

# The cAMP response element binding protein-2 (CREB-2) can interact with the C/EBP-homologous protein (CHOP)

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**Abstract** cAMP response element binding protein-2 (CREB-2) is a basic leucine zipper (bZIP) factor that was originally described as a repressor of CRE-dependent transcription but that can also act as a transcriptional activator. Moreover, CREB-2 is able to function in association with the viral Tax protein as an activator of the human T-cell leukemia virus type I (HTLV-I) promoter. Here we show that CREB-2 is able to interact with C/EBP-homologous protein (CHOP), a bZIP transcription factor known to inhibit CAAT/enhancer-dependent transcription. Co-transfection of CHOP with CREB-2 results in decreased activation driven by the cellular CRE motif or the HTLV-I proximal Tax-responsive element, confirming that CREB-2 and CHOP can interact with each other in vivo. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Basic leucine zipper factor; cAMP response element binding protein-2; C/EBP-homologous protein; cAMP response element site; Human T-cell leukemia virus type I

## 1. Introduction

The basic leucine zipper (bZIP) transcription factors play an important role in regulating the expression of various genes. This group of transcription factors contain a leucine zipper as a common motif required for protein dimerization. Homo- or heterodimerization is a prerequisite for specific DNA binding. DNA binding specificity is determined by a DNA interaction surface being provided by a basic region of about 20 amino acids that precedes the leucine zipper. The different members were originally classified into families

by their ability to bind specific sites. For instance, the members of the activating transcription factor/cAMP response element binding protein (ATF/CREB) family specifically bind to the palindromic cAMP response element (CRE) TGACGTCA. Thus, CREB-2 has been isolated from a Jurkat T-cell cDNA library by using as a probe the human T-cell receptor  $\alpha$  enhancer CRE motif [1] or the related CRE site TGACGTCT corresponding to the distal human T-cell leukemia virus type I (HTLV-I) Tax-responsive element (TxRE) [2].

CREB-2, also called ATF-4 or TAXREB-67 [2,3], together with the mouse mATF-4, mTR67, C/ATF, and the *Aplysia* ApCREB-2, represents a subfamily of the ATF/CREB proteins [4–6]. Although CREB-2 was originally described as a repressor of CRE-dependent transcription [1,7,8], it contains a constitutive activation domain of transcription [6,9,10] and can directly interact with the transcriptional coactivator CREB binding protein [6,10]. We and others have demonstrated that CREB-2 cooperates with the viral Tax protein to enhance the transcription of the HTLV-I promoter [11,12], Tax enhancing the binding of CREB-2 to the three TxREs [10] present in the HTLV-I long terminal repeat (LTR).

CREB-2 is known to form heterodimers in vitro with other bZIP transcription factors such as members of the activator protein-1 (AP-1) [13,14] and CCAAT/enhancer-binding protein (C/EBP) [4,15] families. To characterize other bZIP factors that can interact with CREB-2, we performed a *Saccharomyces cerevisiae* two-hybrid approach using a cDNA library previously synthesized by ourselves [12] from mRNA of the MT2 cell line, a T-cell line infected by HTLV-I. By this approach, we isolated three independent cDNAs encoding the C/EBP-homologous protein (CHOP) that is also known as GADD153 [16,17]. In this study, we demonstrate that CREB-2 directly interacts with CHOP, CREB-2 and CHOP associating via their bZIP domains. In addition, we show by cotransfection assays in CEM cells that CHOP is able to down-regulate CREB-2-dependent transcription confirming the interaction of CHOP with CREB-2 in vivo.

## 2. Materials and methods

### 2.1. Plasmids

For interaction experiments with yeast, cDNA encoding the CREB-2 bZIP domain [10] was ligated as an *EcoRI-SalI* fragment into the LEXA DNA binding domain of the pBTM116 vector [18] to give pLEXA-CREB-2 bZIP. The wild-type CHOP and the truncated mutant were cloned in frame with the GAL4 activation domain of

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**Abbreviations:** CREB, cAMP response element binding protein; ATF, activating transcription factor; bZIP, basic leucine zipper; HTLV-I, human T-cell leukemia virus type I; C/EBP, CCAAT/enhancer binding protein; CHOP, C/EBP-homologous protein; GADD, growth arrest- and DNA damage-inducible protein; TxRE, Tax-responsive element

pGAD424 (Clontech). CHOP cDNA was generated by PCR amplification on the vector pCMV-Neo-CHOP, digested by *EcoRI* and subcloned into pGAD424 to give pGAD-CHOP. The CHOP deletion mutant without the leucine zipper (pGAD-CHOP  $\Delta$ ZIP) was obtained by digesting pGAD-CHOP by *SmaI* and the resulted digest was religated. The eukaryotic vectors pCI-CREB-2, pSG-Tax, pBg312HIV-1Lai-Tat, and pCMV-Neo-CHOP expressing CREB-2, Tax, Tat, and CHOP, respectively, have been described elsewhere [10,18,19]. The plasmid pCMV-Neo-CHOP  $\Delta$ ZIP, which contains the entire coding sequence of CHOP except for its ZIP domain, was constructed by digesting pCMV-Neo-CHOP with *NheI*; the resulting digest was treated with Klenow and religated as blunt ends. For the assays with the GAL4 binding site promoter-reporter plasmid, the vector containing the CREB-2 C-terminal domain (amino acids 263–351) fused in frame with the DNA binding domain of GAL4 (cloned into pBIND vector, Promega) has already been described [10].

## 2.2. Screening for proteins that interact with the CREB-2 bZIP domain

MT2 cDNA fused to the GAL4 activation domain of the pGAD10 vector [12] was screened using the CREB-2 bZIP domain as bait fused to the LEXA DNA binding domain of the pBTM116 vector. The two-hybrid screen was performed as already described [12]. Briefly, pLEXA-CREB-2 bZIP and the fusion cDNA library were cotransformed into the L40 *S. cerevisiae* strain by the lithium acetate method [20]. The L40 yeast strain possesses the *His* synthase gene (*His3*) and the *lacZ* gene under the control of LEXA binding sites. From approximately  $9 \times 10^6$  clones screened, we selected robust colonies growing on agar medium lacking Trp, Leu and His, for those that contained both types of plasmids (*Leu*<sup>+</sup> and *Trp*<sup>+</sup>) and that also expressed interacting hybrid proteins (*His*<sup>+</sup>). Selected transformants were assayed for the expression of *lacZ* by the  $\beta$ -galactosidase filter assay as described in the Clontech protocol. Plasmid DNA of the clones that were strongly positive for  $\beta$ -galactosidase activity was extracted, analyzed by digestion with restriction enzymes and sequenced. The  $\beta$ -galactosidase assay with *O*-nitrophenyl- $\beta$ -D-galactoside (ONPG) as substrate was carried out on three independent colonies per transformation as described in the Clontech protocol. The  $\beta$ -galactosidase activity was calculated in Miller units [21].

## 2.3. Glutathione S-transferase (GST) pull-down assay

CREB-2 cDNA cloned into pCI-neo [12] was transcribed and translated in the presence of [<sup>35</sup>S]methionine using the TNT T7 coupled reticulocyte lysate system of Promega, and incubated at 4°C with equal amounts of GST-CHOP or GST immobilized on glutathione Sepharose beads (Bulk GST purification module of Pharmacia) in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40. After 2 h incubation, the beads were washed five times with incubation buffer and the bound proteins were analyzed by SDS-PAGE followed by autoradiography.

## 2.4. Confocal microscopy analysis

COS7 cells were transfected using the calcium phosphate-mediated transfection method with 10  $\mu$ g of the plasmid pEGFP-CREB-2 expressing a GFP (green fluorescent protein)-CREB-2 fusion protein and 10  $\mu$ g of the CHOP expression vector pCMV-Neo-CHOP. Cells were cultivated on glass slides and then analyzed by fluorescence 24 h after transfection as already described [12,18]. CHOP was detected using anti-CHOP mAb (Santa Cruz Biotechnology) and goat anti-mouse IgG antibody coupled to rhodamine (Pierce). Analysis of the green, red, and yellow fluorescence was performed with a Bio-Rad MRC 1024 confocal microscope.

## 2.5. Transfections and luciferase assays

The lymphoblastoid CEM cell line was obtained from the American Type Culture Collection (Bethesda, MD). Cells were cultured in RPMI 1640 medium supplemented with 1% penicillin-streptomycin antibiotic mixture, 1% glutamax (Life Technologies, Eragry, France) and 10% FCS (Life Technologies), to a density of  $5 \times 10^5$  cells/ml in a 5% CO<sub>2</sub> atmosphere. CEM cells were transiently cotransfected according to the previously published procedure [22]. 5  $\mu$ g of a  $\beta$ -galactosidase-containing plasmid (pAC $\beta$ 1) was included in each transfection to check the transfection efficiency. The total amount of DNA in each series of transfections was equal, the balance being made up with empty vectors. Cell extracts equalized for protein content were used for luciferase and  $\beta$ -galactosidase assays [22].

## 3. Results and discussion

To identify proteins interacting with CREB-2 by the two-hybrid approach in *S. cerevisiae*, we first cloned the complete coding sequence of CREB-2 in frame with the cDNA encoding the LEXA DNA binding domain. However, when this fusion protein was expressed in *S. cerevisiae*, reporter gene expression was activated as already described in T cells [6,12]. For this reason, the activation domain of CREB-2 was removed and the cDNA expression library was screened using the CREB-2 bZIP (amino acids 263–351) domain as a bait. Several positive clones were found to correspond to the transcriptional factor CHOP (Fig. 1A).

CHOP is a small bZIP factor that is expressed in response to metabolic stresses. CHOP dimerizes with members of the C/EBP family [17,19,23] and the resulting heterodimers are no

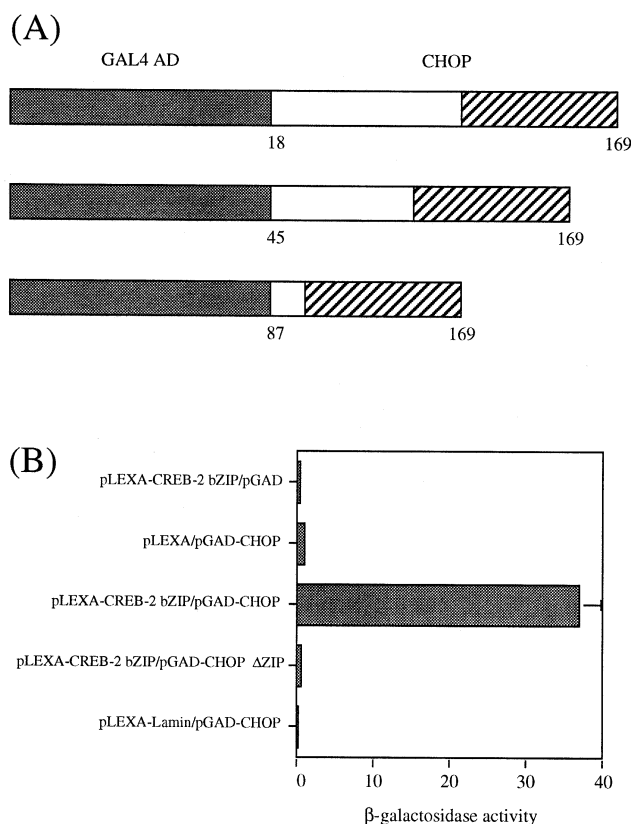


Fig. 1. CREB-2 interacts with CHOP in the yeast two-hybrid system. A: Clones isolated corresponding to CHOP. The MT2 cDNA library was screened using the bZIP domain of CREB-2 as a bait fused to the LEXA DNA binding domain. Three different cDNAs encoding CHOP were obtained. The numbers under the diagrams are CHOP amino acid positions. The bZIP domain is indicated by a striped box. B: Analysis of the interaction between CREB-2 bZIP and CHOP in *S. cerevisiae*. The L40 yeast cells were transformed with an expression vector containing the entire coding sequence of the CHOP cDNA clone fused to the GAL4 activation domain (pGAD-CHOP) together with plasmids expressing either the LEXA DNA binding domain alone (pLEXA), or fused to CREB-2 bZIP (pLEXA-CREB-2 bZIP), or fused to lamin (pLEXA-lamin). pLEXA-CREB-2 bZIP was also cotransformed with a plasmid expressing either the GAL4 activation domain alone (pGAD), or fused to a truncated form of CHOP without leucine zipper (pGAD-CHOP  $\Delta$ ZIP). The  $\beta$ -galactosidase assay with ONPG as substrate was carried out on three independent colonies per transformation. The mean values expressed in Miller units are shown.

longer capable of binding to CAAT/enhancer consensus sites [17]. However, the CHOP-C/EBP heterodimer can bind to a specific CHOP-responsive element distinct from the C/EBP motif [24,25]. CHOP, when bound to the promoter, is able to activate transcription, phosphorylation of CHOP by p38 mitogen-activated protein kinase enhancing its transcriptional activity [26]. CHOP is also able to form heterodimers with other bZIP family members such as AP-1 proteins and ATF-3 [27,28] but has not been yet demonstrated to interact with CREB-2.

All the isolated cDNAs of CHOP encoded the leucine zipper suggesting that this domain is required for the interaction between CREB-2 and CHOP. To test this possibility, we produced a truncated mutant of CHOP without leucine zipper, which was analyzed by using the yeast two-hybrid system as already described [10]. For this analysis, the full-length product of CHOP and its mutated form were fused at their amino-termini to the activation domain of the yeast transcription factor GAL4 (pGAD-CHOP and pGAD-CHOP  $\Delta$ ZIP) and were tested in *S. cerevisiae* in the presence of the CREB-2 bZIP domain fused at its amino-terminus to the LEXA DNA binding domain (pLEXA-CREB-2 bZIP). As shown in Fig. 1B, CHOP was no longer able to interact with CREB-2 when its leucine zipper was deleted. Moreover, to be sure of the specificity of the test, CHOP was also tested in the presence of an unrelated protein (pLEXA-lamin). In these conditions, no significant  $\beta$ -galactosidase activity was detected (Fig. 1B).

It could not be excluded on the basis of the results obtained in yeast that the interaction between CHOP and CREB-2 was not direct but rather required yeast components. To eliminate this possibility, we analyzed the interaction between CHOP and CREB-2 in vitro using recombinant proteins. A fusion protein of CHOP with GST was produced in *Escherichia coli*

and the binding to [ $^{35}$ S]methionine-labeled CREB-2 produced in rabbit reticulocyte lysate was analyzed. As shown in Fig. 2, [ $^{35}$ S]CREB-2 bound to GST-CHOP but not to GST alone. This result demonstrates that CREB-2 interacts with CHOP also in vitro.

To confirm the in vivo relevance of the interaction between CREB-2 and CHOP, we first studied the localization of both proteins inside cells. COS cells were cotransfected with the plasmid pEGFP-CREB-2 expressing a GFP-CREB-2 fusion protein and the CHOP expression vector pCMV-Neo-CHOP. Cells were examined under the confocal microscope (Fig. 3A). Colocalization of CREB-2 and CHOP in the nucleus was visualized in yellow color (Fig. 3A, c) corresponding to the merging of the green fluorescence of the GFP-CREB-2 fusion protein (Fig. 3A, a) and the red staining of CHOP detected by indirect immunofluorescence (rhodamine) (Fig. 3A, b). This result shows that both proteins localized in the nucleus and raises the possibility that CREB-2 might interact with CHOP in the nucleus. To confirm this possibility, we used a transient transfection assay performed in CEM cells where the bZIP domain of CREB-2 was directly bound to the promoter of the reporter gene. For this assay, we used a GAL4-CREB-2 bZIP fusion protein in which the GAL4 DNA binding domain was linked in frame to the C-terminal domain of CREB-2 encompassing the amino acid residues from position 263 to 351. This CREB-2 region has lost the activation domain [10] but still contains the bZIP domain involved in the interaction with CHOP. GAL4-CREB-2 bZIP was assayed using the reporter plasmid pG5luc encoding luciferase under the control of five GAL4 binding sites upstream of a minimal TATA box. As expected, after cotransfection with pG5luc, GAL4-CREB-2 bZIP was unable to stimulate the luciferase expression (Fig. 3B) since no activation domain was present. On the other hand, if the GAL4-CREB-2 bZIP was able to interact with CHOP in vivo, the luciferase expression should be stimulated in the presence of CHOP, GAL4-CREB-2 bZIP supplementing the absence of the CREB-2 activation domain by recruiting CHOP. Indeed, when CHOP was added, luciferase expression was stimulated about 135-fold (Fig. 3B). When the same experiment was carried out with CHOP in the presence of the GAL4 DNA binding domain without the CREB-2 bZIP or with CHOP  $\Delta$ ZIP in the presence of GAL4-CREB-2 bZIP, no stimulation was detected confirming that CHOP enhanced luciferase expression by interacting with the CREB-2 bZIP domain bound to the GAL4 binding sites. Taken together, our results demonstrate that CREB-2 and CHOP interact in vivo.

CHOP has been demonstrated to act as a negative regulator of C/EBP proteins by forming heterodimers that are unable to bind to CAAT/enhancer consensus sites [17]. Similarly, CHOP negatively modulates the activity of ATF-3, which like CREB-2 is a member of the ATF/CREB family. Indeed, CHOP/ATF-3 heterodimers are unable to bind to the CRE motif [27]. This result predicts that if CHOP interacted with CREB-2 in vivo, CHOP should also block CREB-2 transcriptional activity on promoters containing CRE. To test this possibility, CEM cells were cotransfected with a luciferase reporter construct carrying a synthetic promoter containing three tandemly repeated copies of the cellular palindromic CRE site, the CREB-2 expression vector pCI-CREB-2, and increasing amounts of pCMV-Neo-CHOP. Expression of CREB-2 caused a four-fold increase in CRE-dependent tran-

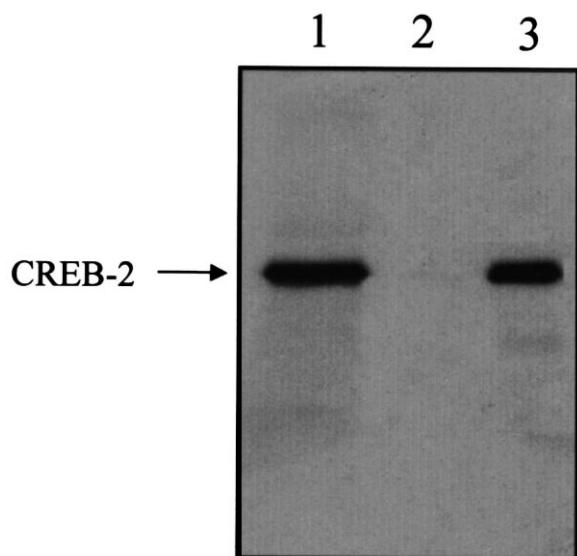


Fig. 2. Binding of CREB-2 to CHOP in vitro. GST-CHOP fusion protein was purified from *E. coli*. In vitro translated CREB-2 in the presence of [ $^{35}$ S]methionine (lane 1) was incubated with equal amounts of GST (lane 2) or GST-CHOP fusion protein (lane 3) immobilized on glutathione Sepharose beads. After incubation, the bound proteins were analyzed by SDS-PAGE followed by autoradiography.

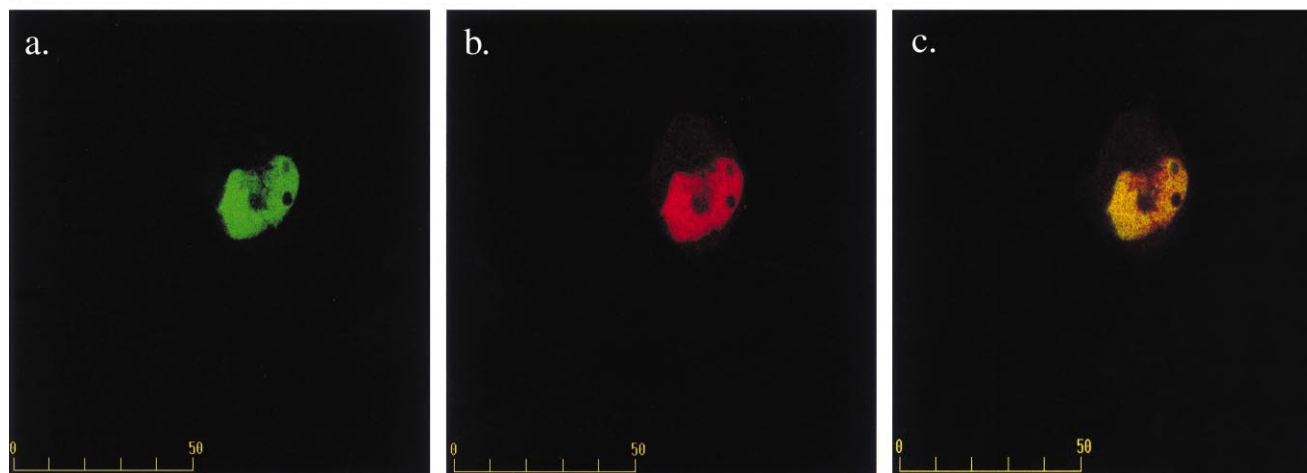
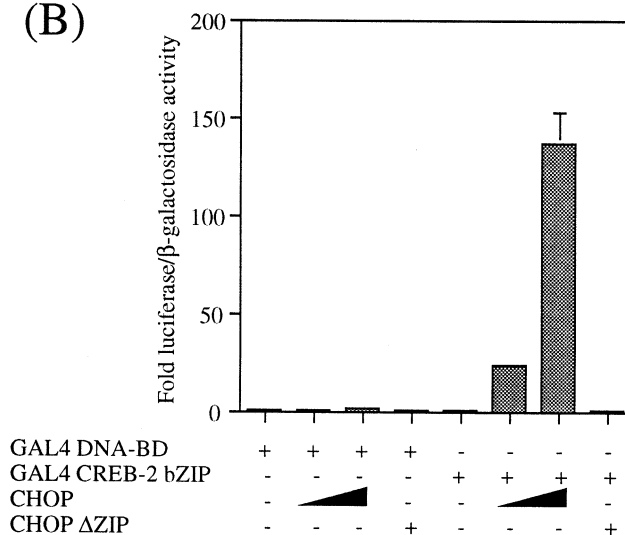
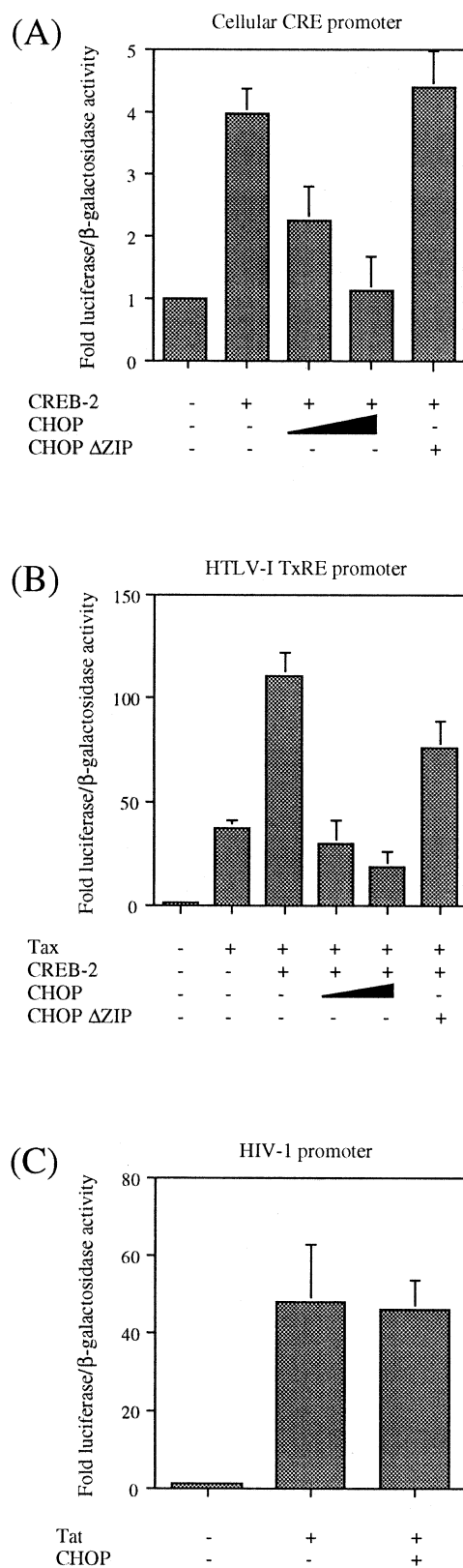
**A****(B)**

Fig. 3. Binding of CREB-2 to CHOP in vivo. A: Analysis by confocal microscopy of the colocalization of CREB-2 and CHOP in vivo. COS cells were cotransfected with pEGFP-CREB-2 and pCMV-Neo-CHOP. Analysis of the green (a), red (b) and merged (c) fluorescence was performed with confocal microscopy. CHOP was detected using anti-CHOP monoclonal antibody and goat anti-mouse IgG antibody coupled to rhodamine. B: CREB-2 and CHOP interact in vivo. CEM cells were cotransfected with 2  $\mu$ g of the luciferase reporter vector pG5luc, 2.5  $\mu$ g of pBIND expressing the GAL4 DNA binding domain fused (GAL4 CREB-2 bZIP) or not (GAL4 DNA-BD) to the CREB-2 bZIP domain, and increasing amounts of pCMV-Neo-CHOP (0.1 and 0.5  $\mu$ g) or pCMV-Neo-CHOP  $\Delta$ ZIP (0.5  $\mu$ g). The total amount of DNA in each series of transfections was equal, the balance being made up with empty plasmids. The luciferase values were normalized for  $\beta$ -galactosidase activity and expressed as fold increase relative to that of cells transfected with pG5luc, pBIND and pCMV-Neo. Values represent the mean  $\pm$  S.D. ( $n=3$ ).

scription (Fig. 4A). This result was in accord with previous studies [4,10]. On the other hand, expression of CHOP suppressed luciferase transcription back to baseline levels (Fig. 4A). In addition, we also analyzed whether CHOP could down-regulate the HTLV-I transcription in a cotransfection assay. Indeed, CREB-2 is able to transactivate the HTLV-I promoter [11,12] when its binding to the TxRE motifs is enhanced by the viral Tax protein [10]. CEM cells were cotransfected with a luciferase reporter construct carrying a synthetic promoter containing three tandem copies of the promoter-proximal TxRE, the Tax expression vector pSG-Tax, pCI-CREB-2, and increasing amounts of pCMV-Neo-CHOP. Fig. 4B shows that the reporter was effectively stimulated 110-fold with CREB-2 and Tax, but this stimulation was inhibited in the presence of CHOP (only a 30-fold stimulation). As shown in Fig. 4A with the cellular palindromic CRE site,

when the experiment was performed with CHOP  $\Delta$ ZIP, such a repression was not observed (Fig. 4B). In order to be sure that this inhibition was not due to a non-specific effect of CHOP on cellular transcription, CHOP was also tested in CEM cells with the human immunodeficiency virus type 1 (HIV-1) promoter in the presence of the HIV-1 activator Tat. Under these conditions, CHOP did not down-regulate the HIV-1 transcription stimulated by Tat (Fig. 4C), confirming that the decrease in transactivation by CHOP from the cellular CRE in the presence of CREB-2 and from the HTLV-I TxRE in the presence of Tax and CREB-2 was specific. Lastly, we also checked that CHOP was unable to interact directly with Tax (data not shown). In conclusion, as predicted, CHOP negatively modulates CREB-2 activity on a promoter containing CRE or related motifs. However, we do not rule out the possibility that the CHOP-CREB-2 heterodimer can bind to DNA sequences



unrelated to the CRE motif, this interaction possibly resulting in activation of transcription.

In this paper, we demonstrate that CREB-2 can interact with another bZIP protein, the transcriptional factor CHOP, although the two proteins are distinguished by their consensus

Fig. 4. CHOP down-regulates CREB-2-dependent transcription. CEM cells were cotransfected with (A) 2  $\mu$ g of luciferase gene driven by three tandem copies of the cellular palindromic CRE, 1  $\mu$ g of pCI-CREB-2, and pCMV-Neo-CHOP (0.1 and 0.5  $\mu$ g) or pCMV-Neo-CHOP  $\Delta$ ZIP (0.5  $\mu$ g), the luciferase values being expressed as fold increase relative to that of cells transfected with pCI-neo, pCMV-Neo, and CRE-luciferase; (B) 2  $\mu$ g of luciferase gene driven by three tandem copies of the viral promoter-proximal TxRE, 1  $\mu$ g of pSG-Tax, 1  $\mu$ g of pCI-CREB-2, and pCMV-Neo-CHOP (0.1 and 0.5  $\mu$ g) or pCMV-Neo-CHOP  $\Delta$ ZIP (0.5  $\mu$ g), the luciferase values being expressed as fold increase relative to that of cells transfected with pSG-5, pCI-neo, pCMV-Neo, and HTLV-I TxRE-luciferase; (C) 2  $\mu$ g of HIV-1 LTR-luciferase, 1  $\mu$ g of the Tat expression vector pBg312HIV-1Lai-Tat, and 0.5  $\mu$ g of pCMV-Neo-CHOP. The luciferase values are expressed as fold increase relative to that of cells transfected with HIV-1 LTR-luciferase without Tat and CHOP. For all the cotransfections, the total amount of DNA in each series of transfections was equal, the balance being made up with empty plasmids, and the luciferase values were normalized for  $\beta$ -galactosidase activity. Values represent the mean  $\pm$  S.D. ( $n = 3$ ).

recognition sites. In addition, we find that CREB-2 in the presence of CHOP is no longer able to activate the transcription from CRE or related sites such as TxREs. From these results, it is tempting to postulate that balance between the production of CHOP and CREB-2 could be involved in the regulation of HTLV-I transcription in infected T cells. In the same way, CHOP could control its own expression since CHOP promoter activity is controlled by CREB-2 through a C/EBP-ATF composite site [29]. However, all these hypotheses remain speculative for the moment and should be investigated further to clearly demonstrate the physiological importance of the CREB-2/CHOP heterodimer in the cell.

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