Aberrant expression of cAMP-response-element-binding protein ('CREB') induces apoptosis

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We have reported previously that cAMP-response-element-binding protein (CREB) was phosphorylated in a cell-cycle-dependent manner, showing that it was phosphorylated at early S-phase at casein kinase II target sites. To assess the possible involvement of CREB in cell cycle progression, CREB expression vector was transiently transfected into various cells. Unexpectedly we found that transfection with CREB expression vector resulted in an abundance of dead cells. Morphological examination revealed that these cells had undergone apoptosis. The coincidence of CREB overexpression and apoptosis induction at the individual cell level was confirmed by a immunohistochemical study. To confirm that overexpression of CREB was the cause of apoptosis, a dominant-negative mutant of CREB, KCREB, was co-expressed with the wild type. The co-existence of KCREB effectively rescued CREB-mediated apoptosis in a dose-dependent manner, verifying that apoptosis was truly a specific effect of overexpressed CREB and not an artifact of the transfection procedure. Deletion analysis indicates that neither the Q1 transactivation domain, which functions in transcription, nor the kinase-inducible domain, in which a cluster of various kinase targets exists, is necessary; however, the Q2 transactivation domain is required for the induction of apoptosis. A more precise study indicates that the four-residue stretch Glu-Glu-Ala-Ala at the most C-terminal region of the Q2 domain is especially important for the induction of apoptosis. Thus overexpressed CREB induces apoptosis by transmitting certain signals from the C-terminal portion of the Q2 domain. Possible roles of cell-cycle-regulated phosphorylation and also an elevation of the intracellular cAMP level in CREB-induced apoptosis are suggested.

Key words: casein kinase II, DNA affinity precipitation assay, phosphorylation, S-phase.

INTRODUCTION

cAMP-response-element-binding protein (CREB) regulates cAMP response element (CRE)-mediated transcriptions [1] through phosphorylation at Ser^{133} by cAMP-dependent protein kinase [2] or Ca^{2+}/calmodulin-dependent protein kinase [3]. Although the biological significance of CREB has been well explained in terminally differentiated tissues such as cerebrum [4,5] and retina [6], the involvement of CREB in cell proliferation is also suggested in T-lymphocytes [7] and melanoma cells [8,9].

As we have reported previously, CREB is phosphorylated at casein kinase II (CKII) target sites besides Ser^{133} [10]. We showed that three CKII target serines, Ser^{108}, Ser^{111} and Ser^{114}, were phosphorylated in early S-phase. This cell-cycle-dependent phosphorylation by CKII, which is known as a growth-promoting kinase, seems to imply the possible involvement of CREB in cell cycle progression and also in cell growth.

The progression of the cell cycle is strictly controlled by a cascade of phosphorylation-dependent protein signals. For example, the activities of the cyclin-dependent kinase family proteins such as cdc2 and cdk2, and the tumour suppresser gene products such as p53 and Rb, are stringently regulated by their specific phosphorylations. Moreover, overexpressions or aberrant expressions of these proteins often cause apoptosis through an impaired cell cycle progression, as with $p34^{cdc2}$ [11] and p53 [12]. To assess the biological significance of the cell-cycle-dependent phosphorylation of CREB, a CREB expression vector was transiently transfected into various cells and the effect of CREB overexpression on cell survival was examined.

We show that an overexpression of CREB results in the induction of apoptosis with its impaired cell-cycle-dependent phosphorylation. The mechanism of CREB-mediated apoptosis is discussed.

EXPERIMENTAL

Plasmids, cells, transfection and agents

The expression plasmid for CREB (pCG-CREB) was described previously [13]. Human amnion FL cells, simian COS-7 cells and Chinese hamster ovary cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, U.S.A.) supplemented with 5% (v/v) fetal calf serum. Transfection procedure was performed by the standard calcium phosphate method with approx. 3×10^5 cells in 60 mm culture plates with the glycerol shock procedure [20% (v/v) solution] for 3 min after 4 h from transfection [14]. 8-Bromo-cAMP (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in water, and both rolipram (Sigma) and vinpocetine (Sigma) were suspended in DMSO.

Antiserum, Western blotting and immunostaining

Anti-CREB antiserum was prepared as described previously [13]. In brief, rabbits were immunized by keyhole limpet haemocyanin-conjugated synthetic C-terminal CREB peptide (QNKTLIEELKALKDLYCHKSD). The titre of antiserum was evaluated by ELISA. Immunoblotting was performed as described previously [15]. To perform the immunostaining, adherent and detached FL cells were collected separately. The former cells

Abbreviations used: ATF, activating transcription factor; b-Zip, basic domain and leucine zipper; CHO, Chinese hamster ovary; CKII, casein kinase II; CRE, cAMP response element; CREB, cAMP-response-element-binding protein; PDE, phosphodiesterase.

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were collected by treatment with 1 mM EDTA containing PBS; the latter were collected by centrifugation of the culture supernatant. Each sample was fixed on slide glasses by using a cytospin apparatus (Cytospin2; Shandon, Pittsburgh, PA, U.S.A.). After refixing with a mixture of acetone/methanol (1:3) for 5 min at room temperature, samples were incubated with first antibody solution [anti-CREB antiserum (diluted 1:100) and fetal calf serum (diluted 1:200) in PBS] for 90 min at 37 °C. After they had been washed vigorously with PBS, samples were then incubated with second antibody solution [rhodamine-conjugated rabbit IgG (Chemicon International, Temecula, CA, U.S.A.) (diluted 1:50) and fetal calf serum (diluted 1:100) in PBS] for 60 min at 37 °C. Absorption of antiserum was performed as described previously [10].

Evaluation of apoptosis by DNA fragmentation and cell morphology

At 1 h after glycerol shock in the transfection procedure, the culture medium was exchanged to wash out the cells damaged by glycerol shock. After incubation for a further 24 h, both the adherent and detached cells were collected and mixed; low-molecular-mass DNA was extracted as described previously [16]. Marker 4 (Wako Pure Chemicals Industries, Osaka, Japan) was used as a DNA molecular marker. The nuclear morphology was examined by fluorescence light microscopy after incubation with H33342 (Calbiochem, La Jolla, CA, U.S.A.) as described previously [16]. For electron microscopic study, detached cells were directly fixed with 2% (v/v) formaldehyde/2.5% (v/v) glutaraldehyde, and the ultrastructure was examined as described [17].

Preparation of CREB mutants

A dominant-negative mutant of CREB, KCREB, was constructed by oligonucleotide-directed mutagenesis as described previously [10]. KCREB has a replacement of Arg^{301} by Leu^{301} [18]. We constructed a KCREB expression vector from a CREB expression vector by replacing the 1037th residue (guanine) in CREB cDNA by cytosine, causing a change of CGT to CCT in the 301st codon. The sequence of the oligonucleotide probe used was 5′-AGGGAAGCAGCTCGTGAGTGTCTTAGAAAGA-AGAAA-3′, in which the two mutation sites are underlined. The first T did not cause any amino acid mutation because both CGA and CGT encode Arg. However, it destroyed a restriction enzyme *Xho*I recognition site in CTCGAG. We could therefore easily select the correctly mutated clone by checking the cutting pattern by *Xho*I (Takara Shuzo Co., Shiga, Japan). The second T caused the replacement of Arg^{301} by Leu^{301}. A series of deletion mutants of CREB were constructed by using the Erase-a-base system (Promega, Madison, WI, U.S.A.). The nucleotide sequence of each mutant was checked with a ABI PRISMTM 377 Genetic Analyzer (Perkin-Elmer, Norwalk, CT, U.S.A.) by the dye terminator method.

DNA affinity precipitation assay

A DNA affinity precipitation assay was performed as described [19], with minor modifications. At 20 h after transfection, cells were collected and washed with PBS. They were suspended in 400 μl of hypotonic buffer containing 10 mM Tris/HCl, pH 7.5, and 2 mM $MgCl_2$, then placed on ice for 15 min. Cells were then homogenized with Dounce's homogenizer. The nuclear pellets were collected by centrifugation at 800 ***g***, washed twice with hypotonic buffer and suspended in 100 μl of binding buffer containing 10 mM Hepes/KOH, pH 7.8, 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$ and 10% (v/v) glycerol. After mild sonication, insoluble material was removed by centrifugation at 15000 ***g*** and the supernatant was used as nuclear lysate. A 20 μl sample of the nuclear lysate was mixed with 2 × Laemmli's sample buffer, boiled and used for Western blotting as an input fraction. The residual lysate was used for the DNA precipitation assay. The 36 bp CRE probe [20] was prepared by annealing the biotin-treated sense oligonucleotide (5′-CTC GGG GCG CCT CCT TGG CTG ACG TCA GAG AGA GAG-3′) and non-biotinated anti-sense oligonucleotide (5′-CTC TCT CTC TGA CGT CAG CCA AGG AGG CGC CCC CAG-3′). A 3 pmol sample of the probe was added to 80 μl of the nuclear lysate and placed on ice for 40 min. Then 20 μl of streptavidin-conjugated magnetic beads (Promega) was added, and the sample was incubated for a further 30 min at 4 °C, with rotation. After separation of the magnetic beads from the supernatant by centrifugation, the beads were washed five times with binding buffer, suspended with 40 ml of sample buffer, boiled and used for Western blotting as a DNA-bound fraction. The supernatant was mixed with an equal volume of 2 × sample buffer, boiled and used for Western blotting as an unbound fraction. For competition, a 10-fold excess of unlabelled probe was added to the nuclear lysate and incubated on ice for 30 min before addition of the labelled probe.

Synchronization of the cells after transfection

Synchronization of cells in early S-phase was performed after transfection by hydroxyurea treatment as described previously [10]. FL cells were transfected with an empty vector or an expression vector for CREB with a glycerol shock procedure at 4 h after transfection. After incubation for a further 2 h at 37 °C in Dulbecco's modified Eagle's medium medium, 1 mM hydroxyurea (Sigma) was added to the culture medium. The cells were incubated for a further for 40 h at 37 °C to be arrested at early S-phase.

RESULTS

Overexpression of CREB induces apoptosis

In the course of transient transfection experiments with human amnion FL cells, we found that transfection with CREB expression vector resulted in the emergence of a larger number of

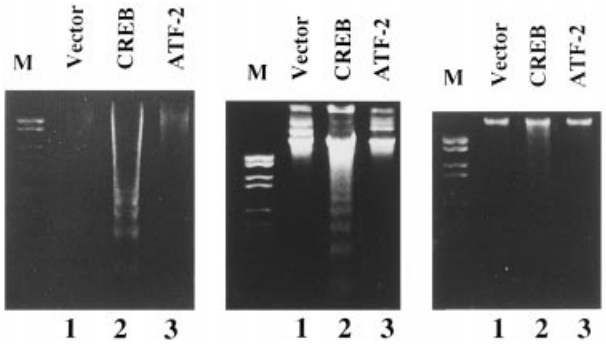

Figure 1 Transfection of CREB expression vector causes apoptosis in various cells

A 0.5 μg sample of an empty vector or an expression vector for CREB or ATF-2 was transfected into FL cells (left), COS-7 cells (middle) and CHO cells (right). After 24 h, low-molecular-mass DNA was extracted from the total cells, including the adherent and detached population. Lane M, DNA molecular marker.

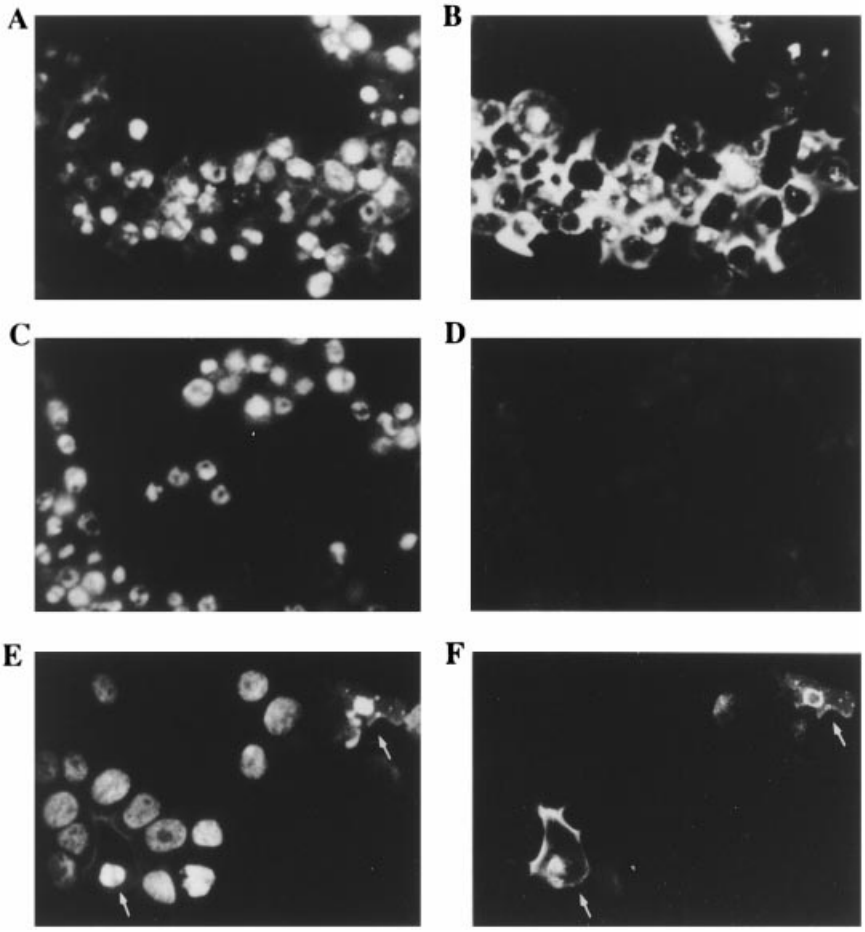

Figure 2 Cells overexpressing CREB specifically undergo apoptosis

A 0.5 μg sample of a CREB expression vector was transfected into FL cells. At 24 h after glycerol shock, stratum-detached cells (**A**–**D**) or adherent cells (**E**, **F**) were collected separately [(**A**) and (**B**) were taken from the same microscopic visual field, as were (**C**) and (**D**)]. Cells were stained with H33342 fluorochrome to examine nuclear morphology (**A**, **C**, **E**) or immunostained with anti-CREB antiserum (**B**, **F**). In (**D**), antiserum had previously been incubated with synthetic CREB peptides. Arrows in (**E**) and (**F**) indicate apoptotic cells with nuclear condensation and fragmentation.

detached cells than in controls. When 3×10^5 FL cells were transfected with 0.5 μg of an empty vector, CREB expression vector or an expression vector for activating transcription factor 2 (ATF-2), one of the CREB/ATF family of proteins, the mean percentages of detached cells were 0.07%, 5.7% and 0.08% respectively. As the transfection efficiency was 5–6%, it seems that the transfection-mediated CREB-overexpressing cells had selectively detached from the stratum. Because of the shrunken morphology of these detached cells (results not shown; see also Figure 2), we hypothesized that the overexpression of CREB induces apoptosis. To examine this possibility, we first checked the existence of fragmented DNA in the CREB expression-vector-transfected sample. The low-molecular-mass DNA was extracted from the mixture of the adherent and detached cells after transfection. The fragmented DNA was only detectable in a CREB-transfected sample but not in samples transfected with empty vector or ATF-2 expression vector (Figure 1, left panel). Similar results were also obtained in simian COS-7 and CHO cells (Figure 1, middle and right panels respectively), indicating that apoptosis was specifically induced by transfection with CREB expression vector. To confirm the coincidence of CREB overexpression and the induction of apoptosis in individual cells, cells were doubly stained with anti-CREB antiserum and nuclear stain agent. Most of the stratum-detached cells, all of which had pyknotic or condensed nuclei characteristic of apoptosis (Figures 2A and 2C), was densely stained by anti-CREB antiserum (Figure 2B); this stain was effectively blocked by synthetic CREB peptide (Figure 2D). The electron microscopic examination proved that these cells underwent apoptosis, with condensation of chromatin and the formation of apoptotic bodies (Figure 3). There were indeed a small number of apoptotic-featured cells in the adherent population (Figure 2E, arrows). Immunostaining with anti-CREB antiserum ensured that these cells, but not the other viable cells, actually expressed a high level of CREB (Figure 2F, arrows). This finding not only confirms the co-incidence of CREB overexpression and the induction of apoptosis at an individual cell level but also suggests that cells over-expressing CREB first underwent apoptosis and that the loss of adhesiveness was a subsequent phenomenon.

To confirm that the overexpression of CREB was indeed the cause of apoptosis (i.e. to exclude the possibility that the apoptosis was an artifact of the transfection procedure), an expression vector for a dominant-negative mutant of CREB, KCREB, was co-transfected with the wild-type. This mutant has a defective

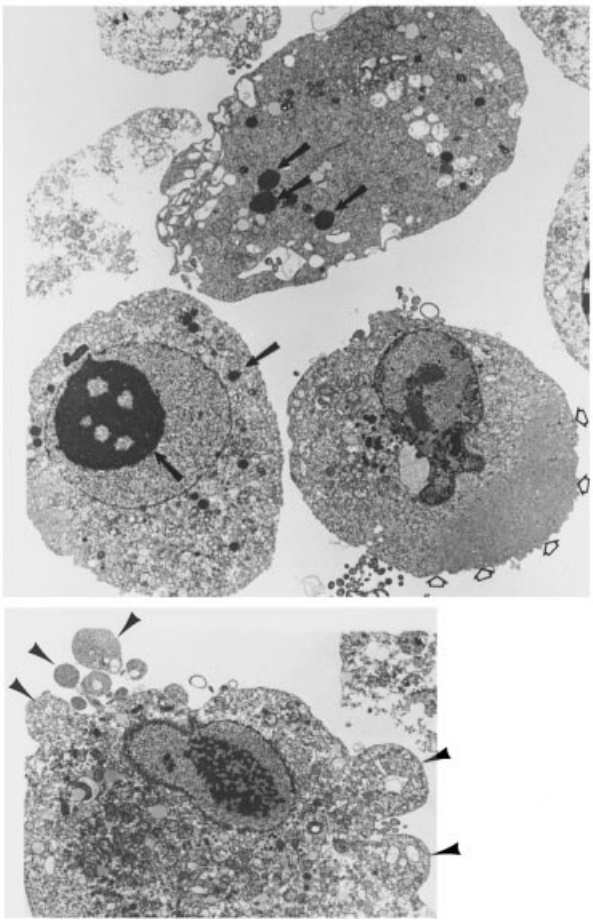

Figure 3 Ultrastructure of detached cells in a sample transfected with CREB

Electron micrograph of detached cells collected from a sample transfected with CREB expression vector. The condensation or fragmentation of nuclei (filled arrows), the condensation of cytoplasm (open arrows) and the formation of apoptotic bodies (arrowheads) are shown.

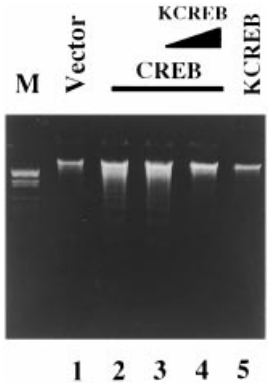

Figure 4 Co-expression of a dominant-negative mutant, KCREB, effectively inhibits CREB-mediated apoptosis

FL cells were transfected with 0.2 μg of an empty vector (lane 1), 0.2 μg of a CREB expression vector (lanes 2–4), 0.2 μg of a KCREB expression vector (lane 5) or an increasing amount of KCREB expression vector (lane 3, 0.2 μg; lane 4, 6 μg). The low-molecular-mass DNA was extracted as described in the legend to Figure 1. The total amount of transfected plasmids was adjusted with an empty vector. Lane M, DNA molecular marker.

DNA-binding activity but retains a normal dimerization activity, so its co-existence outcompetes the wild type for DNA and thus exhibits its dominant negativity [20]. As shown in Figure 4, co-expression of KCREB effectively suppressed CREB-mediated apoptosis in a dose-dependent manner (lanes 2–4), confirming that the observed apoptosis was indeed caused by the over-expressed CREB. We also confirmed that transfection of KCREB-expression vector alone did not cause apoptosis (Figure 4, lane 5). These findings also imply that DNA-binding activity is required for the induction of apoptosis. Thus overexpressed CREB binds to DNA and induces apoptosis.

Specific amino acid residues are important for the induction of apoptosis

To elucidate the mechanism of CREB-mediated apoptosis, we performed a deletion analysis. Because DNA-binding activity, which locates at the C-terminal basic and leucine zipper domain (b-Zip), is required for apoptosis induction, as shown by KCREB, a series of N-terminal deletion mutants and one internal deletion mutant were constructed (Figure 5A) and their expression vectors were transfected into FL cells. Deletion of the Q1 domain, which functions in transcriptional enhancement, and the kinase-inducible domain ('KID'), in which is located a cluster of phosphorylation sites including the cell-cycle-dependent phosphorylation sites of Ser^{108}, Ser^{111} and Ser^{114} and also the conventional cAMP-dependent phosphorylation site of Ser^{133}, did not eliminate apoptosis inducibility (Figure 5B, lane 3), indicating that these two domains are dispensable for apoptosis induction. In contrast, deletion of the Q2 transactivation domain entirely eliminated apoptosis inducibility (Figure 5B, lane 10), indicating that the Q2 domain is indispensable for apoptosis. For further identification of the minimum essential sequence for apoptosis induction, the Q2 domain was deleted stepwise from its N-terminus (Figure 5A, #176 to #b-Zip), and the apoptosis-inducing activity of these deletion mutants was evaluated by DNA fragmentation assay (Figure 5B, lanes 3–10). Deletion to the 280th residue (mutant #280) did not abolish apoptosis inducibility (Figure 5B, lanes 5–8), whereas deletion to the 284th residue (mutant #b-Zip) completely eliminated apoptosis inducibility (Figure 5B, lane 9), suggesting that the four-residue stretch Glu-Glu-Ala-Ala is critical for apoptosis induction. The protein expression of each mutant was clearly detected by Western blotting including mutants #b-Zip and d-Q2 (Figure 5C), which excludes the possibility that the loss of apoptosis inducibility in mutant #b-Zip or d-Q2 was due to the lack of protein expression. To clarify whether or not the loss of apoptosis inducibility in mutant #b-Zip resulted from the loss of DNA-binding activity, we performed a DNA affinity precipitation assay with a CRE probe. As shown in Figure 6, mutants #280 and #b-Zip as well as the wild type were detected in the DNA-bound fraction (Figure 6A, lanes 6–8) and not at all in unbound fractions, where only the background protein bands were detected (Figure 6A, lanes 10–12). Furthermore, the binding of mutants #280 and #b-Zip with probe DNA was competed for effectively by an excess of unlabelled DNA (Figure 6B, lanes 5 and 7). These results indicate that mutant #b-Zip has sufficient DNA-binding activity, which excludes the possibility that the loss of apoptosis inducibility was due to the lack of DNA-binding activity. Thus an excess of DNA-bound CREB induces apoptosis and the specific residues Glu-Glu-Ala-Ala are important for apoptosis induction.

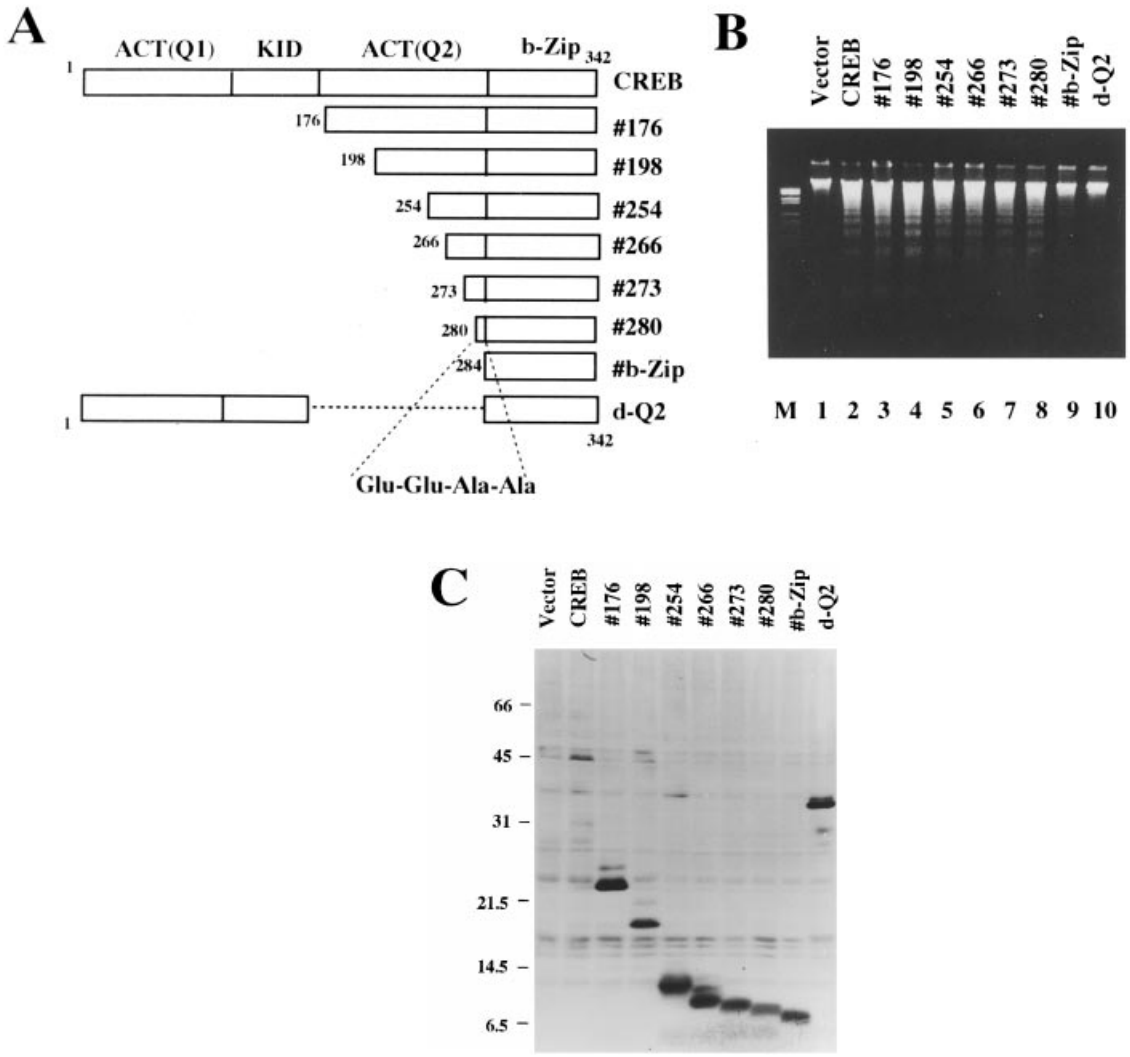

Figure 5 Deletion analysis of CREB in apoptosis induction

(**A**) The structures of a series of deletion mutants of CREB. (**B**) FL cells were transfected with 0.5 μg of an empty vector (lane 1) or an expression vector for each indicated mutant (lanes 2–10). The low molecular mass DNA was extracted as described in the legend to Figure 1. (**C**) Transfection was performed as in (**B**). After 24 h, Western blotting was performed with anti-CREB antiserum with the use of 15% (w/v) polyacrylamide gel. The positions of molecular mass markers are indicated (in kDa) at the left.

Normal cell-cycle-dependent phosphorylation of CREB is disturbed by transfection with CREB expression vector

As we have reported previously, CREB is phosphorylated in early S-phase at CKII target sites [10]. Because CKII functions in cell cycle progression in various situations [21,22] and is required for cell growth [23], the question of whether phosphorylation at these CREB sites is accurately executed in a CREB-transfected sample is a matter of interest. Because the phosphorylated form can easily be distinguished from the unphosphorylated form by a shift in apparent molecular mass from 45 to 45.5 kDa [10], we examined the phosphorylation level of CREB by Western blotting. Although the phosphorylation level of endogenous CREB was drastically changed at early S-phase with an electrophoretic mobility shift from 45 to 45.5 kDa in cells transfected with empty vector (Figure 7, lanes 1 and 2) [10]; the alternation in phosphorylation was impaired in cells transfected with CREB expression vector with an inappropriate increment of the unphosphorylated form at early S-phase (Figure 7, lanes 3 and 4). Thus the normal cell-cycle-dependent phosphorylation of CREB is disturbed by overexpression.

The involvement of an intracellular elevation of cAMP levels in the induction of apoptosis

To evaluate the physiological relevance of CREB in apoptosis induction, the effect of intracellular cAMP-elevating agents on the induction of apoptosis was examined. A non-hydrolysable cAMP analogue of 8-bromo-cAMP, an adenylate cyclase activator of forskolin, a specific inhibitor of phosphodiesterase 4 (PDE4) of rolipram and a specific inhibitor of PDE1 of vinpocetine were added to FL, COS-7 and CHO cells, and a DNA fragmentation assay was performed (Figure 8). DNA fragmentation was not induced in FL or COS-7 cells by these agents (Figure 8, left and middle panels respectively), indicating that cAMP-mediated signals are not involved in apoptosis induction in these cells. In CHO cells, however, 8-bromo-cAMP and forskolin did induce apoptosis (Figure 8, right panel, lanes

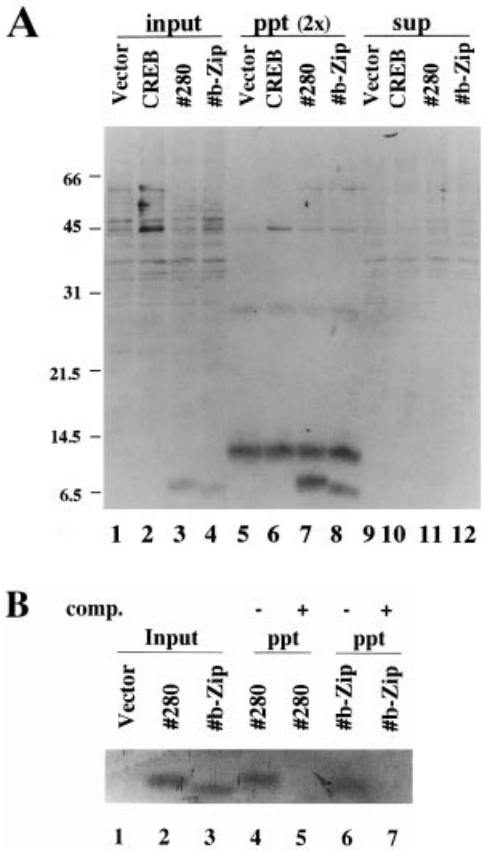

Figure 6 Mutant #b-Zip retains DNA-binding activity

(**A**) FL cells were transfected with 0.5 μg of an empty vector (lanes 1, 5 and 9) or an expression vector for CREB (lanes 2, 6 and 10), mutant #280 (lanes 3, 7 and 11) or mutant #b-Zip (lanes 4, 8 and 12). After 24 h, nuclear lysates were prepared and used either for Western blotting (lanes 1–4) or DNA affinity precipitation assay with CRE probe (lanes 5–12). The DNA-bound fractions (ppt) (lanes 5–8) and unbound fractions (sup) (lanes 9–12) were denatured with Laemmli's sample buffer and used for Western blotting. Crude lysates and the unbound fractions were applied in equal volumes and the bound fractions in double the volume. The broad band at approx. 13 kDa (lanes 5–8) was derived from magnetic beads. The positions of molecular mass markers are indicated (in kDa) at the left. (**B**) Nuclear lysate of cells transfected with empty vector (lane 1), mutant #280 expression vector (lanes 2, 4 and 6) or mutant #b-Zip expression vector (lanes 3, 5 and 7) was used in a DNA affinity precipitation assay with (lanes 5 and 7) or without (lanes 4 and 6) an excess of unlabelled probe (comp.).

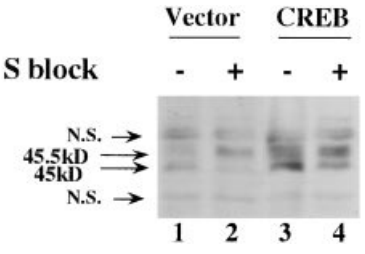

Figure 7 Impaired cell-cycle-dependent phosphorylation in a sample transfected with CREB expression vector

FL cells were transfected with 0.5 μg of empty vector or CREB expression vector. At 2 hours after glycerol shock, cells were treated with 1 mM hydroxyurea to arrest at early S-phase (S block; lanes 2 and 4). After incubation for a further 40 h, cell lysate was prepared and Western blotting was performed. Samples not treated with hydroxyurea were also cultured in the same way (lanes 1 and 3). The upper (45.5 kDa) and lower (45 kDa) arrows indicate the expression of the phosphorylated and unphosphorylated forms of CREB respectively. Abbreviation: N. S., non-specific bands.

3 and 5), indicating the involvement of cAMP-mediated signals, some of which could cause the activation of CREB, in apoptosis induction. The lack of apoptosis induction by PDE1 and PDE4 inhibitors suggests that other phosphodiesterases might mainly catalyse the breakdown of intracellular cAMP in CHO cells. Thus the effect of an increase in intracellular cAMP levels on cell viability differs with the cell type, despite the similar effects of overexpressed CREB, suggesting that the upstream mechanism of CREB-induced apoptosis does not depend totally on the intracellular level of cAMP and that distinct signal transduction pathways are involved in the CREB-mediated induction of apoptosis depending on the type of cell.

DISCUSSION

We have demonstrated that an overexpression of CREB results in apoptosis in various cell lines. This is, to our knowledge, the first observation that CREB might induce apoptosis. A protein-overexpressing condition by transfection is not a physiological state and sometimes gives a misleading result through competition between the exogenous proteins and their endogenous counterparts. In our system, however, apoptosis induction by CREB overexpression is not a result of this kind of competition, because inhibition of the endogenous CREB function by an overexpression of a dominant-negative mutant, KCREB, did not result in apoptosis; instead it suppressed the apoptosis caused by overexpressed CREB. This suppressive effect of KCREB also indicates that DNA-binding activity is required for apoptosis induction. It therefore seems highly possible that overexpressed, DNA-bound CREB had a certain function that would result in apoptosis.

It is often observed that an enforced expression of cell-cycle-regulating proteins induces apoptosis as a result of impaired cell cycle progression, as with $p34^{cdc2}$ or p53 [11,12]. If CREB has roles in cell cycle progression, its aberrant expression or overexpression would result in apoptosis. We have reported previously that CREB expression is strictly controlled in a cell-cycle-dependent manner, with specific phosphorylations at early S-phase at the CKII target site [10]. As shown in Figure 7, the cell-cycle-dependent phosphorylation of CREB was severely impaired in a CREB-transfected sample with an inappropriate increment of an unphosphorylated form during S-phase. It seems that the overexpression of CREB might cause apoptosis by an altered or impaired cell cycle progression.

The molecular mechanism of CREB-mediated apoptosis requires elucidation. As we showed in a mutation analysis, the four-residue stretch Glu-Glu-Ala-Ala is important for apoptosis induction. It is worth noticing that this region corresponds to the site that is important for the binding of CREB with Tax, a viral oncoprotein of human T cell leukaemia virus I [24,25]. Interestingly, Tax is known to be a mediator of apoptosis [26]. We speculate that overexpressed, DNA-bound CREB might incorporate a certain factor adjacent to chromosomal DNA; the apoptosis-inducing signals might then be transmitted. We have found that a 50 kDa nuclear protein specifically binds to synthetic Glu-Glu-Ala-Ala peptide (K. Saeki, unpublished work). We are now trying to isolate and the identify this factor. Whether this protein does indeed mediate apoptosis-inducing signals, as a cellular homologue of Tax, is a matter of great interest.

The physiological relevance of CREB in apoptosis induction remains to be evaluated. As we showed, the effect of an increase in intracellular cAMP levels differs greatly between the cell types. We could not detect any sign of apoptosis in FL or COS cells but apoptosis was potently induced in CHO cells, as reported by others [27], suggesting the involvement of CREB in apoptosis

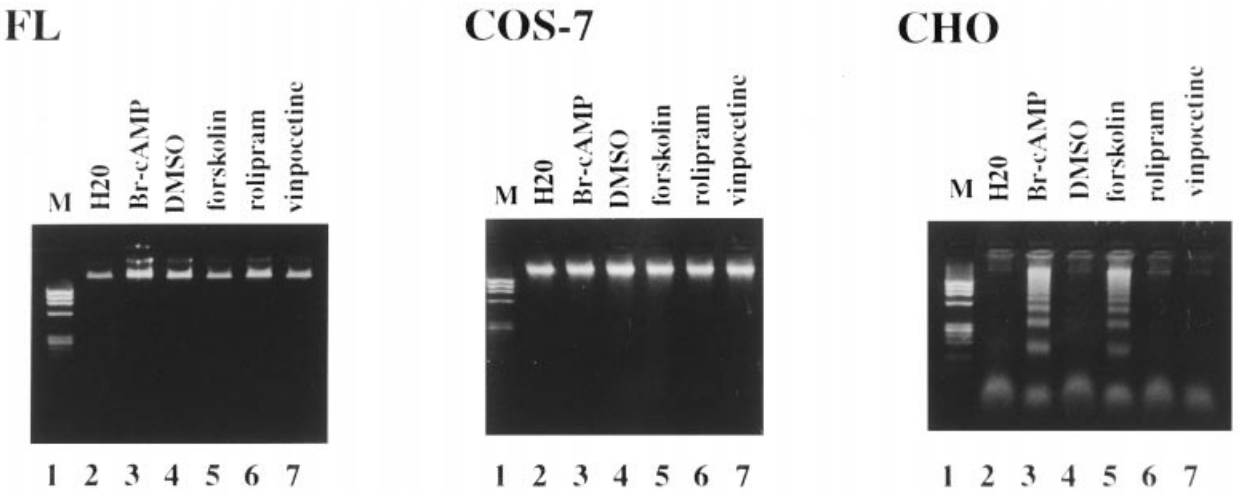

Figure 8 Effect of an increase in intracellular cAMP levels on apoptosis induction

FL (left), COS-7 (middle) and CHO (right) cells were treated with water (H20, lanes 2), 1 mM 8-bromo-cAMP (Br-cAMP) (lanes 3), 0.1% (v/v) DMSO (lane 4), 3 μM forskolin (lane 4), 100 μM rolipram (lane 6) and 30 μM vinpocetine (lane 7). After 32 h, cytoplasmic DNA was extracted. Lane 1, DNA molecular marker (M).

induction. Interestingly, the effects of an increase in intracellular cAMP levels on cell viability reportedly differ between an induction of apoptosis in some cells [28–30] and an inhibition of apoptosis in other cells [31–33]. Similarly, different results have been reported for the effects of CREB on cell survival. The involvement of CREB in cell survival has been shown in some cases [8,9,34], whereas its involvement in apoptosis induction has also been suggested [30]. Despite the diverse effects of an increase in intracellular cAMP levels and the expression of CREB on cell death, it nevertheless seems that cAMP-mediated signals and also the activation of CREB severely affect the viability of the cells. In addition to a cAMP-related pathway that might be linked to cAMP-dependent protein kinase, CKII might also be significant in the CREB-mediated control of cell viability. Further investigations are required for the precise evaluation of the biological significance of CREB in cellular apoptosis.

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