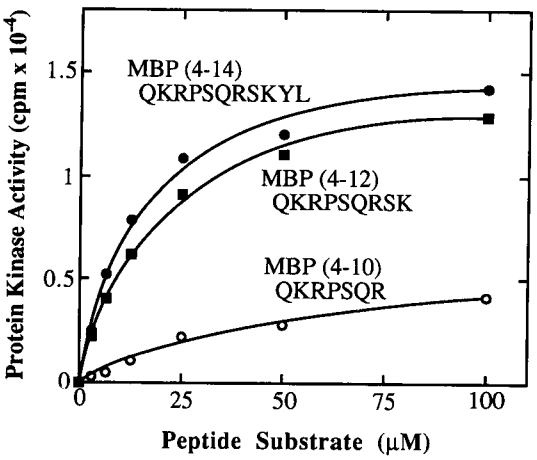

Fig. 3
Requirement of amino acid residues in the carboxyl-terminal of MBP(4-14) for phosphorylation by protein kinase C. Each synthetic peptide at various concentrations was phosphorylated by purified protein kinase C type II (0.001 unit) for 6 min in the presence of phosphatidylserine, 1,2-diolein, and Ca^{2+}. (●), MBP(4-14); (■), MBP(4-12); (○), MBP(4-10).

As shown in Fig. 2, the phosphate is incorporated exclusively into Ser-8 but not into Ser-11. Essentially identical results are obtained using type I and II enzymes.

Peptide and substrate specificity

Fig. 3 shows the effects of deletion of carboxyl-terminal basic region of MBP(4-14) on its phosphorylation by protein kinase C. MBP(4-12) lacking Tyr-13 and Leu-14 was phosphorylated efficiently as MBP(4-14), but MBP(4-10) further lacking Ser-11 and Lys-12 was poorly phosphorylated, indicating that Ser-11 and Lys-12 are important for substrate recognition by protein kinase C. *Vmax* for the phosphorylation of MBP(4-14) was larger than any other substrates tested in this study (Table 2). Under comparable conditions the velocity of the phosphorylation of MBP(4-14) was about two to three times as fast as that obtained with H1 histone and myelin basic protein.

Table 2 Relative phosphorylation velosities of various substrates by protein kinase C and protein kinase A.

Protein kinase C type II (0.003 unit) and protein kinase A catalytic subunit (0.008 unit) were assayed under the standard conditions of each enzyme for 6 min using either MBP(4-14) at 25 μM, myeline basic protein at 25 μM, H1 histone at 10 μM, EGFR(650-658) at 25 μM, or S6(232-239) at 25 μM as substrate.

Substrate	Protein kinase C		Protein kinase A
MBP(4-14)	7.50 [a]	0.30 [b]	<0.01
myelin basic protein	4.20 [a]	1.02 [b]	2.00
H1 histone	3.00 [a]	0.48 [b]	10.71
EGFR(650-658)	6.48 [a]	1.08 [b]	1.08
S6(232-239)	5.64 [a]	2.67 [b]	5.31

[a] in the presence of phosphatidylserine, 1,2-diolein, and Ca^{2+}.
[b] in the presence of EGTA instead of phosphatidylserine, 1,2-diolein, and Ca^{2+}.

Two synthetic peptides, S6(232-239) and EGFR(650-658), which correspond to the phosphorylation site of ribosomal S6 protein and epidermal growth factor receptor by protein kinase C, respectively, have often been used as substrates to assay protein kinase C activity.[6,21] These peptides both showed low *Km* values,[6,21] but their reaction velocities were much slower than that for MBP(4-14). Table 2 also showed that these two peptides and other substrate proteins listed served as substrates for protein kinase C as well as protein kinase A, whereas

MBP(4-14) was not phosphorylated significantly by protein kinase A. MBP(4-14) was not phosphorylated by none of other protein kinases tested, including Ca^{2+}/calmodulin-dependent protein kinase II, casein kinases I and II, and phosphorylase kinase (data not shown). Consistent with the observations by House and Kemp,[5] the phosphorylation of MBP(4-14) catalyzed by protein kinase C was strongly inhibited by the addition of 5 μM pseudosubstrate, whereas phosphorylation of H1 histone was not effected by this inhibitory peptide (Fig. 4).

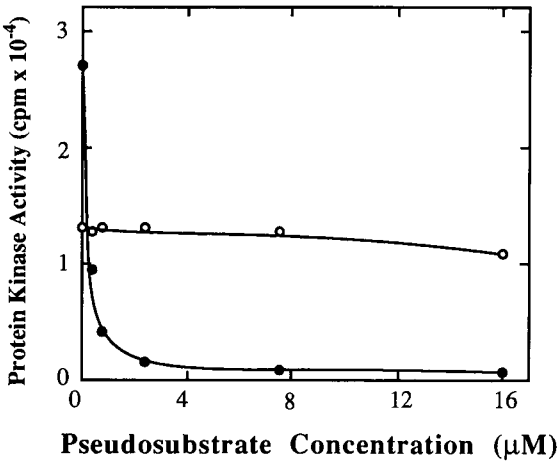

Fig. 4
Effect of pseudosubstrate peptide on phosphorylation of MBP(4-14) and H1 histone by protein kinase C. MBP(4-14) and H1 histone were phosphorylated by purified protein kinase C type II (0.003 unit) for 6 min with various concentrations pseudosubstrate peptide of in the presence of phosphatidylserine, 1,2-diolein, and Ca^{2+}. (●), MBP(4-14); (○), H1 histone.

Assay for crude extract

The time course of phosphorylation of MBP(4-14) by rat brain crude soluble fraction is given in Fig. 5A. The reaction was dependent on Ca^{2+}, diacylglycerol and phosphatidylserine, and proceeded in a time-dependent manner for at least five minutes. Comparison of MBP(4-14) and H1 histone for crude enzyme assay is shown in Fig. 5B. When H1 histone is employed as a substrate, enzyme activity dependent on Ca^{2+}, diacylglycerol, and phosphatidylserine was much lower than that for MBP(4-14) and the reaction reached a plateau quickly. With the extract obtained from cultured HL-60 cells essentially same results were obtained.

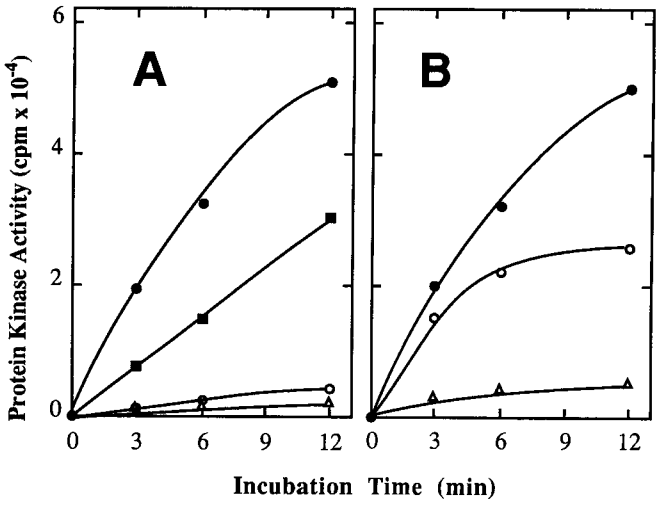

Fig. 5
Time course of phosphorylation of MBP(4-14) and H1 histone by a crude protein kinase C preparation. Rat brain crude extract was prepared and diluted 20 times with 20 mM Tris/HCl at pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, and 10% glycerol. A 5-µl aliquot was assayed under the standard condition with either MBP(4-14) or H1 histone as substrate. A, Effects of phosphatidylserine, 1,2-diolein, and Ca^{2+}. (●), in the presence of phosphatidylserine, 1,2-diolein, and Ca^{2+}; (■), in the presence of phosphatidylserine, 1,2-diolein, and EGTA; (○), in the presence of Ca^{2+} alone; (△), in the absence of phosphatidylserine, 1,2-diolein, and Ca^{2+}. B, Phosphorylation of MBP(4-14) and H1 histone. (●), MBP(4-14); (○), H1 histone; (△), no substrate.

DISCUSSION

The synthetic peptide, MBP(4-14), can be used for selective assay of type I, II, and III protein kinase C, which correspond to γ-, βI- and βII-, and α-subspecies. Ser-8 of MBP(4-14) is phosphorylated by these protein kinase C subspecies, like Hl histone, in a manner dependent on Ca^{2+}, phosphatidylserine and diacylglycerol. Many protein and peptide substrates for protein kinase C have been reported, but most of these substrates are phosphorylated also by other kinases such as protein kinase A.[3,6,13] MBP(4-14) is not phosphorylated by any other protein kinases thus far tested than protein kinase C. It has been generally aggreed that protein kinases C and A phosphorylate seryl- and threonyl-residues in proteins located in the amino- and carboxy-terminal side of a basic residues, respectively.[6-8,10] In the case of the peptide S6(232-239),

Ser-236, that is surrounded by basic amino acid residues such as Arg-232, Arg-233, and Arg-238 on both amino- and carboxy-terminal sides like MBP(4-14), is phosphorylated both by protein kinases C and A (reference 6 and this report). Ser-8 of myelin basic protein is surrounded by basic amino acids on both amino- and carboxy-terminal sides such as Lys-5, Arg-6, Arg-10, and Lys-12. When myelin basic protein is phosphorylated by protein kinase C *in vitro*, this serine residue is the most preferable phosphorylation site and its phosphorylation velocity is highest among phosphorylation sites in this protein.[8] In addition, protein kinase A also phosphorylates Ser-8 to some extent in intact myelin basic protein.[8] However, MBP(4-14) is not phosphorylated by protein kinase A as shown in this study.

MBP(4-14) is a useful substrate for protein kinase C, especially in crude extract as shown in this study. Schaap *et al.*[18] have shown that ε-subspecies expressed in COS cells does not efficiently phosphorylate H1 histone. MBP(4-14) appeared to be a poor substrate for δ-, ε-, and ζ-subspecies (data not shown). Thus, type I, II, and III protein kinase C activity present in crude extracts could be measured using this peptide with low background. Selective assay for protein kinase C using this substrate peptide will be employed for quantitation and characterization of protein kinase C subspecies of various cells and tissues.

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REFERENCES

1. Bazzi, M.D., and Nelsestuen, G.L.: Biochemistry 1987. 26. 1974/1982. Role of substrate in imparting calcium and phospholipid requirements to protein kinase C activation.

2. Bechtel, P.J., Beavo, J.A., and Krebs, E.G.: J. Biol. Chem. 1977. 252. 2691/2697. Purification and characterization of catalytic subunit of skeletal muscle adenosine 3':5'-monophosphate-dependent protein kinase.
3. Ferrari, S., Marchiori, F., Borin, G., and Pinna, L.A.: FEBS Lett. 1985. 184. 72/77. Distinct structural requirements of Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) and cAMP-dependent protein kinase as evidenced by synthetic peptide substrates.
4. Hodges, R.S., and Merrifield, R.B.: Anal. Biochem. 1975. 65. 241/272. Monitoring of solid phase peptide synthesis by an automated spectrophotometric picrate method.
5. House, C., and Kemp, B.E.: Science 1987. 238. 1726/1728. Protein kinase C contains a pseudosubstrate prototope in its regulatory domain.
6. House, C., Wettenhall, R.E.H., and Kemp, B.E.: J. Biol. Chem. 1987. 262. 772/777. The influence of basic residues on the substrate specificity of protein kinase C.
7. Kemp, B.E., Graves, D.J., Benjamini, E., and Krebs, E.G.: J. Biol. Chem. 1977. 252. 4888/4894. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase.
8. Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y., and Nishizuka, Y.: J. Biol. Chem. 1985. 260. 12492/12499. Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3':5'-monophosphate-dependent protein kinase.
9. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and Nishizuka, Y.: J. Biol. Chem. 1980. 255. 2273/2276. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover.
10. Kondo, H., Baba, Y., Takaki, K., Kondo, K., and Kagamiyama, H.: Biochem. Biophys. Res. Commun. 1987. 142. 155/161. Phosphorylation by protein kinase C of a synthetic heptapeptide bearing a lysine residue on the C terminal side of serine.

11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.: J. Biol. Chem. 1951. 193. 265/275. Protein measurement with the Folin phenol reagent.
12. Nishizuka, Y.: Science 1986. 233. 305/312. Studies and perspsectives of protein kinase C.
13. Nishizuka, Y.: Nature 1988. 334. 661/665. The molecular heterogeneity of protein kinase C and its implications for cellular regulation.
14. O'Brian, C.A., Lawrence, D.S., Kaiser, E.T., and Weinstein, I.B.: Biochem. Biophys. Res. Commun. 1984. 124. 296/302. Protein kinase C phosphorylates the synthetic peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val in the presence of phospholipid plus either Ca^{2+} or a phorbol ester tumor promoter.
15. Oliver, D., Sommer, K.R., Panyim, S., Spiker, S., and Chalkley, R.: Biochem. J. 1972. 129. 349/353. A modified procedure for fractionating histones.
16. Oshiro, Y. and Eylar, E.H.: Arch. Biochem. Biophys. 1970. 138. 392/396. Allergic encephalomyelitis: preparation of the encephalitogenic basic protein from bovine brain.
17. Romhányi, T., Seprődi, J., Antoni, F., Mészáros, G., and Faragó, A.: Biochim. Biophys. Acta 1985. 827. 144/149. Specific substrate for histone kinase II: a synthetic nonapeptide.
18. Schaap, D., Parker, P.J., Bristol, A., Kriz, R., and Knopf, J.: FEBS Lett. 1989. 243. 351/357. Unique substrate specificity and regulatory properties of PKC-ε: a rationale for diversity.
19. Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U., and Nishizuka, Y.: J. Biochem. 1988. 103. 759/765. Mode of activation and kinetic properties of three distinct forms of protein kinase C from rat brain.
20. Turner, R.S., Kemp, B.E., Su, H., and Kuo, J.F.: J. Biol. Chem. 1985. 260. 11503/11507. Substrate specificity of phospholipid/Ca^{2+}-dependent protein kinase as probed with synthetic peptide fragments of the bovine myelin basic protein.
21. Woodgett, J.R., Gould, K.L., and Hunter, T.: Eur. J. Biochem. 1986. 161. 177/184. Substrate specificity of

protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements.

22. Yamamoto, M., Criss, W.E., Takai, Y., Yamamura, H., and Nishizuka, Y.: J. Biol. Chem. 1979. 254. 5049/5052. A hepatic soluble cyclic nucleotide-independent protein kinase. Stimulation by basic polypeptides.