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PHOSPHORYLATION OF CRE-BP1, A CYCLIC AMP RESPONSE ELEMENT BINDING PROTEIN, BY PROTEIN KINASE C AND CYCLIC AMP-DEPENDENT PROTEIN KINASE

ATSUSHI SAKURAI

Second Division, Department of Biochemistry
Kobe University School of Medicine

INDEXING WORDS

cyclic AMP response element binding protein; CRE-BP1; protein kinase C; cyclic AMP-dependent protein kinase

ABBREVIATED TITLE

PHOSPHORYLATION OF CRE-BP1 IN VITRO

This article is the dissertation submitted by Atsushi Sakurai to Kobe University School of Medicine for the requirements of Doctor of Medical Sciences.

Abbreviations used are: CRE, cyclic AMP response element; HPLC, high performance liquid chromatography; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; SDS/PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate.

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Author's name in Japanese: 櫻井敦志

SYNOPSIS

CRE-BP1 is a transcriptional activator binding to the cyclic AMP response element, which has a putative metal finger structure and the leucine zipper motif linked to a cluster of basic amino acids in the amino and carboxyl-terminal regions, respectively. The activities of a number of transcription factors are known to be controlled through phosphorylation and dephosphorylation. At the first step for understanding of the regulation of CRE-BP1, phosphorylation of CRE-BP1 was studied *in vitro*. The human recombinant CRE-BP1 was phosphorylated by protein kinase C and cyclic AMP-dependent protein kinase. These two protein kinases recognized distinct seryl residues of CRE-BP1. Amino acid sequence analysis after phosphopeptide map indicated that two seryl residues, Ser-340 and Ser-367, located in the basic region of CRE-BP1 were identified as the major protein kinase C phosphorylation sites, whereas Ser-62 downstream of the metal finger structure was determined as the phosphorylation site by cyclic AMP-dependent protein kinase. The phosphorylation of CRE-BP1 by these two protein kinases may regulate the function of this transcriptional activator protein.

INTRODUCTION

Signal transduction pathways converge ultimately at the level of transcriptional activation to produce specific patterns of gene expression in response to environmental stimuli.[4,28] Transcription of a number of cellular genes is activated by second messengers like cyclic AMP and phorbol esters that act through specific protein kinases, cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC), respectively. Many genes that are transcriptionally regulated by cyclic AMP have been shown to contain a conserved sequence TGACGTCA, the cyclic AMP response element (CRE) or its similar palindromic sequence in the 5' flanking region.[8,27] The TPA response element (TRE; TGACTCA) has been identified as an inducible enhancer of genes, which can be transcribed in response to phorbol esters.[1,20] The CRE and TRE are structurally related closely to each other.

Multiple proteins such as CREB,[13,15] CRE-BP1,[25] and ATFs,[14] have been isolated and characterized by their sequence-specific binding to the CRE. All of these proteins contain a carboxyl-terminal positively charged basic region adjacent to a leucine zipper motif involved in sequence-specific DNA binding and protein-protein interactions, respectively. This indicates that these proteins bind to the CRE as a homodimer or heterodimer. CREB is phosphorylated both *in vivo* and *in vitro* at Ser-133 by the catalytic subunit of PKA, thereby activates transcription

upon stimulation with cyclic AMP.[12,35] The same site of CREB is also phosphorylated by calmodulin-dependent protein kinases I and II, indicating that CREB functions to integrate Ca^{2+} and cyclic AMP signals.[10,33] However, little is known about the relationship between the function of other CRE-binding proteins and the phosphorylation by PKA. In the case of the TRE, the transcription factors Fos and Jun were indicated to form a heterodimer through their leucine zipper motifs and bind to the TRE.[1,5,7,20,29,31] Activation of PKC results in the dephosphorylation of c-Jun at sites which are phosphorylated *in vitro* by glycogen synthetase kinase 3, suggesting that a protein phosphatase or the c-Jun protein kinase is involved in the pathway initiated by activation of PKC.[6] Thus, the CRE- and TRE-binding proteins appear to be regulated by different protein kinases. Recently, however, one of the CRE-binding proteins, CRE-BP1, was demonstrated to make a heterodimer with c-Jun.[17,23] CRE-BP1 can bind to CRE with high affinity as a homodimer or heterodimer with c-Jun, but with relatively low affinity to TRE. This indicates another level of complexity to the nuclear signal transduction processes.

CRE-BP1 consists of 505 amino acids[25] and can activate transcription through CRE as a homodimer or heterodimer with c-Jun.[26] For *trans*-activation, the DNA-binding domain, consisting of a basic amino acid cluster linked to the leucine zipper, and the amino-terminal transcriptional activation domain containing metal finger structure are required.[26] CRE-BP1 (also designated as ATF-2) is also involved in the transcriptional activation by the adenovirus E1A protein.[21,24] E1A does not bind to DNA directly in a sequence-specific manner, and appears to operate indirectly through interactions with CRE-BP1.[37] CRE-BP1 is fairly abundant in the brain, especially hippocampus. The level of its mRNA is induced in the regenerating liver, and is higher in some human tumors than in normal tissues. These facts suggest that CRE-BP1 is important for both cellular proliferation and signal transduction in the brain.[34] At the first step to understand the regulation of function of CRE-BP1, phosphorylation of CRE-BP1 *in vitro* by PKC and PKA was studied.

MATERIALS AND METHODS

Materials and Chemicals

PKC (specific activity, 1,000-1,500 units/mg protein) was purified from rat brain and separated into three distinct fractions, type I, II, and III, by hydroxyapatite column chromatography.[32] The catalytic subunit of PKA (specific activity, 2,900 units/mg protein) was purified from rabbit skeletal muscle as

described[2] or was kindly donated from Dr. T. Kuno, Kobe University School of Medicine, which is purified from bovine heart. Trypsin treated with *L*-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone, thermolysin, and (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride were purchased from Worthington, Seikagaku Co., and Wako Chemical, respectively. [γ-^{32}P]ATP was from New England Nuclear. Bovine brain phosphatidylserine and 1,2-diolein were obtained from Serdary Research Laboratories.

Expression and purification of CRE-BP1 protein

CRE-BP1 protein was expressed in *Escherichia coli* by the T7 expression vector system[30] using the whole insert of a human CRE-BP1 cDNA[25] as described.[34] The whole region of CRE-BP1 with additional 15 amino acids on its amino-terminal having an approximate molecular weight of 66-kDa on SDS/PAGE was expressed and was purified by successive chromatographies on Mono S HR5/5 (0.5 x 5 cm) and HiLoad Superdex 200 (1.6 x 60 cm) columns which were connected to an FPLC system (Pharmacia-LKB). Purification procedures were carried out at 0-4°C. The urea/Triton X-100 extract of *E. coli* was applied to a Mono S column equilibrated with Buffer A (20 mM Tris/HCl at pH 7.5, 0.1 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride, 6 M urea) containing 40 mM NaCl, and CRE-BP1 was eluted by application of a linear concentration gradient of NaCl (40 to 280 mM) in Buffer A. The fractions of CRE-BP1 were pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane, and was applied to gel filtration on a HiLoad Superdex 200 column equilibrated with Buffer A containing 400 mM NaCl. The fractions of CRE-BP1 eluted were pooled and dialyzed extensively 20 mM Tris/HCl at pH 7.5 containing 0.1 mM EDTA, 10% glycerol, and 10 mM 2-mercaptoethanol. CRE-BP1 thus obtained was apparently homogenous as judged by SDS/PAGE.

Phosphorylation of CRE-BP1

Phosphorylation of CRE-BP1 was carried out in 0.5 ml of the reaction mixture containing 20 mM Tris/HCl at pH 7.5, 5 mM magnesium acetate, 10 μM [γ-^{32}P]ATP (3-6 x 10^5 cpm), CRE-BP1 (80 μg, 1.2 nmol), and either the type II PKC (0.15 unit) or the catalytic subunit of PKA (0.22 unit). When phosphorylated by PKC, 0.3 mM $CaCl_2$, 10 μg/ml phosphatidylserine, and 1 μg/ml 1,2-diolein were added to the reaction mixture. After incubation at 30°C, an aliquot of the reaction mixture was removed at each time and spotted onto a phosphocellulose paper (P-81, Whatman), and the paper was washed for 5 min five times with 75 mM phosphoric acid, and the ^{32}P-radioactivity was determined.[36] One unit of protein kinase was

defined as the amount of enzyme which incorporates 1 nmol of phosphate from ATP into H1 histone for PKC and whole histone for PKA per minute under the conditions specified above.

Analysis of phosphopeptides

CRE-BP1 was phosphorylated either by the type II PKC or the catalytic subunit of PKA in 0.5 ml of the reaction mixture as described above. After incubation for 2 hr, each ^{32}P-labeled CRE-BP1 was precipitated by 50% ammonium sulfate, and was digested with trypsin (1.6 μg) in 0.5 ml of 0.1 M ammonium bicarbonate for 15 hr at 37°C. After ammonium bicarbonate was removed by lyophilization, the recovered tryptic peptides were dissolved in a small volume of 0.1% TFA. Aliquots of the solution were injected onto an Aquapore RP-300 C8 column (0.12 x 3 cm, Applied Biosystems) connected with a DfB-HPLC system (Pharmacia-LKB), which was equilibrated with 0.1% TFA. After washing with 2 ml of 0.1% TFA, peptides were eluted with a 10-ml linear concentration gradient of acetonitrile (0 to 40%) in 0.1% TFA at a constant flow rate of 0.2 ml/min. Fractions of 0.2 ml each were collected. Phosphopeptides C1 and C2 and Phosphopeptides A1 and A2 were recovered from the phosphorylated CRE-BP1 by PKC and PKA, respectively. Phosphopeptide C1 was lyophilized, dissolved in 20 mM ammonium bicarbonate containing 10% acetonitrile, and injected onto a Mono Q column HR5/5 (0.5 x 5 cm, Pharmacia-LKB), and was eluted with a 19.5-ml linear concentration gradient of ammonium bicarbonate (0.02 to 1.0 M) in 10% acetonitrile using the DfB-HPLC system. Phosphopeptide C2 was further purified by rechromatography on the reverse phase column, lyophilized, dissolved in 100 mM ammonium formate, and digested with 4 μg of thermolysin at 37°C for 15 hr. The resultant peptides were resuspended in 0.1% TFA after the lyophilization, and was subjected to rechromatography on the Aquapore RP-300 C8 column and two fractions, Phosphopeptides C2a and C2b, were obtained. Phosphopeptides A1 and A2 were further purified by rechromatography on the Aquapore RP-300 C8 column. The peptides thus purified were subjected to amino acid sequence analysis.

Other Procedures

Phosphoamino acid analysis was performed under the conditions described by Cooper *et al.*[9] Amino acid sequence analysis was carried out on an Applied Biosystems 477A pulsed liquid protein sequenator with an Applied Biosystems 120A on-line phenylthiohydantoin analyzer, using the manufacturer's standard program. Protein was determined by the method of Lowry *et al.* with bovine serum albumin as

a standard.[22] Radioactivity was measured by Cerenkov counting. SDS/PAGE was carried out by the method of Laemmli.[19]

RESULTS AND DISCUSSION

Recombinant human CRE-BP1 protein expressed in *E. coli* was purified to homogeneity and was incubated with either the type II PKC or the catalytic subunit of PKA in each phosphorylation reaction mixture. The recombinant CRE-BP1 was phosphorylated by both PKC and PKA (Fig. 1). Approximately 0.7 mol of phosphate was incorporated into one mol of CRE-BP1 when phosphorylated by type II PKC, and the Km value for PKC was 0.2 μM. Similar results were obtained for types I and III PKC subspecies. On the other hand, the affinity of CRE-BP1 for PKA was much lower than that for PKC. The maximum reaction velocity for the phosphorylation of CRE-BP1 by PKA was not obtained under the conditions employed, and 0.2 mol of phosphate at most was incorporated into each mol of CRE-BP1 even after prolonged incubation. The reason for this low stoichiometry of the phosphorylation is not clear presently.

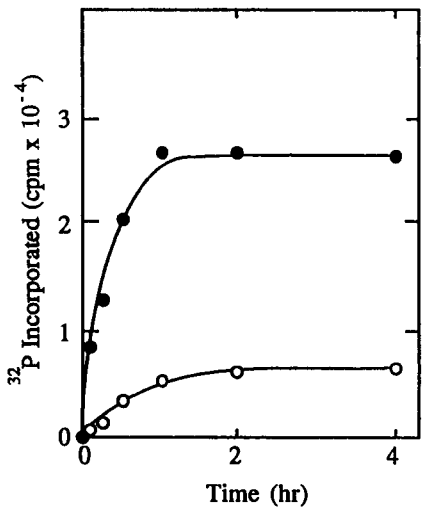

Fig. 1 Phosphorylation of CRE-BP1 by PKC and PKA. CRE-BP1 was phosphorylated each time by either PKC or PKA as described in "Materials and Methods." (●), phosphorylation by PKC; (○), phosphorylation by PKA.

^{32}P-labelled CRE-BP1 was digested with trypsin and the resulting peptides were separated by HPLC to determine the phosphorylation sites in CRE-BP1 (Fig. 2). Tryptic phosphopeptide maps of CRE-BP1 phosphorylated by PKC and PKA showed profiles distinct one another, suggesting that these two enzymes recognized different amino acid residues. Two major phosphopeptides (C1 and C2) were isolated from the CRE-BP1 phosphorylated by PKC with recovery of ^{32}P-radioactivity of 70%.

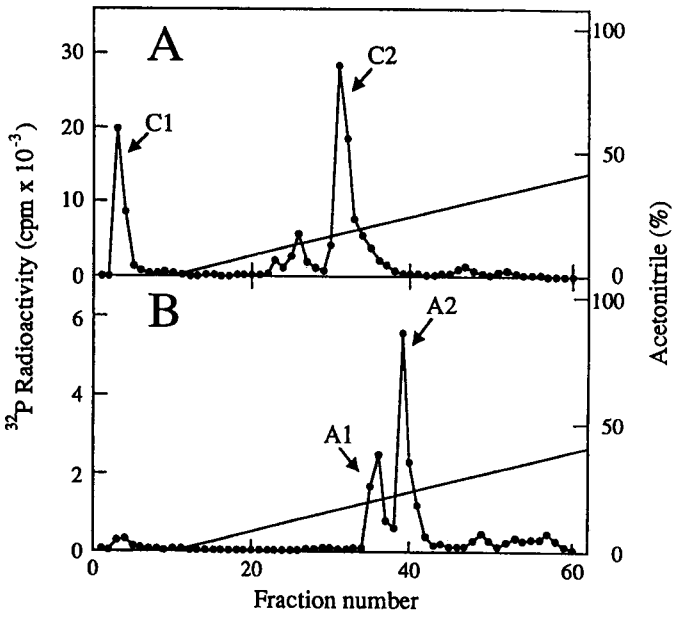

Fig. 2 Separation of radioactive tryptic phosphopeptides. CRE-BP1 phosphorylated by either PKC or PKA was digested with trypsin and the resultant phosphopeptides were separated as described in "MATERIALS AND METHODS." A, with PKC; B, with PKA. (●), radioactivity. (———), acetonitrile concentration. The positions of Phosphopeptides C1, C2, A1, and A2, are indicated.

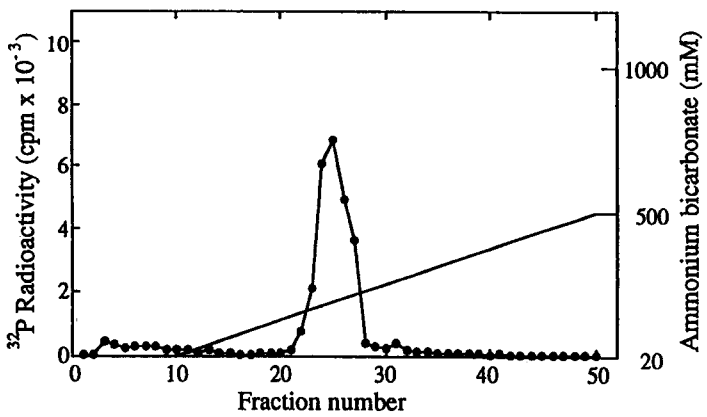

Fig. 3 Purification of Phosphopeptide C1. Phosphopeptide C1 (Fractions 3 and 4 in Fig. 2A) was purified by Mono Q column chromatography as described in "MATERIALS AND METHODS." (●), radioactivity. (———), ammonium bicarbonate concentration.

Two major phosphopeptides (A1 and A2) were recovered from the CRE-BP1 protein phosphorylated by PKA, which accounted for about 60% of the total radioactivity of the original sample. Phosphoamino acid analysis of these peptides showed that all of these peptides contained solely phosphoserine.

Phosphopeptide C1 was not retained to the reverse-phase column under the conditions employed and was further purified by Mono Q column chromatography. A single peak of phosphopeptide was eluted from the column at about 150 mM ammonium bicarbonate (Fig. 3), and the peptide was subjected to amino acid sequence analysis after desalting. A sequence of five amino acid residues corresponding to Ala-364 to Arg-368 of CRE-BP1[25] including a single seryl residue of Ser-367 was obtained (Table I). Thus, the seryl residue, Ser-367, was determined as one of the major phosphorylation site by PKC. The amino-terminal sequence of Phosphopeptide C2, which was further purified by rechromatography on the reverse-phase column of HPLC, gave a sequence starting at the amino acid 305 in the human CRE-BP1. The first basic amino acid residue in the deduced amino acid sequence of the human CRE-BP1 is Arg-308, which is followed by Pro-309 and the peptide bond between arginine and proline is known to be resistant to trypsin. Thus, Phosphopeptide C2 was judged as a quite long peptide starting from Thr-305 to Arg-342, and was subjected to the cleavage with thermolysin followed by purification on the reverse-phase column (Fig. 4).

Table I. Amino acid sequence of Phosphopeptide C1.

The purified Phosphopeptide C1 was subjected to amino acid sequence analysis. No obvious amino acid was detected from the 6th cycle indicating that the arginyl residue at the 5th cycle was the carboxyl-terminal of Phosphopeptide C1.

Phosphopeptide C1			Sequence of CRE-BP1[a]	
Cycle number	Amino acid	Yield	Amino acid	Residue No.
		(pmol)		
			Arg	363
1	Ala	32.0	Ala	364
2	Ala	19.8	Ala	365
3	Ala	21.3	Ala	366
4	Ser	—[b]	Ser	367
5	Arg	6.2	Arg	368
			Cys	369
			Arg	370

[a] From Reference 25.
[b] Not quantified.

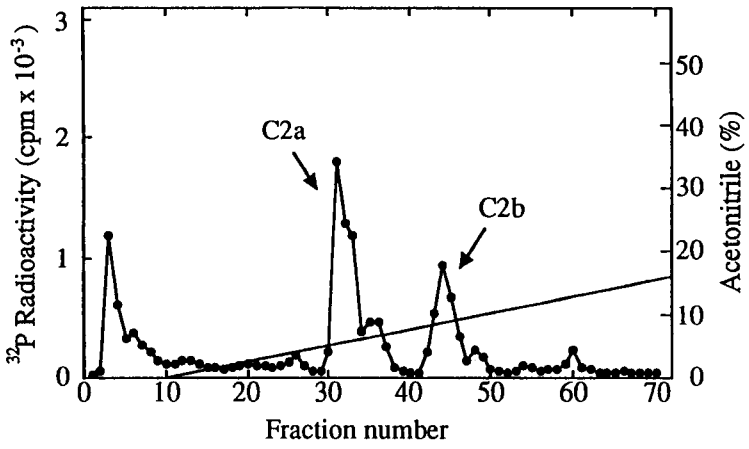

Fig. 4 Separation of radioactive thermolytic phosphopeptides derived from Phosphopeptide C2. Phosphopeptide C2 (Fractions 31 and 32 in Fig. 2A) was digested with thermolysin and the resultant peptides were purified by Aquapore RP-300 column chromatography as described in "MATERIALS AND METHODS." (●), radioactivity. (———), acetonitrile concentration. The positions of Phosphopeptides C1a and C1b are indicated.

The resulting two phosphopeptides, C2a and C2b, showed overlapping sequence and corresponded to the basic region of CRE-BP1, Ala-327 to Arg-342 and Ala-319 to Arg-342, respectively, and Phosphopeptide C2b appeared to be a product of incomplete digestion (Table II). Although these sequences contained three or four seryl residues, the yield of seryl residue corresponding to Ser-340 was extremely lower than those of other seryl residues. In addition, analysis of the phosphorylated seryl residue of Phosphopeptide C2a with sequential Edman degradation by the method of Bengur *et al.*[3] indicated that the phosphate was incorporate into this seryl residue. Thus, Ser-340 in Phosphopeptide C2 was concluded as another major phosphorylation site by PKC. Ser-340 in Phosphopeptide C2 and Ser-367 in Phosphopeptide C1 are located on the amino-terminal side of the cluster of basic amino acid residues, which agree with the consensus sequence for the phosphorylation by PKC.[16,18] Ser-121 has arginine and lysine at both its amino- and carboxyl-terminal sides, and been regarded as a candidate for the phosphorylation site by PKC,[25] however, the phosphopeptide including this seryl residue was not detected.

Table II. Amino acid sequences of Phosphopeptide C2a and C2b.

The purified Phosphopeptide C2a and C2b were subjected to amino acid sequence analysis. No obvious amino acid was detected from the 17th and 26th cycles of Phosphopeptides C2a and C2b, respectively, indicating that the arginyl residues at the 16th and 25th cycle were the carboxyl-terminals of these peptides, respectively.

Phosphopeptide C2a			Phosphopeptide C2b			Sequence of CRE-BP1[a]	
Cycle number	Amino acid	Yield	Cycle number	Amino acid	Yield	Amino acid	Residue No.
		(pmol)			(pmol)		
			1	Ala	40.0	Ala	319
			2	Thr	35.1	Thr	320
			3	Ser	17.3	Ser	321
			4	Thr	18.7	Thr	322
			5	Thr	16.3	Thr	323
			6	Glu	18.6	Glu	324
			7	Thr	15.3	Thr	325
			8	Pro	15.7	Pro	326
1	Ala	—[b]	9	Ala	17.0	Ala	327
2	Ser	10.1	10	Ser	5.6	Ser	328
3	Pro	6.7	11	Pro	13.8	Pro	329
4	Ala	10.2	12	Ala	11.2	Ala	330
5	His	3.4	13	His	2.0	His	331
6	Thr	4.3	14	Thr	6.3	Thr	332
7	Thr	4.4	15	Thr	5.0	Thr	333
8	Pro	5.5	16	Pro	7.8	Pro	334
9	Gln	4.5	17	Gln	3.5	Gln	335
10	Thr	2.3	18	Thr	3.9	Thr	336
11	Gln	3.6	19	Gln	3.3	Gln	337
12	Ser	2.4	20	Ser	4.3	Ser	338
13	Thr	1.5	22	Thr	3.0	Thr	339
14	Ser	—[b]	23	Ser	—[b]	Ser	340
15	Gly	2.3	24	Gly	1.7	Gly	341
16	Arg	1.7	25	Arg	1.0	Arg	342
						Arg	343
						Arg	344

[a] From Reference 25.
[b] Not quantified.

Phosphopeptides, A1 and A2, were subjected to rechromatography on the reverse-phase column. Each appeared as a single peak and amino acid sequence analysis revealed that Phosphopeptides A1 and A2 contained sequences corresponding to Asn-60 to Arg-74 and Phe-55 to Arg-74 of the amino-terminal region of CRE-BP1, respectively (Table III). Both peptides shared an overlapping sequence and Phosphopeptide A2 appeared to be a product of incomplete digestion.

Table III. Amino acid sequences of Phosphopeptides A1 and A2.

The purified Phosphopeptides A1 and A2 were subjected to amino acid sequence analysis. No obvious amino acid was detected from the 16th and 21st cycles of Phosphopeptides A1 and A2, respectively, indicating that the arginyl residues at the 15th and 20th cycle were the carboxyl-terminals of these peptides, respectively.

Phosphopeptide A1			Phosphopeptide A2			Sequence of CRE-BP1[a]	
Cycle number	Amino acid	Yield	Cycle number	Amino acid	Yield	Amino acid	Residue No.
		(pmol)			(pmol)		
						Lys	54
			1	Phe	17.0	Phe	55
			2	Gly	13.8	Gly	56
			3	Pro	10.1	Pro	57
			4	Ala	14.5	Ala	58
			5	Arg	2.7	Arg	59
1	Asn	25.4	6	Asn	6.0	Asn	60
2	Asp	25.8	7	Asp	11.8	Asp	61
3	Ser	—[b]	8	Ser	—[b]	Ser	62
4	Val	26.9	9	Val	2.3	Val	63
5	Ile	32.9	10	Ile	3.1	Ile	64
6	Val	16.5	11	Val	2.3	Val	65
7	Ala	20.6	12	Ala	3.2	Ala	66
8	Asp	8.6	13	Asp	4.5	Asp	67
9	Gln	12.3	14	Gln	1.5	Gln	68
10	Thr	6.5	15	Thr	1.0	Thr	69
11	Pro	11.7	16	Pro	2.2	Pro	70
12	Thr	4.1	17	Thr	0.6	Thr	71
13	Pro	6.3	18	Pro	1.8	Pro	72
14	Thr	3.0	19	Thr	0.6	Thr	73
15	Arg	3.7	20	Arg	0.2	Arg	74

[a] From Reference 25.
[b] Not quantified.

As these tryptic peptides contained phosphoserine but no phosphothreonine as described above and the yield of the seryl residue corresponding to Ser-62 was extremely lower than those of other residues, the single seryl residue Ser-62 in these phosphopeptides was determined as the major phosphorylation site on CRE-BP1 by PKA. Ser-62 is located on the downstream of an arginyl residue (Arg-59), which agrees with the consensus sequence for the phosphorylation by PKA.[11] Ser-100 has been regarded as a potential phosphorylation site by PKA,[25] as the sequence of Lys-Lys-Ala-Ser at amino acids 97-100 of this protein is also consistent with the consensus sequence. However, no phosphopeptide corresponding this sequence was recovered. When the amino-terminal truncated form of CRE-BP1 lacking amino acids 1-94[25] was incubated with PKA, no obvious phosphorylation by PKA was

detected. Thus, Ser-62 but not Ser-100 is the phosphorylation site by PKA in CRE-BP1.

The phosphorylation sites of CRE-BP1 by PKC and PKA were determined in this study. PKC recognizes those located in the basic region that is a DNA-binding domain, whereas PKA phosphorylates the seryl residue near the putative metal finger structure, which is a transcriptional activation domain.[21] Thus, it seems possible that transcriptional and DNA-binding activities of CRE-BP1 are regulated by phosphorylation by these protein kinases. Further studies of the role of phosphorylation of CRE-BP1 will be necessary to understand the physiological functions of this protein.

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