J. Biochem. 119, 626-632 (1996)

Phosphorylated Sites of M_r 25,000 Protein, a Putative Protein Phosphatase 2A Modulator, and Phosphorylation of the Synthetic Peptide Containing These Sites by Protein Kinase C[1]

Eikichi Hashimoto,*,[2] Naoki Kobayashi,* Norika Kubota,* Yukie Tanaka,† and Hirohei Yamamura‡,§,[3]

**Department of Pathological Biochemistry, School of Life Sciences, Faculty of Medicine, Tottori University, Nishimachi 86, Yonago 683; †Central Research Laboratories and ‡Department of Biochemistry, Fukui Medical School, Matsuoka, Fukui 910-11; and §Biosignal Research Center, Kobe University, Kobe 657*

Received for publication, August 11, 1995

The M_r 25,000 protein isolated from *Xenopus laevis* oocytes was shown to be an effective phosphate acceptor for Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C) [Hashimoto, E. *et al.* (1995) *J. Biochem.* 118, 453-460]. In this study, the sites of this protein phosphorylated by protein kinase C were determined and the mechanism of substrate recognition was studied using a synthetic peptide containing the phosphorylation sites. After incorporation of about 2 mol of phosphate per mol of this protein, the radioactive protein was digested with trypsin and the phosphopeptides were purified by a series of column chromatographies. The amino acid sequence of the major radioactive peptide was shown to be Ser-Arg-Val-Ser-Lys-Arg. This and previous results suggest that the two serine residues at the amino-terminal region were phosphorylated by protein kinase C. To confirm this, the phosphorylated protein was directly analyzed for the amino acid sequence. The percent distribution of dithiothreitol adduct of the phenylthiohydantoin derivative of serine (PTH-serine) compared with that of PTH-serine increased at the first and fourth cycles of the sequence analysis. When the synthetic peptide composed of the amino-terminal eleven amino acids was employed as phosphate acceptor, the K_m value was unexpectedly high (1.1 mM) compared with that of the native protein (0.5 μM). A stimulatory effect of M_r 25,000 protein on the activity of protein phosphatase 2A was further enhanced after phosphorylation by protein kinase C. These results suggest that the two serine residues recognized by protein kinase C may have some role in the regulation of this M_r 25,000 protein.

Key words: phosphorylated site, protein kinase C, protein phosphatase 2A, synthetic peptide, *Xenopus laevis* oocytes.

Protein kinase C is known to play pivotal roles in various signal transduction pathways (*1*). Reflecting the biological and medical importance of this enzyme, many intracellular proteins were shown to be candidates for its natural substrate (*2*). In parallel with these analyses, the phosphorylated sites of effective phosphate acceptors such as H1 histone (*3*), myelin basic protein (*4, 5*), and ribosomal S6 protein (*6*) were determined in order to elucidate the amino acid sequence preferably recognized by this enzyme. Based on these results, the consensus sequence in substrate phosphorylation by protein kinase C was presented as well as those for other protein serine/threonine kinases (*7*).

More recently, we isolated a M_r 25,000 protein from the cytosolic fraction of *Xenopus laevis* oocytes as an effective phosphate acceptor for both protein kinase C and casein kinase II (*8*). In this report, the sites of this M_r 25,000 protein phosphorylated by protein kinase C were determined and the phosphorylation of a synthetic peptide containing these sites was studied in order to know whether primary structure is a definitive factor in making this M_r 25,000 protein an effective phosphate acceptor. The results obtained suggest the importance of the higher order structure of the substrate protein as well as the primary structure around the phosphorylated site(s). In addition, the possible role of the phosphorylation of M_r 25,000 protein by protein kinase C was examined from the standpoint on the modulation of protein phosphatase 2A activity by this protein.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—M_r 25,000 protein was purified from the cytosolic fraction of *X. laevis* oocytes as

[1] This work was supported in part by Grants-in-Aid for General Scientific Research, for Scientific Research on Priority Areas, and for Co-operative Research from the Ministry of Education, Science and Culture of Japan.

[2] To whom correspondence should be addressed.

[3] Present address: Department of Biochemistry, Kobe University School of Medicine, Kobe 650.

Abbreviations: protein kinase A, cAMP-dependent protein kinase; protein kinase C, Ca^{2+}-phospholipid-dependent protein kinase; PTH, phenylthiohydantoin; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; EGF, epidermal growth factor.

described previously (*8*) or by the simplified procedure using heat-treatment and chromatography on an anion-exchange column. The detailed procedure of the latter method will be described elsewhere. Protein kinase C (type II and III enzymes) was obtained from rat brain as indicated previously (*9*). Protein phosphatase 2A (polycation-stimulated protein phosphatase, PCS_L) was purified as described previously (*10*) till the purification step of Polylysine-Agarose. Sephadex G-15, Sephacryl S-200 and S-300 were purchased from Pharmacia. A reverse-phase C18 column (0.39×15 cm, μBondasphere 5 μm C18-100A) was purchased from Waters. A TSKgel QAE-2SW column (0.46×25 cm) and a TSKgel SP-2SW column (0.46×25 cm) were obtained from Tosoh. Calf thymus H1 histone was prepared as described previously (*11*). Bovine serum albumin (fatty acid-free), trypsin (treated with L-1-tosylamide-2-phenylethylchloromethyl ketone), and *p*-nitrophenyl phosphate were obtained from Sigma. Phosphatidylserine (bovine brain) and diolein were purchased from Serdary Research Laboratories. [γ-^{32}P]ATP was obtained from ICN. Okadaic acid was purchased from Wako. The synthetic peptide SRVSKRQQEKN (Peptide I, the eighth amino acid, Q, was tentatively identified), derived from the amino-terminal eleven amino acids of M_r 25,000 protein, was obtained from TANA Laboratories. This peptide was HPLC-purified and the amino acid composition of the peptide was shown in molar ratio as follows, D/N, 1.07; S, 2.14; E/Q, 3.24; V, 0.93; K, 1.89; R, 2.10.

Enzyme Assays—Protein kinase C was assayed as described previously (*12*) except that bovine serum albumin was added to a final concentration of 0.3 mg/ml. After stopping the reaction by addition of 25 μl of 5% (w/v) trichloroacetic acid, the radioactivity incorporated into H1 histone was determined as indicated previously (*12*). One unit of the kinase was defined as the amount of enzyme which incorporated 1 nmol of phosphate per min under the conditions described. The specific activity of rat brain type II protein kinase C mainly used in this study was 1,480 units/mg protein. Phosphorylation of Peptide I (final concentration was 2 mM, usually) was performed under the reaction conditions used for the phosphorylation of M_r 25,000 protein (*8*). After stopping the reaction, the radioactivity incorporated into the peptide was determined as described above. The specific activity of [γ-^{32}P]ATP was 4.4–7.0×10^5 cpm/nmol and incubation was performed for 4.5–10 min at 30°C. Other detailed reaction conditions and modifications are indicated in each experiment.

Protein phosphatase 2A was assayed as described previously (*10*) by measuring the *p*-nitrophenyl phosphatase activity, except that the concentration of *p*-nitrophenyl phosphate was decreased to 5 mM. The specific activity of the purified enzyme was 760 units/mg protein. Other modifications of reaction conditions of this phosphatase and the definition of enzyme unit were indicated in the corresponding experiment and the previous report (*10*), respectively.

Phosphorylation of M_r 25,000 Protein in Large Scale and Tryptic Digestion—Large-scale phosphorylation of M_r 25,000 protein (114 μg) by protein kinase C (0.4 unit) was performed under the reaction conditions described previously (*8*), except that (i) the volume of the reaction mixture was scaled up by 100-fold (5 ml); (ii) bovine serum albumin for stabilizing the enzyme was not included; (iii) the specific activity of [γ-^{32}P]ATP was 1.1×10^5 cpm/nmol. After incubation for 1.25 h at 30°C, the reaction mixture was kept on ice and 2 M NaCl was added to give a final concentration of 0.4 M. To remove unreacted [γ-^{32}P]ATP, the reaction mixture was directly applied to a Sephacryl S-300 column (2.5×90 cm) equilibrated with buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 0.5 mM EGTA, 0.4 M NaCl, and 0.02% (w/v) NaN_3. Flow rate was 31 ml/h and fractions of 5.6 ml each were collected. The peak of radioactive M_r 25,000 protein was eluted in fractions from 53 to 62, and unreacted [γ-^{32}P]ATP was continuously eluted in later fractions. The former fractions were pooled and the solution containing the radioactive M_r 25,000 protein (56 ml) was dialyzed against 5 liters of distilled water for 4 h at 4°C. This dialysis was repeated. Judging from the radioactivity recovered, about 2 mol of phosphate was incorporated per mol of the M_r 25,000 protein. This dialyzed solution was freeze-dried and the residue was dissolved in about 0.7 ml of 0.1 M ammonium bicarbonate. The M_r 25,000 protein was then digested with trypsin (1:50 by weight ratio) for 4 h at 37°C with shaking. The tryptic digest was freeze-dried and the radioactive peptides were further purified as described in the text.

In the experiment to examine the effect of the phosphorylated and nonphosphorylated M_r 25,000 protein on protein phosphatase 2A activity, the protein (1 mg each) was also phosphorylated by protein kinase C (type III enzyme, 1.1 units) under the reaction conditions (*8*) except that (i) the volume of the reaction mixture was 6 ml (120-fold scale-up); (ii) 20 mM Tris-HCl, pH 7.8, was employed; (iii) $CaCl_2$ was added to exceed the concentration of EGTA and EDTA derived from enzyme and M_r 25,000 protein; (iv) 0.17 mM nonradioactive ATP was employed; (v) the enzyme was not included in the control reaction for the preparation of the nonphosphorylated M_r 25,000 protein. After incubation for 5 h at 30°C, about 1.23 mol of phosphate was incorporated per mol of M_r 25,000 protein. This value was obtained from the small-scale incubation mixture in which radioactive ATP was employed. Each reaction mixture was separately concentrated to about 1.5 ml using Molcut II (Millipore) and then applied to a Sephacryl S-200 column (1.6×117.5 cm) equilibrated with the buffer described above except that NaN_3 was not included. Flow rate was 4.7 ml/h and fractions of 2 ml each were collected. The elution position of M_r 25,000 protein was confirmed by SDS-PAGE and protein staining. The phosphorylated and nonphosphorylated M_r 25,000 protein (about 16 ml) eluted from the column were concentrated to about 2 ml using Amicon aparatus equipped with PM 10 membrane and further concentrated by freeze-drying to about 0.4 ml. After determination of protein concentration, these samples of M_r 25,000 protein were employed for the experiment.

Other Procedures and Determinations—SDS-PAGE was performed as described previously (*13*) utilizing a 12.5% acrylamide running gel and a 4.5% stacking gel. Protein was determined by the method of Bradford (*14*) with bovine serum albumin as a standard. The concentration of M_r 25,000 protein was determined as described previously (*8*). Radioactivity was determined with an Aloka LSC-950 or a Beckman LS-5801 liquid scintillation counter with Cerenkov radiation. The amino acid sequence was determined by automated Edman degradation using an Applied

Biosystems gas-phase protein sequencer, model 477A, equipped with an on-line reverse-phase chromatography system for identification of PTH-amino acids. For identification of phosphoserine residues, the areas of both peaks of PTH-serine and DTT adduct of PTH-serine were calculated according to the previous report (*15*).

RESULTS

Purification of Radioactive Peptides—Freeze-dried tryptic digest of M_r 25,000 protein was dissolved in 0.4 ml of 50% acetic acid (7.50 nmol ^{32}P) and applied to a Sephadex G-15 column (1.3×95 cm) equilibrated with 50% acetic acid. As shown in Fig. 1, one major peak (A, 5.35 nmol ^{32}P) and one minor peak (B, 1.31 nmol ^{32}P) were recovered. The radioactive materials in each peak were concentrated by freeze-drying and dissolved in small amount of 0.2% TFA. When peak A was applied to a reverse-phase C18 column and eluted with an increasing linear concentration of acetonitrile in 0.2% TFA, one major radioactive peak was eluted around fractions 16–18 as shown in Fig. 2. After repeating this analysis five times, the radioactive materials in fraction 16 (A-1) and fractions 17 plus 18 (A-2) were separately pooled and concentrated by freeze-drying. The amounts of radioactive materials in fractions A-1 and A-2 were 0.53 nmol ^{32}P and 2.34 nmol ^{32}P, respectively. The recovery at this step was about 54%. The materials in peak B were not retained on the reverse-phase C18 column under the same conditions and not analyzed further in this study. The materials in fraction 16 and fractions 17 plus 18 were then dissolved in 0.25 ml of 50 mM ammonium bicarbonate. Each fraction was applied to a TSK gel QAE-

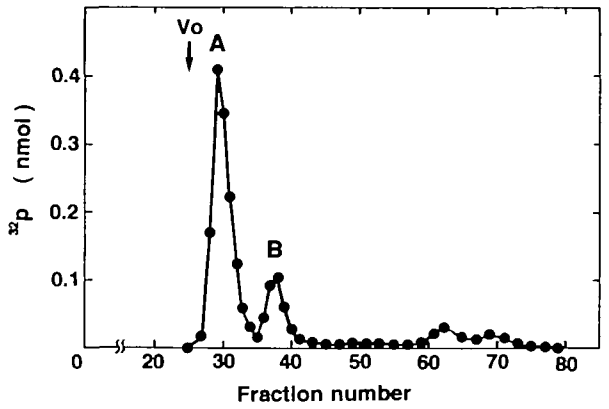

Fig. 1. **Purification of radioactive peptides by gel filtration on a Sephadex G-15 column.** After tryptic digestion, the radioactive materials were applied to a Sephadex G-15 column as described in the text. Flow rate was 6.4 ml/h and fractions of 1.6 ml each were collected. The radioactivity of 0.4 ml of each fraction was determined as described in "EXPERIMENTAL PROCEDURES." Vo shows the position of void volume.

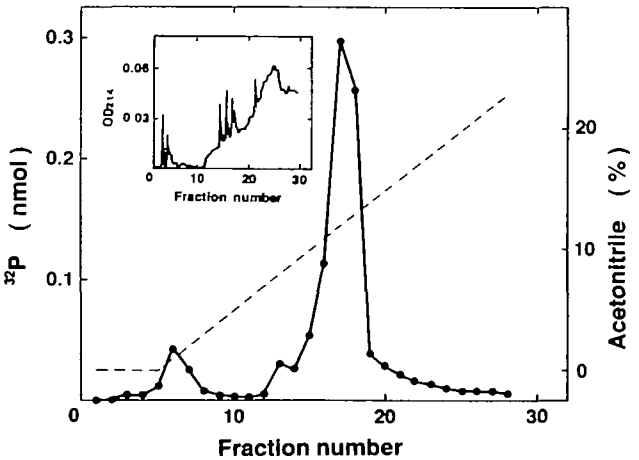

Fig. 2. **Purification of radioactive peptides in peak A on a reverse phase C18 column.** About one-fifth of the radioactive materials in peak A (5.35 nmol ^{32}P) obtained by the gel filtration on the Sephadex G-15 column was further purified by use of a reverse phase C18 column as described in the text. Flow rate was 1 ml/min and fractions of 1 ml each were collected. The radioactivity of the total volume of each fraction was determined as described in the legend to Fig. 1. The dotted line shows the concentration of acetonitrile. This analysis was repeated five times, and a typical result is presented. The insert shows the elution profile of the peptides detected as the optical density at 214 nm.

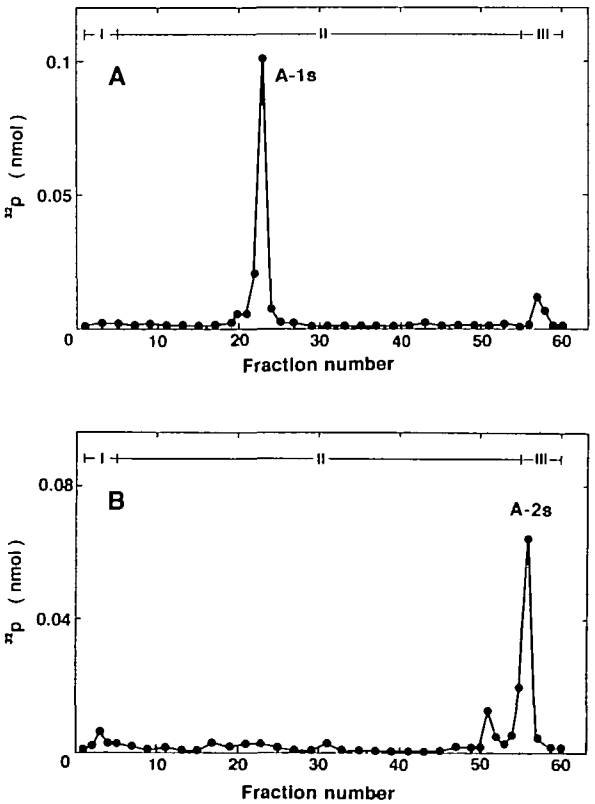

Fig. 3. **Purification of radioactive peptides A-1 and A-2 on a TSK-SP column.** An aliquot of each sample (0.2 nmol ^{32}P and 0.19 nmol ^{32}P for the radioactive peptides A-1 and A-2, respectively) obtained by the reverse phase C18 column was further purified by use of a TSK-SP column as described in the text. The radioactive peptides were eluted as follows: I, 0.1 M pyridine-acetic acid buffer (pH 3.1); II, a linear concentration gradient between 0.1 M pyridine-acetic acid buffer (pH 3.1) and 0.25 M pyridine-acetic acid buffer (pH 3.25); III, 0.25 M pyridine-acetic acid buffer (pH 3.25). Flow rate was 1 ml/min and fractions of 1 ml each were collected. The radioactivity of the total volume of each fraction was determined as described in the legend to Fig. 1. Each purification was repeated twice and a typical result is presented. A, the radioactive peptides in peak A-1; B, the radioactive peptides in peak A-2.

2SW column equilibrated with the same solution, but all of the radioactive materials were recovered in pass-through fractions. These fractions were freeze-dried again and dissolved in 0.25 ml of 0.1 M pyridine-acetic acid buffer, pH 3.1. Each radioactive sample was applied to a TSK gel SP-2SW column and eluted as indicated in Fig. 3. The major radioactive material from A-1 sample was eluted at about 0.15 M pyridine-acetic acid buffer (peak A-1s) and the major radioactive material from A-2 sample was eluted at 0.25 M pyridine-acetic acid buffer (peak A-2s). The radioactive peptides thus purified were 0.19 nmol ^{32}P and 0.96 nmol ^{32}P with A-1s and A-2s samples, respectively. The total recovery of radioactive materials in these two fractions was 22%. These fractions were separately concentrated by freeze-drying and subjected to the sequence analysis.

Determination of the Phosphorylated Sites—The amino acid sequences of these radioactive peaks were determined as follows. The sequence of A-1s was X-R-V- X-K, in which X was not identified conclusively. As indicated in Fig. 4, the sequence of A-2s was determined to be S-R-V-S-K-R. These results suggest that the two radioactive peptides were derived from the same part of the M_r 25,000 protein, and the carboxy-terminal arginine residue in A-2s was due to the incomplete digestion by trypsin. In addition, the sequence of S-R-V-S-K-R was completely identical with that of the amino-terminal region of this protein (*8*). From the fact that the phosphorylated amino acid was serine (*8*) and that about 2 mol of phosphate was incorporated in this protein, the two serine residues located at the amino-terminal region seem to be phosphorylated by protein kinase C.

To confirm this inference, the amino acid sequences of the amino-terminal domain of nonphosphorylated and phosphorylated M_r 25,000 protein were directly analyzed, and the percent distributions of PTH-serine and DTT adduct of PTH-serine appearing at the first and fourth cycles of sequence analyses were compared, because the percent distribution of DTT adduct of PTH-serine is usually increased when serine residue is phosphorylated (*15*). The results shown in Table I indicate that the percent distribution of two serine derivatives was about 50% when nonphosphorylated M_r 25,000 protein was analyzed. However, the percentage of DTT adduct of PTH-serine clearly increased at the first and fourth cycles of the sequence analyses of the phosphorylated protein. These results further support the phosphorylation by protein kinase C of the two serine residues at the amino-terminal region.

Phosphorylation of Synthetic Peptide Containing the

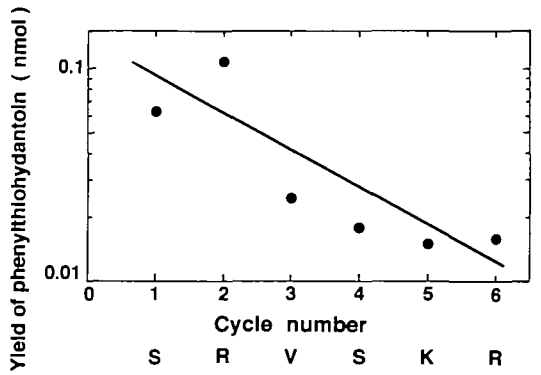

Fig. 4. **Sequence analysis of the tryptic phosphopeptide A-2s derived from M_r 25,000 protein phosphorylated by protein kinase C.** An aliquot of the purified phosphopeptide was subjected to analysis of amino acid sequence as described under "EXPERIMENTAL PROCEDURES." The identified amino acid was indicated at the bottom.

TABLE I. **Percent distribution of PTH-serine and DTT adduct of PTH-serine obtained at the first and fourth cycles of the sequence analyses of unphosphorylated and phosphorylated M_r 25,000 protein.** The peak areas of PTH-serine and DTT adduct of PTH-serine at the first and fourth cycles were calculated and % distribution of each derivative was determined as described under "EXPERIMENTAL PROCEDURES." The values in this table were averages of seven and two repeated sequence analyses of unphosphorylated and phosphorylated forms of M_r 25,000 protein, respectively.

M_r 25,000 protein	First cycle		Fourth cycle	
	PTH-serine (%)	DTT adduct (%)	PTH-serine (%)	DTT adduct (%)
Unphosphorylated form	44.8	55.2	40.2	59.8
Phosphorylated form	24.3	75.7	7.7	92.3

TABLE II. **Ca^{2+}- and phospholipid-requirement for phosphorylation of Peptide I.** Peptide I (final concentration 2 mM) was phosphorylated by protein kinase C (2.4 milliunits) for 6 min under the conditions given under "EXPERIMENTAL PROCEDURES," except that Ca^{2+} and phospholipid were included or not as indicated. When Ca^{2+} was not included, the final concentration of EGTA was 0.64 mM. The activity was expressed as pmol of phosphate incorporated per min.

Effectors	Activity (pmol phosphate/min)
$+Ca^{2+}$ + phospholipid	5.60
$+Ca^{2+}$ − phospholipid	0.14
+EGTA + phospholipid	0.94
+EGTA − phospholipid	0.07

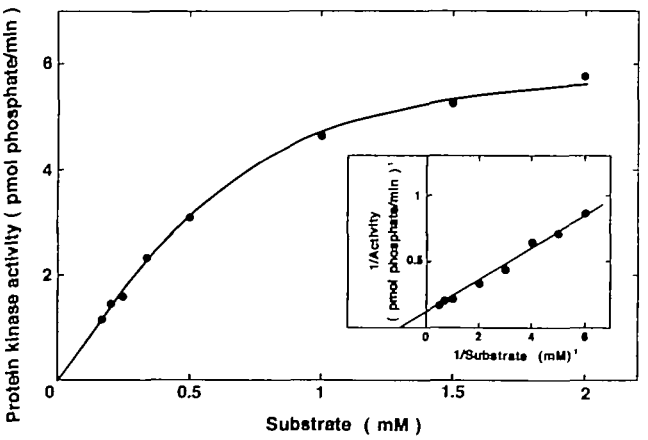

Fig. 5. **Effect of increasing concentration of Peptide I in phosphorylation by protein kinase C.** Peptide I was phosphorylated by protein kinase C (2.4 milliunits) for 6 min under the reaction conditions given under "EXPERIMENTAL PROCEDURES," except that the concentration of Peptide I was changed from 0.167 to 2 mM. The protein kinase activity was presented as pmol of phosphate incorporated per min. The insert shows the double reciprocal plot for the phosphorylation of Peptide I by protein kinase C. Experiments were repeated 4 times, and a typical result is presented.

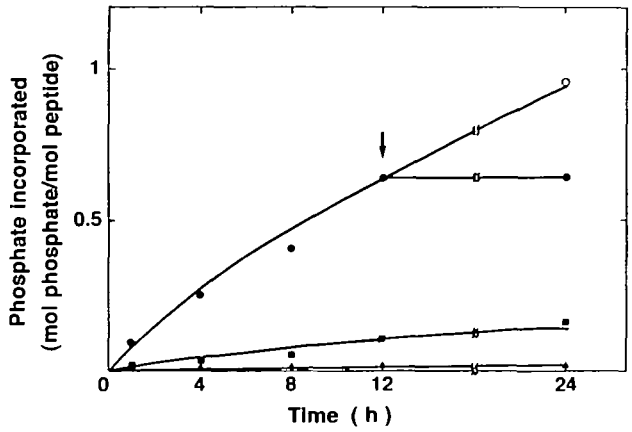

Fig. 6. **Time course of phosphorylation of Peptide I by protein kinase C.** Peptide I (final concentration 0.1 mM) was phosphorylated by protein kinase C (10.4 milliunits) under the reaction conditions described in the legend to Fig. 5, except that (i) the reaction conditions were changed as indicated below; (ii) ATP concentration was increased to 0.41 mM (530-800 cpm/μmol). The incubation was performed for a longer period as indicated, and an aliquot (5 μl) was removed and mixed with 2.5 μl of 5% trichloroacetic acid. After spotting 5 μl of this mixture onto phosphocellulose paper, the radioactivity incorporated into the peptide was determined as described under "EXPERIMENTAL PROCEDURES." At the time indicated by an arrow, a reaction mixture (21 μl) consisting of the complete system from which imidazole-HCl buffer, magnesium acetate, Peptide I and bovine serum albumin were omitted, was further added to 25 μl of the reaction mixture of the complete system. ●, complete system (not modified at 12 h); ○, complete system modified at 12 h as indicated above; ■, minus Ca^{2+} and phospholipid; ▲, minus Peptide I.

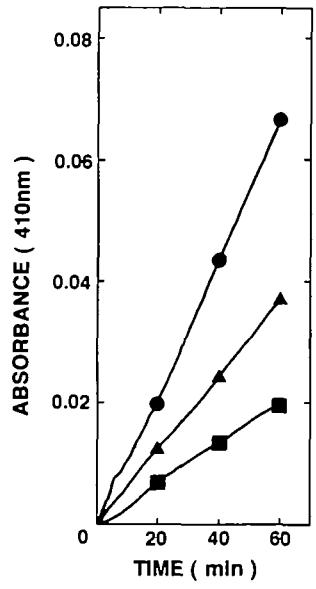

Fig. 7. **Effect of M_r 25,000 protein and its phosphorylated form by protein kinase C on the modulation of *p*-nitrophenyl phosphatase activity of protein phosphatase 2A.** The activity of protein phosphatase 2A was measured as described under "EXPERIMENTAL PROCEDURES," except that (i) the volume of reaction mixture was 0.3 ml; (ii) nonphosphorylated and phosphorylated M_r 25,000 protein by protein kinase C (26 μg protein each) were added as indicated; (iii) the absorbance at 410 nm, reflecting the liberation of *p*-nitrophenol, was recorded using a Hitachi spectrophotometer model 181. The liberation of *p*-nitrophenol in the absence of protein phosphatase 2A was negligible. ●, in the presence of phosphorylated M_r 25,000 protein; ▲, in the presence of non-phosphorylated M_r 25,000 protein; ■, in the presence of the buffer.

Phosphorylated Sites—Kinetic analysis of the phosphorylation of M_r 25,000 protein showed this protein to be an effective phosphate acceptor for protein kinase C (*8*). In the next experiment, the peptide (Peptide I) containing the eleven amino acids of the amino-terminal region of M_r 25,000 protein (containing the two phosphorylated sites) was synthesized and its effectiveness as a phosphate acceptor was examined. The result shown in Table II indicated that this peptide was phosphorylated by protein kinase C in a Ca^{2+}- and phospholipid-dependent manner. The optimum concentration of Mg^{2+} was around 10 mM and the optimum pH was around 8 (pH 7.8-8.5). In contrast to the low K_m (0.5 μM) obtained using M_r 25,000 protein (*8*), the maximum phosphorylation was observed at millimolar concentration of this peptide as shown in Fig. 5. The K_m for Peptide I was 1.12 ± 0.03 mM (mean $\pm$ SE) and apparent V_{max} was calculated to be $5,780 \pm 120$ nmol/min/mg (mean $\pm$ SE). Reflecting the lower affinity of protein kinase C for this peptide, about 1 mol of phosphate was incorporated per mol of this peptide after incubation for 24 h, as presented in Fig. 6. Because of the limitation of the enzyme and the high K_m for this peptide, it seemed to be hard to demonstrate the stoichiometric phosphorylation of this peptide. However, the reaction proceeded in a Ca^{2+}- and phospholipid-dependent manner and the radioactivity incorporated in the absence of the peptide was negligible.

Effect of Phosphorylation by Protein Kinase C on the Functional Activity of M_r 25,000 Protein—It has recently been observed in our laboratory that M_r 25,000 protein has an ability to modulate protein phosphatase 2A activity (Kobayashi, N. and Hashimoto, E., manuscript in preparation). In the last experiment, the effect of phosphorylation by protein kinase C on this activity of M_r 25,000 protein was examined using the protein phosphatase 2A highly purified from *Xenopus* oocytes. When the phosphatase activity was monitored in terms of *p*-nitrophenyl phosphate hydrolysis, the stimulatory effect of M_r 25,000 protein on this phosphatase was further enhanced after phosphorylation by protein kinase C, as shown in Fig. 7. This *p*-nitrophenyl phosphatase activity was strongly inhibited by a low concentration (10 nM) of okadaic acid (data not shown).

DISCUSSION

In an attempt to elucidate the mechanism of substrate recognition by protein kinase C, the amino acid sequences surrounding the phosphorylated sites have been determined on many substrate proteins, and various peptides including the phosphorylated sites have been synthesized to examine the substrate activity in place of the original protein. Part of these studies is summarized in Table III. In general, the affinity of the synthetic peptide tends to be lower than that of its original protein. However, the peptides derived from ribosomal S6 protein and troponin I showed essentially same affinity as the original protein. Based on these analyses by many investigators, the consensus sequence in substrate recognition by protein kinase C revealed the essential role of one or more basic amino acids

TABLE III. **Summary of kinetic analyses on phosphorylation of protein and peptide substrates by protein kinase C.**[a]

Protein substrate	K_m (μM)	Reference	Peptide substrate[b]	K_m (μM)	Reference
M_r 25,000 protein	0.5	*8*	S(1)RVS(4)KRQQEKN[c]	1,100	This report
MARCKS[d]	0.011	*17*	K_5RFSFK$_2$SFKLSGFSFK$_2$NK$_2$	0.95	*17*
H1 histone	1-20	*18-23*	RRKAS(38)GPPV	130	*21*
			AAAS(103)FKAKK	220	*22*
			ASGS(106)FKL	100	*23*
Protamine	3.6	*19*	GSRRRRRRY	11.6	*24*
Myelin basic protein	1	*4*	GKGRGLSLSRFS(115)WGA	14	*4*
	50 μg/ml	*5*	QKRPS(8)QRSKYL	7	*16*
Ribosomal S6 protein	0.8	*18*	AKRRRLSS(236)LRA	0.51	*27*
Troponin I	1	*20*	FDLRGKFKRPT(144)LRRVRISADA	1	*20*
Troponin T	1	*20*	QKVSKTRGKAKVT(280)GRWK	15	*20*
Glycogen synthase	0.25 mg/ml	*25*	PLSRTLS(7)VSS	33	*27*
EGF-receptor	—[e]	*26*	VRKRT(654)LRRL	1.4	*27*
			//	48	*28*

[a]This table was made with reference to that presented in the previous report (*16*). [b]In the peptide substrate, the serine or threonine residues underlined with numbering in parentheses were reported to be phosphorylated. [c]The eighth amino acid, Q, was tentatively identified. It is not clear at this time whether one or both serine residues in Peptide I were phosphorylated. [d]Myristoylated alanine-rich C kinase substrate. [e]Not reported.

in the vicinity of the phosphorylated serine/threonine residue (*7*). These basic amino acids were sometimes detected on both sides of the phosphorylated amino acid and usually observed on at least one side of the site (*7*).

The M_r 25,000 protein purified from the cytosolic fraction of *Xenopus laevis* oocytes was shown to be an effective phosphate acceptor for both protein kinase C and casein kinase II (*8*). The K_m value for this protein was determined to be below 1 μM (*8*) and the high affinity sites were identified as the two serine residues present in the amino-terminal region, as shown in this report. The amino acid sequence Ser(1)-Arg-Val-Ser(4)-Lys-Arg- seems to satisfy the consensus sequence for protein kinase C (*7*). However, the synthetic peptide containing the phosphorylation sites showed high K_m value (1.1 mM) compared with the M_r 25,000 protein itself, although apparent V_{max} values were not so different between these two substrates (*8*). Although the exact reason for the low affinity for this peptide is not yet clear, we suppose that the presence of an amino acid as a spacer between basic amino acid(s) and phosphorylated serine residues may be important in the case of Peptide I, as already reported for protein kinase A. Feramisco *et al.* reported using protein kinase A that a peptide possessing one Ala between Arg-Arg and Ser showed micromolar order of K_m, but this value increased to millimolar order when this Ala was omitted (*29*). In Table III, the peptide substrates having K_m values below 10 μM contain generally at least one amino acid between basic amino acid(s) and the phosphorylated site. The results of the previous (*8*) and present studies suggest that the interaction of the region containing the phosphorylation site(s) with other domains of substrate protein or the higher order structure around these site(s) is important for the preferred recognition of the substrate in addition to the primary structure around the phosphorylated serine or threonine residue(s).

Although the exact function of this M_r 25,000 protein has not yet been conclusively identified, it was observed recently that this protein has the ability to modulate protein phosphatase 2A activity in an *in vitro* reaction system (Kobayashi, N. and Hashimoto, E., manuscript in preparation). In an attempt to elucidate the role of the phosphorylation of this protein by protein kinase C, we showed in this report that the stimulatory effect of this protein on the activity of protein phosphatase 2A was further enhanced after phosphorylation. This result suggests that the phosphorylation of the two serine residues on the amino-terminal domain of M_r 25,000 protein by protein kinase C may play a role in alteration of the activity of this protein, although the physiological significance of this phenomenon is not yet clear.

We are grateful to Prof. Y. Nishizuka, Kobe University, for his encouragement and support.

REFERENCES

1. Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **9**, 484-496
2. Azzi, A., Boscoboinik, D., and Hensey, C. (1992) The protein kinase C family. *Eur. J. Biochem.* **208**, 547-557
3. Jakes, S., Hastings, T.G., Reimann, E.M., and Schlender, K.K. (1988) Identification of the phosphoserine residue in histone H1 phosphorylated by protein kinase C. *FEBS Lett.* **234**, 31-34
4. Turner, R.S., Kemp, B.E., Su, H., and Kuo, J.F. (1985) Substrate specificity of phospholipid/Ca^{2+}-dependent protein kinase as probed with synthetic peptide fragments of the bovine myelin basic protein. *J. Biol. Chem.* **260**, 11503-11507
5. Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y., and Nishizuka, Y. (1985) Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **260**, 12492-12499
6. Sakanoue, Y., Hashimoto, E., Mizuta, K., Kondo, H., and Yamamura, H. (1987) Comparative studies on phosphorylation of synthetic peptide analogue of ribosomal protein S6 and 40-S ribosomal subunits between Ca^{2+}/phospholipid-dependent protein kinase and its protease-activated form. *Eur. J. Biochem.* **168**, 669-677
7. Kennelly, P.J. and Krebs, E.G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**, 15555-15558
8. Hashimoto, E., Takeuchi, F., Tanaka, Y., and Yamamura, H. (1995) Purification and phosphorylation of a M_r 25,000 protein, an effective phosphate acceptor for casein kinase II and protein kinase C, detected in the cytosolic fraction of *Xenopus laevis* oocytes. *J. Biochem.* **118**, 453-460
9. Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U., and Nishizuka, Y. (1988) Mode of activation and kinetic properties of three distinct forms of protein kinase C from rat brain. *J. Biochem.*

103, 759-765
10. Hermann, J., Cayla, X., Dumortier, K., Goris, J., Ozon, R., and Merlevede, W. (1988) Modulation of the substrate specificity of the polycation-stimulated protein phosphatase from *Xenopus laevis* oocytes. *Eur. J. Biochem.* **173**, 17-25
11. Hashimoto, E., Takeda, M., Nishizuka, Y., Hamana, K., and Iwai, K. (1976) Studies on the sites in histones phosphorylated by adenosine 3′:5′-monophosphate-dependent and guanosine 3′:5′-monophosphate-dependent protein kinases. *J. Biol. Chem.* **251**, 6287-6293
12. Hashimoto, E., Takeuchi, F., and Yamamura, H. (1990) Protease-activated form of protein kinase C with M_r 80,000 generated from rat liver plasma membrane by trypsin-like protease. *Biochem. Int.* **21**, 949-957
13. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
14. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
15. Meyer, H.E., Hoffmann-Posorske, E., Korte, H., and Heilmeyer, L.M.G., Jr. (1986) Sequence analysis of phosphoserine-containing peptides. *FEBS Lett.* **204**, 61-66
16. Yasuda, I., Kishimoto, A., Tanaka, S., Tominaga, M., Sakurai, A., and Nishizuka, Y. (1990) A synthetic peptide substrate for selective assay of protein kinase C. *Biochem. Biophys. Res. Commun.* **166**, 1220-1227
17. Fujise, A., Mizuno, K., Ueda, Y., Osada, S., Hirai, S., Takayanagi, A., Shimizu, N., Owada, M.K., Nakajima, H., and Ohno, S. (1994) Specificity of the high affinity interaction of protein kinase C with a physiological substrate, myristoylated alanine-rich protein kinase C substrate. *J. Biol. Chem.* **269**, 31642-31648
18. Le Peuch, C.J., Ballester, R., and Rosen, O.M. (1983) Purified rat brain calcium- and phospholipid-dependent protein kinase phosphorylates ribosomal protein S6. *Proc. Natl. Acad. Sci. USA* **80**, 6858-6862
19. Ferrari, S., Marchiori, F., Marin, O., and Pinna, L.A. (1987) Ca^{2+} phospholipid-dependent and independent phosphorylation of synthetic peptide substrates by protein kinase C. *Eur. J. Biochem.* **163**, 481-487
20. Noland, T.A., Jr., Raynor, R.L., and Kuo, J.F. (1989) Identification of sites phosphorylated in bovine cardiac troponin I and troponin T by protein kinase C and comparative substrate activity of synthetic peptides containing the phosphorylation sites. *J. Biol. Chem.* **264**, 20778-20785
21. O'Brien, C.A., Lawrence, D.S., Kaiser, E.T., and Weinstein, I.B. (1984) 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. *Biochem. Biophys. Res. Commun.* **124**, 296-302
22. Romhányi, T., Seprődi, J., Antoni, F., Mészáros, G., and Faragó, A. (1985) Specific substrate for histone kinase II: A synthetic nonapeptide. *Biochim. Biophys. Acta* **827**, 144-149
23. Kondo, H., Baba, Y., Takaki, K., Kondo, K., and Kagamiyama, H. (1987) Phosphorylation by protein kinase C of a synthetic heptapeptide bearing a lysine residue on the C terminal side of serine. *Biochem. Biophys. Res. Commun.* **142**, 155-161
24. Ferrari, S., Marchiori, F., Borin, G., and Pinna, L.A. (1985) Distinct structural requirements of Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) and cAMP-dependent protein kinase as evidenced by synthetic peptide substrates. *FEBS Lett.* **184**, 72-77
25. Ahmad, Z., Lee, F.-T., DePaoli-Roach, A., and Roach, P.J. (1984) Phosphorylation of glycogen synthase by the Ca^{2+}- and phospholipid-activated protein kinase (protein kinase C). *J. Biol. Chem.* **259**, 8743-8747
26. Hunter, T., Ling, N., and Cooper, J.A. (1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. *Nature* **311**, 480-483
27. House, C., Wettenhall, R.E.H., and Kemp, B.E. (1987) The influence of basic residues on the substrate specificity of protein kinase C. *J. Biol. Chem.* **262**, 772-777
28. Woodgett, J.R., Gould, K.L., and Hunter, T. (1986) Substrate specificity of protein kinase C. *Eur. J. Biochem.* **161**, 177-184
29. Feramisco, J.R., Glass, D.B., and Krebs, E.G. (1980) Optimal spatial requirements for the location of basic residues in peptide substrates for the cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **255**, 4240-4245