Cell-cycle-regulated phosphorylation of cAMP response element-binding protein: identification of novel phosphorylation sites

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We report that the cAMP response element binding protein (CREB) undergoes cell-cycle-regulated phosphorylation. In human amnion FL cells, CREB was expressed as two forms with different molecular masses, 45 and 45.5 kDa. Although asynchronous cells contained predominantly the 45 kDa forms, this form shifted to 45.5 kDa when the cells were synchronized with the early S-phase. Furthermore the expression of the 45.5 kDa band was increased when cells were treated with okadaic acid, confirming that the 45.5 kDa band was a phosphorylated form of the 45 kDa band. Mutation analysis indicated that neither Ser¹³³, the target of cAMP-dependent protein kinase and calcium calmodulin kinase, nor Ser¹²⁹, the target of glycogen synthetase kinase 3, was responsible for the expression of the 45.5 kDa

band, but that Ser¹⁰⁸, Ser¹¹¹ and Ser¹¹⁴, located in a region matching the consensus sequence for the casein kinase II target, were required. A mutant in which Ser¹¹¹ and Ser¹¹⁴ were each replaced by a glutamic residue, mimicking a phosphorylated state, had a higher activation potential in cAMP response element-mediated transcription. These results strongly suggest that the casein kinase II target region is involved in cell cycle-regulated phosphorylation of the CREB protein and also in transcriptional enhancement.

Key words: casein kinase II, early S-phase, transcriptional enhancement.

INTRODUCTION

cAMP response element-binding protein (CREB) is a transcription factor that activates cAMP response element (CRE)-mediated transcriptions [1]. The activity of CREB is regulated by multiple kinases after various kinds of stimulation. The confirmed phosphorylation sites of CREB are Ser¹³³ and Ser¹²⁹, both of which have been shown to be required for stimulation-dependent transcriptional enhancement. Ser¹³³ is phosphorylated in response to an increase in intracellular cAMP concentration [2] and Ca²⁺ concentration [3] by cAMP-dependent protein kinase (PKA) and by calcium and calmodulin-dependent protein kinases, respectively. Ser¹²⁹ is phosphorylated by glycogen synthetase kinase 3 after the initial phosphorylation at Ser¹³³ [4].

CREB, however, seems to be phosphorylated without any kind of stimulation. It is reported that exogenously introduced CREB protein, as a fusion form of GAL4-CREB, is phosphorylated under unstimulated conditions in simian COS cells [5]. Furthermore the deletion of the pDE-1 domain, a heptadecapeptide sequence from Ala¹⁰⁶ to Gln¹²², resulted in a severe loss of [³²P]P_i uptake into the fusion protein [5]. The pDE-1 domain coincides with the consensus sequence for the target of casein kinase II (CKII). A synthetic peptide containing pDE-1 domain was shown to be phosphorylated at every serine residue by CKII *in vitro*; its involvement in transcriptional activation was suggested [5]. However, the biological significance and the precise target site *in vivo* of this 'stimulation-independent phosphorylation' remain unsolved.

We independently observed that the endogenous CREB protein is phosphorylated without any exogenous stimulation in human amnion FL cells (K. Saeki and A. Yuo, unpublished

work), suggesting that there exists some self-controlling mechanism that regulates a phosphorylation state of this protein. The cell cycle and its related events might be one such mechanism. In this context, the roles of protein phosphorylation in cell cycle progression are of interest.

Here we show that CREB protein is phosphorylated in a cell cycle-dependent manner and that the pDE-1 domain is required for this phosphorylation. We also discuss the possible involvement of the CREB protein in cell cycle progression.

EXPERIMENTAL

Plasmids, cells and transfection

The expression plasmids for CREB (pCG-CREB) and empty vector (pCG-UT) were prepared as described previously [6]. The expression plasmid for CKII α was generated by inserting its coding sequence, prepared by PCR with a 5' primer of ATGT-CGGGACCCGTGCCAAGCAGGGCCAGAGTT and a 3' primer of CTGCTGAGCGCCAGCGGCAGCTGGAACAGGCAT (GenBank accession number HSACKII) with HeLa cells first-strand DNA as template, into a pCG-expression vector (pCG-CKII). Human amnion FL cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY, U.S.A.) supplemented with 5% (v/v) fetal calf serum. Transfection into these cells was performed in 60 mm culture plates by the standard calcium phosphate method [7].

Antiserum

Antiserum against CREB was prepared as described previously

Abbreviations used: CREB, cAMP response element-binding protein; CRE, cAMP response element; CKII, casein kinase II; DMEM, Dulbecco's modified Eagle's medium.

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Figure 1 Detection of CREB protein in FL cells

(A) FL cells were transfected with 1 μ g of an empty vector (lanes 1 and 3) or CREB expression vector (lanes 2 and 4). After 21 h, cell lysate was prepared and immunoblotting was performed with anti-CREB antiserum. In lanes 3 and 4, antiserum had previously been incubated with synthetic CREB peptide before it was used for immunoblotting. (B) The lysate of empty-vector-transfected cells (lane 1) or CREB-expression vector-transfected cells (lane 2) was prepared and immunoblotted in a similar way to (A) but was exposed for a longer time. Abbreviation: kD, kDa.

[6]. In brief, rabbits were immunized by keyhole limpet haemocyanin-conjugated synthetic peptide (QNKTLEELKALKD-LYCHKSD). The titre of antiserum was evaluated by ELISA.

Immunoblotting and immunoprecipitation

After the cells had been washed with PBS to exclude the dead and floating populations, adherent FL cells were collected by trypsin treatment. Cells were then lysed with RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 % (v/v) Triton X-100, 1 % (w/v) SDS and 1 % deoxycholate and were sonicated on ice. For immunoblotting, the cell lysate (10 μ g of protein equivalent) was mixed with an equal volume of 2 $\times$ Laemmli's sample buffer then boiled, separated by SDS/PAGE [12.5 % (w/v) gel] (acrylamide-to-bisacrylamide ratio 40:0.8) and transferred to PVDF membrane (Millipore, Bedford, MA, U.S.A.). Blocking was performed with 5 % (w/v) skimmed milk-containing PBS for 30 min at room temperature. The first antibody reaction was performed with anti-CREB antiserum [diluted 1:200 in 5 % (w/v) skimmed milk/PBS] for 90 min at room temperature, and the second antibody reaction was with 1:4000-diluted horseradish peroxidase-conjugated protein A (Amersham International, Little Chalfont, Bucks., U.K.) for 30 min at room temperature. The detection was performed with ECL[®] Western blotting detection reagents (Amersham). For the absorption test, 10 μ l of anti-CREB antiserum was incubated with 1 μ g of synthetic CREB peptide for 60 min on ice. In the experiments shown in Figure 1, the second antibody reaction was performed by 1:2000-diluted alkaline phosphatase-conjugated anti-(rabbit IgG) (Promega, Madison, WI, U.S.A.), followed by a detection method described elsewhere [8]. There was no significant difference between the two second-antibody systems in their protein detection patterns (results not shown).

For immunoprecipitation, 300 μ l of cell lysate (600 μ g of protein equivalent) was mixed with 10 μ l of anti-CREB antiserum and rotated at 4 °C for 2 h. This was rotated for a further 1 h with 20 μ l of 50 % Protein A-Sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden). For immunoblotting, Protein A-Sepharose beads were washed three times with RIPA buffer and suspended in 20 μ l of 2 $\times$ Laemmli's buffer, boiled and separated on a 12.5 % (w/v) polyacrylamide gel. For alkaline phosphatase treatment, beads were washed with alkaline phosphatase buffer (Takara Shuzo Co., Shiga, Japan) and suspended in 150 μ l of the same buffer. We added 0.5 or 5 μ l of bacterial

alkaline phosphatase (Takara) to this suspension and incubated the mixture for 1 h at 37 °C.

Synchronization of cells

Cell synchronization was performed as described originally [9]. For arrest at early S-phase, cells were cultured at 37 °C in DMEM containing 1 mM hydroxyurea (Sigma, St. Louis, MO, U.S.A.). To release them from arrest, cells were washed with serum-free DMEM three times and cultured in DMEM containing 5 % (v/v) serum and 7.5 μ g/ml Hoechst 33342 fluorochrome (Calbiochem, La Jolla, CA, U.S.A.) to avoid progression beyond the G2 phase.

Oligonucleotide-directed mutagenesis

A series of CREB mutants was constructed by Kunkel's method [10]. The oligonucleotide used in mutant 1 was CTT TCA AGG AGG CCA gCC TAC AGG AAA ATT (mutated nucleotides are shown in lower-case letters), which hybridized to CTT TCA AGG AGG CCT TCC TAC AGG AAA ATT, giving replacement of Ser¹³³ by Ala along with the destruction of the underlined *StuI* site. The oligonucleotide used in mutant 2 was CAG TCT gCa TGc TTG GAC T, which hybridized to CAG TCT TCC TGT TTG GAC T, giving replacement of Ser⁸⁹ by Ala along with the generation of the underlined *SphI* site. The oligonucleotide used in mutant 3 was GAG TCA GTG GAa GcT tTA gCT GAT TCC CAG, which hybridized to GAG TCA GTG GAT AGT GTA ACT GAT TCC CAG, giving replacement of Ser¹¹⁷ by Ala, Thr¹¹⁹ by Ala, Asp¹¹⁶ by Glu and Val¹¹⁸ by Leu along with the generation of the underlined *HindIII* site. The oligonucleotide used in mutant 4 was AGG GAA ATT CTT gCt AGc AGG CCT TCC TAC, which hybridized to AGG GAA ATT CTT TCA AGG AGG CCT TCC TAC, giving replacement of Ser¹²⁹ by Ala along with the generation of the underlined *NheI* site. The oligonucleotide used in mutant 5 was T AGT GTA ACT GAT gCg CAA AAG CGA AGG GA, which hybridized to T AGT GTA ACT GAT TCC CAA AAG CGA AGG GA, giving replacement of Ser¹²¹ by Ala along with the generation of the underlined *FspI* site. The oligonucleotide used in mutant 6 was GAA AAT CAA AAC AAa gCt TTG ATT GAG GAG CTA, which hybridized to GAA AAT CAA AAC AAG ACA TTG ATT GAG GAG CTA, giving replacement of Thr 325 by Ala along with the generation of the underlined *HindIII* site. The

oligonucleotide used in mutant 7 was ATT GCA GAA gCt GAA GAT gCg CAG GAG gCA GTG GAT AGT, which hybridized to ATT GCA GAA AGT GAA GAT TCA CAG GAG TCA GTG GAT AGT, giving replacement of Ser¹⁰⁸, Ser¹¹¹ and Ser¹¹⁴ by Ala along with the generation of the underlined *FspI* site. The oligonucleotide used in mutant 8 was A CAG ATT TCA ACa ATT GCA GAA gCt GAA GAT TCA CA, which hybridized to A CAG ATT TCA ACT ATT GCA GAA AGT GAA GAT TCA CA, giving replacement of Ser¹⁰⁸ by Ala along with the generation of the underlined *MunI* site. The oligonucleotide used in mutant 9 was GAA AGT GAA GAT gCg CAG GAG TCA GTG, which hybridized to GAA AGT GAA GAT TCA CAG GAG TCA GTG, giving replacement of Ser¹¹¹ by Ala along with the generation of the underlined *FspI* site. The oligonucleotide used in mutant 10 was GAT TCA CAG GAG gCA GTc GAc AGT GTA ACT GAT, which hybridized to GAT TCA CAG GAG TCA GTG GAT AGT GTA ACT GAT, giving replacement of Ser¹¹⁴ by Ala along with the generation of the underlined *SaI* site. The oligonucleotide used in mutant 11 was GAA AGT GAA GAT gaA CAG GAG gaA GTc GAc AGT GTA ACT GAT, which hybridized to GAA AGT GAA GAT TCA CAG GAG TCA GTG GAT AGT GTA ACT GAT, giving replacement of Ser¹¹¹ and Ser¹¹⁴ by Glu along with the generation of the underlined *SaI* site. The oligonucleotide used in mutant 12 was GAA AGT GAA GAT gCg CAG GAG gCA GTG GAT AGT, which hybridized to GAA AGT GAA GAT TCA CAG GAG TCA GTG GAT AGT, giving replacement of Ser¹¹¹ and Ser¹¹⁴ by Ala along with the generation of the underlined *FspI* site. The mutated clones were selected by checking the cutting pattern of the restriction enzymes.

Luciferase assay

A CRE-carrying luciferase reporter plasmid, pCRE-TK-Luc, was constructed by inserting the synthetic 25 bp fragment (–56 to –32) of the rat somatostatin CRE [11] and the 156 bp fragment of *BamHI*–*Bgl*II (–105 to +51) of HSV-1 thymidine kinase promoter [12] upstream of the luciferase gene into the pGL3-basic vector (Promega, Madison, WI, U.S.A.). FL cells were transfected with 1 µg of reporter plasmid together with 0.1 µg of an expression vector for CREB or mutant and incubated at 37 °C. After 21 h, cell lysate was prepared and a luciferase assay was performed with the luciferase assay system (Promega). Luciferase activity was normalized by the content of total protein in each sample (relative light units/µg of protein).

RESULTS

CREB protein is expressed in two forms in FL cells

We performed immunoblotting to identify the expression of CREB protein in human amnion FL cells. In the lysate of the cells transfected with an empty vector, three bands (47, 45.5 and 45 kDa) were specifically detected by antiserum against CREB (Figure 1A, lane 1; Figure 1B, lane 1). The specificity was confirmed by the fact that preincubation of the antiserum with antigenic peptide blocked the detection of the three bands (Figure 1A, lane 3). To confirm the expression of CREB, we performed immunoblotting with the lysate of cells transfected with a CREB-expression vector. We found that the expression of the 45 and 45.5 kDa bands in particular was increased, in contrast with an insignificant alteration in the 47 kDa band (Figure 1A, lane 2). Preincubation of antiserum with antigenic peptide again blocked the expression of the 45 and 45.5 kDa bands (Figure 1A, lane 4), confirming that these two bands represented CREB protein. In accordance with this, the 45 and 45.5 kDa bands were the only

Figure 2 Cell-cycle-regulated expression of CREB protein

(A) Asynchronous FL cells (lane 1) or cells arrested in early S-phase by hydroxyurea treatment (lane 2) were lysed and immunoprecipitated with anti-CREB antiserum. The precipitates were immunoblotted with the same antiserum. (B) FL cells that had been arrested at early S-phase (lane 1) were released by intensive washing to remove hydroxyurea. After 3 h (lane 2) or 6 h (lane 3), cell lysate was prepared from adherent cells and immunoblotting was performed. Lane 4, cells detached from the stratum after 6 h from the release were collected. The lysate of the empty-vector-transfected cells (lane 5) or CREB-expression vector-transfected cells (lane 6) were used for comparison. In both panels the upper arrow indicates 45.5 kDa and the lower arrow 45 kDa.

Figure 3 The 45.5 kDa band is a phosphorylated form of the 45 kDa band

(A) The lysate of asynchronous cells (lane 1) or early-S-arrested FL cells (lane 2) was immunoprecipitated with anti-CREB antiserum; the precipitates were then immunoblotted with the same antiserum. The precipitated materials from early-S-arrested cells were further incubated for 30 min at 37 °C with buffer alone (lane 3) or with 0.5 or 5 µl of bacterial alkaline phosphatase (BAP) (lanes 4 and 5 respectively), and were then immunoblotted. (B) FL cells were transfected with CREB expression vector and incubated with (+) or without (lane 2) 0.1 mg/ml okadaic acid. After 21 h, cell lysate was prepared. The expression of CREB was examined by immunoprecipitation with anti-CREB antiserum followed by immunoblotting with the same antiserum. In both panels the upper arrow indicates 45.5 kDa and the lower arrow 45 kDa.

bands detected in an immunoprecipitation assay with anti-CREB antiserum (results not shown, and see Figures 2 and 3). Thus CREB protein is expressed in FL cells in two forms with different molecular masses: 45 and 45.5 kDa.

Cell-cycle-regulated expression of CREB protein

We found, while investigating the physiological basis of the regulation of the expression of the 45 and the 45.5 kDa bands, that CREB protein expression was controlled in a cell-cycle-dependent manner. As shown in Figure 2(A), the 45 kDa band

Table 1 Mutants of CREB

Amino acid substitutions are represented by, for example, S113A (replacement of Ser¹¹³ by Ala) and S111E (replacement of Ser¹¹¹ by Glu). The consensus column lists the candidate kinases responsible for the phosphorylation of each serine or threonine residue indicated, as suggested by proximal amino acid sequences ('consensus' sequence). Abbreviations: PKA, cAMP-dependent kinase; PKC, protein kinase C; GSK, glycogen synthetase kinase.

Mutant number	Site	Consensus	References
1	S133A	PKA, PKC	[19,20]
2	S89A	PKC	[20]
3	S117A, T119A	CKII	[13]
4	S129A	GSK	[4]
5	S121A	PKC	[20]
6	T325A	CKII	[13]
7	S108A, S111A, S114A	CKII	[13]
8	S108A	CKII	[13]
9	S111A	CKII	[13]
10	S114A	CKII	[13]
11	S111E, S114E	CKII	[13]

was detected predominantly in the lysate of asynchronous cells (lane 1), whereas most of the CREB protein was expressed in the 45.5 kDa form in cells treated with hydroxyurea, an early

S-phase-arresting agent (lane 2). This result indicates that the expression of CREB is strictly controlled in a cell-cycle-dependent manner, with a molecular shift from 45 to 45.5 kDa at early S-phase. After release from arrest, the expression of the 45.5 kDa band decreased gradually (Figure 2B, lanes 2 and 3). Interestingly, expression of the 45 kDa band did not increase either in a major adherent population (lanes 2 and 3) or in a minor stratum-detached population (lane 4), indicating that CREB expression is down-regulated with the progression through S-phase. Thus the expression pattern of CREB protein, including the ratio of its two forms and also its total amount, is strictly regulated in a cell-cycle-dependent manner.

The 45.5 kDa band is a phosphorylated form of the 45 kDa band

Next we investigated the molecular basis for the differences between the two forms of CREB protein. Because phosphorylation sometimes causes a mobility shift in SDS/PAGE, we hypothesized that the 45.5 kDa band might be a phosphorylated form of the 45 kDa band. The lysate of the early-S-arrested cells was immunoprecipitated with anti-CREB antiserum. The precipitant was further treated with alkaline phosphatase, and immunoblotting was performed. As shown in Figure 3(A), the mobility of the 45.5 kDa band was shifted to 45 kDa by treatment with phosphatase, suggesting that the former was a phosphorylated form of the latter. This was further supported by the

Figure 4 Mutation analysis for CREB protein isoforms

(A, B) FL cells were transfected with 1 μ g of an expression vector for the wild type or for various phosphorylation-deficient mutants of CREB (Table 1); mutants 1–7 (A) and mutants 8–10 (B). Immunoblotting was performed 16 h later. (C) FL cells were transfected with 1 μ g of an empty vector (lane 1), or a CREB expression vector (lane 2), or were co-transfected with CREB and CKII α expression vectors (lane 3). Immunoblotting was performed as in (A). In all panels the upper arrow indicates 45.5 kDa and the lower arrow 45 kDa.

Figure 5 Involvement of serine residues in the pDE-1 domain in transcription

FL cells were transfected with 1 μ g of a luciferase reporter carrying CRE enhancer together with 1 μ g of an empty vector (vector) or a wild-type (CREB), mutant 11 (Mut11) or mutant 7 (Mut 7) expression vector. The luciferase assay was performed after 21 h. The results of three independent experiments are shown. * Activity significantly higher than that of CREB ($P < 0.01$); ** Activity not significantly different from that of CREB ($P > 0.05$).

result that the relative expression of the 45.5 kDa band to that of the 45 kDa band was increased when cells were cultured in the presence of okadaic acid, a specific inhibitor of protein phosphatases 1 and 2A (Figure 3B). These results indicate that the 45.5 kDa band is a serine- or threonine-phosphorylated form of the 45 kDa band.

Ser¹⁰⁸, Ser¹¹¹, Ser¹¹⁴, Ser¹¹⁷ and Thr¹¹⁹ within the CKII consensus target region are involved in the expression of 45.5 kDa form

To determine which amino acid was involved in the expression of the 45.5 kDa form, a series of mutants was constructed in which the known or possible phosphorylation sites that matched the consensus sequences for protein kinases were mutated (Table 1). The expression vector for each mutant was transfected into FL cells and immunoblotting was performed. As shown in Figure 4(A), both the 45 and 45.5 kDa bands were clearly detected in cells transfected with mutant 1, mutant 2, mutant 4, mutant 5 or mutant 6 (lanes 3, 4, 6, 7 and 8), indicating that Ser⁸⁹, Ser¹²¹, Ser¹²⁹, Ser¹³³ and Thr³²⁵ are completely dispensable for the expression of the 45.5 kDa band. In contrast, the expression of the 45.5 kDa band was weak in mutant 3-transfected cells (lane 5). Surprisingly, the 45.5 kDa band was not detected in mutant 7-transfected cells (lane 9), indicating that Ser¹⁰⁸, Ser¹¹¹ and Ser¹¹⁴ are essential for 45.5 kDa band expression. These serine residues are located in a region called pDE-1, which coincides with the CKII target consensus sequence and was previously shown to be involved in the phosphorylation of CREB in simian COS cells [5]. To identify the phosphorylation site more precisely, Ser¹⁰⁸, Ser¹¹¹ and Ser¹¹⁴ were individually mutated (mutants 8, 9 and 10) and transfected into FL cells. As shown in Figure 4(B) the 45.5 kDa band was not detected in any of the mutant-

transfected cells, indicating that all three serine residues are indispensable for 45.5 kDa band expression.

We also examined the possible involvement of CKII in the phosphorylation of CREB. The CREB-expression vector was co-transfected into FL cells together with an expression vector for CKII α , a catalytic subunit of CKII. As shown in Figure 4(C), the co-existence of CKII α resulted in an enhanced expression of the 45.5 kDa band (compare lanes 3 and 2). Although the enhancement was not as great, this phenomenon was reproducible (results not shown). Thus CKII might be involved in the cell-cycle-regulated phosphorylation of CREB.

Increase in negative charges in the pDE-1 domain results in higher activation potential in CRE-mediated transcription

Because deletion of the pDE-1 domain resulted in a severe loss of transcriptional activation potential [5], we hypothesized that phosphorylation in this region might have an important role in transcriptional enhancement. A mutant was constructed in which Ser¹¹¹ and Ser¹¹⁴ were each replaced by a glutamic residue, mimicking a phosphorylated state (mutant 11). CRE-carrying luciferase reporter was co-transfected into FL cells together with an expression vector for the wild type, mutant 11 or mutant 7 in which Ser¹⁰⁸, Ser¹¹¹ and Ser¹¹⁴ were replaced by alanine, and a luciferase assay was performed. As shown in Figure 5, the luciferase activity of the mutant 11-transfected sample was significantly higher than that of the wild type ($P < 0.01$). In contrast there was no significance between the luciferase activities of the mutant 7-transfected sample and that of the wild type ($P > 0.05$). Because FL cells contain many potent activators for CRE-mediated transcription besides CREB, the relative luciferase activity in wild-type-transfected cells was rather low. Therefore the lack of inhibition in CRE-mediated transcription by mutant 7 was not incongruous. Thus phosphorylation in the pDE-1 domain is possibly involved in an effective transcriptional enhancement.

DISCUSSION

We have demonstrated that CREB protein is phosphorylated at early S-phase in FL cells, and that Ser¹⁰⁸, Ser¹¹¹ and Ser¹¹⁴ located in the pDE-1 domain are essential for this phosphorylation.

The pDE-1 domain is an acidic helical region of Ala¹⁰⁶-Glu-Ser-Glu-Asp-Ser-Gln-Glu-Ser-Val-Asp-Ser-Val-Thr-Glu-Ser-Gln¹²². Because Ser¹⁰⁸, Ser¹¹¹ and Ser¹¹⁴ all match the consensus phosphorylation site of CKII, which contains a cluster of acidic amino acids especially in the C-terminus-proximal region of serine or threonine [13], it is quite possible that CREB is phosphorylated by CKII at these serine residues at early S-phase. Our result in Figure 4(C), that the co-existence of CKII enhanced the expression of the 45.5 kDa band, an early S-phase-specific form of CREB, supports this idea. The pDE-1 domain contains two additional serine residues and one threonine residue, Ser¹¹⁷, Thr¹¹⁹ and Ser¹²¹. As we showed, Ser¹¹⁷ and Thr¹¹⁹ are not essential for cell-cycle-dependent phosphorylation, although they seem to be partly involved in it. Thr¹¹⁹ matches the consensus phosphorylation site of CKII and might possibly be phosphorylated *in vivo*. Although Ser¹¹⁷ does not match the consensus, the possibility of its phosphorylation might not be excluded because phosphorylation at Thr¹¹⁹ gives a negative charge, like Glu or Asp. In contrast, Ser¹²¹ does not match the consensus and might not be phosphorylated. Our result that mutant 5, in which

Ser¹²¹ was replaced by alanine, showed the same expression level of the 45.5 kDa band as the wild type supports this presumption. Although the transfection procedure provokes a non-physiological state and sometimes gives an inappropriate evaluation of the function of native protein, our mutant analysis would nevertheless provide useful information about the possible phosphorylation of CREB *in vivo*. The occurrence of the transfected protein's phosphorylation suggests the incidence of native CREB's phosphorylation under a condition in which kinases are highly active. However, if phosphorylation can never be observed even in over-expressed mutants, native CREB might not be phosphorylated in all circumstances at the mutated sites.

A time course study showed that CREB protein was scarcely detected at later S-phase, suggesting that the total amount of CREB protein is also regulated by cell-cycle dependence. Although the secondary effect of the agents used in this experiment should be considered carefully, the idea of cell-cycle-regulated induction of the CREB gene might be further supported by our preliminary observation that CREB was detected in half, but not all, of the population of FL cells by immunostaining (K. Saeki, unpublished work). The relationship between cell cycle progression and CREB gene induction is an interesting subject that remains to be investigated.

The functional importance of CKII in cell cycle progression and cell proliferation has been well documented. It is reported that CKII is required for G₀/G₁, early G₁ and the G₁/S transition [14]. It is also reported that anti-sense oligodeoxynucleotides against CKII inhibit the growth of cells [15]. However, the downstream targets of CKII have not been completely elucidated. The only candidate so far determined is p34cdc2, which was shown to be a substrate of CKII at G₁ phase [16]. Whether CREB might be another target of CKII *in vivo* is a matter of great interest.

The possible involvement of CREB protein in cell cycle progression was discussed earlier for human fibroblastic Hs27 cells [17]. The induction of the gene for cyclin A is reportedly stimulated by cAMP agonist via CRE sequences in its promoter region, and CREB is phosphorylated and activated concomitantly with cyclin A gene inducibility by cAMP. Thus CREB must be implicated in the G₁/S transition through Ser¹³³ phosphorylation. In contrast, we identified the phosphorylation at Ser¹⁰⁸, Ser¹¹¹ and Ser¹¹⁴ at early S-phase in human amnion FL cells. We further observed that an anti-sense oligonucleotide against CREB inhibited the proliferation of human haemopoietic cells with an accumulation of S-phase population (K. Saeki, A. Yuo, M. Koizumi, K. Fujiwara and K. Kaneko, unpublished work), suggesting that CREB might function in the progression through S-phase. The difference between the two observations might be due to the use of different cell lines. Further investigations are required for evaluating the contribution of CREB protein to cell cycle progression.

We showed that mutant 11, in which Ser¹¹¹ and Ser¹¹⁴ are each replaced by a glutamic residue, had a higher activation potential in CRE-mediated transcription. Because the replacement of serine by a glutamic residue mimics a phosphorylated state, pDE-1 phosphorylation would activate the function of CREB protein. A previous report showing that the deletion of pDE-1 resulted in a loss of basal and AMP-stimulated transcriptional enhancement [5] supports this idea.

CREB might function in diverse biological phenomena as a housekeeping gene. It is suggested that CREB knockout mice have a disadvantage in surviving to adulthood because the ratio of homozygotic mutant offspring is lower than expected [18]. It is thus of importance to re-evaluate the function of CREB from various points of view, including cellular growth, death and differentiation.

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