Glucocorticoid induction of CRE-binding protein isoform mRNAs in rat C6 glioma cells

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Received October 30, 1991; Revised and Accepted January 21, 1992

ABSTRACT
Mammalian cells express several distinct isoforms of transcription factor CREB (cAMP-responsive element binding protein). At least two forms, α- and ΔCREB, arise through alternative splicing of the CREB gene transcript. In this communication we demonstrate that the mRNAs of several CREB isoforms are expressed in rat C6 glioma cells and that the intracellular levels of these mRNAs are markedly induced by the synthetic glucocorticoid dexamethasone. Nuclear run-off assays show that the induction occurs, at least in part, through a transcriptional mechanism. The enhanced cellular levels of CREB mRNAs are accompanied by increased CREB protein and CRE-binding activity of nuclear extracts as evaluated by immunoblot and Southwestern blot assays.

INTRODUCTION
Steroids are important physiological regulators in cells of the nervous system not only during development but also in maintaining specific differentiated functions in mature brain. The determination of the molecular mechanisms by which hormones regulate the activity of specific genes in nervous tissue has been a major goal of our research efforts. Toward this end, we have chosen to study the expression of the gene for lactate dehydrogenase (LDH) A subunit in the rat C6 glioma cell line. The C6 cells offer an ideal experimental model and offer the advantages of working with a clonal population of cells in a defined environment. C6 cells have receptors which bind glucocorticoids in a specific manner (1) leading to the induction of glycerol phosphate dehydrogenase (2) and LDH A subunit. Furthermore, it has been known for several years that in C6 cells LDH-5 isozyme activity increases in response to β-adrenergic receptor stimulation by catecholamines via a cAMP-mediated mechanism (3,4). Subsequent investigations into the mechanism of cAMP action have revealed that transcriptional as well as posttranscriptional effects are instrumental in the induction of the LDH A subunit (4-6).

Analysis of the promoters of several cAMP-inducible genes, including LDH A subunit (7), has led to the identification of a cis-regulatory sequence referred to as CAMP-regulatory element (CRE) with a palindromic octamer motif 5'-TGACGTCA-3' (8). Mammalian cells contain multiple proteins which exhibit specific binding affinity for CRE (9). One of these CRE-binding proteins, CREB, is a nuclear phosphoprotein which exists in several isoforms (10,11). Several CREB-related genes have been cloned, revealing a super family of similar transcription factors (9). CREB binds as a homodimer to CRE sequences, and its transcriptional activity is modulated by cAMP-dependent protein kinase-mediated phosphorylation.

While phosphorylative modification of CREB determines the transcriptional activity and hence functional levels of CREB, there is no information on the regulation of intracellular CREB protein levels. From recent studies it appears that CREB is expressed at similar levels in a variety of tissues. These findings have been taken as an indication that regulation of CREB protein levels may not be a major determinant of its functional activity. However, previous studies in our laboratory using the Southwestern blot assay have shown that the level (or CRE-binding activity) of two CRE-binding proteins is hormonally regulated in the rat ovary (12).

We have recently cloned and characterized a 47-kDa CRE-binding protein from rat C6 glioma cells (13), whose sequence shows a high degree of similarity with the reported sequence of αCREB from PC12 pheochromocytoma cells (14). The C6 glioma cell CREB, however, lacks amino acids 88 through 101 of the PC12 cell αCREB, which characterizes glioma cell CREB as a ΔCREB isoform (10). During our investigations into the mechanisms of glucocorticoid and cAMP regulation of LDH A subunit induction, we noted a marked effect of glucocorticoid stimulation on glioma cell CREB. In this paper, we report that dexamethasone, at a concentration equivalent to physiological concentrations of glucocorticoids, induces α and Δ CREB mRNA levels in rat C6 glioma cells which results in increased intracellular levels of glioma cell CREB. The increased mRNA levels appear to result, at least in part, from increased transcription of the CREB gene(s).

MATERIALS AND METHODS
Cell culture
Monolayer cultures of rat C6 glioma cells were grown to confluency in 150-cm² Corning tissue culture flasks as described previously (4). Twenty-four hours before induction, the culture medium was decanted and replaced with fresh Ham's F-10 serum-free medium. Cells were stimulated at 37°C by the addition of dexamethasone at a final concentration of 1 µM after which incubation was continued for the time periods indicated in the text.
DNA hybridization probes
To generate the αCREB probe used for the nuclear run-off assays, two complementary oligonucleotides corresponding to the αCREB trans-activating region (10), 5'-GAT CTC TTC CTG TAA GGA CTT AAA AAG ACT TTT CTC CGG AAC ACG T-3' with 5', 3' Mbo I restriction sites were annealed, phosphorylated and ligated. The resulting oligomers ranged from 3-mers to about 25-mers. For Northern blot analysis, the double-stranded αCREB oligonucleotide was not catenated. The ΔCREB probe consisted of an 1100-bp glioma cell ΔCREB cDNA which had been generated from rat C6 glioma cell poly(A)⁺RNA by polymerase chain reaction (13) with primers based on the PC12 cell CREB cDNA sequence (14). A rat α-tubulin DNA probe was obtained from the aCREB fro/is-activating region (10), 5'-GAT CTC TTC CTG TAA GGA CTT AAA AAG ACT TTT CTC CGG AAC ACG T-3'. To generate the aCREB probe used for the nuclear run-off assays, DNA hybridization probes were prepared using the GIBCO/BRL HYBRL-SCREEN kit as described by us (19). The expression of CREB in C6 glioma cell nuclear extracts was assessed by a Western blot analytical procedure using the antibodies to purified α-gal-CREB fusion protein. The cloning and expression of rat C6 glioma cell CREB cDNA preparation of antiserum was done by methods described by us (5) using β-gal-fusion protein. Non-immune control immunoglobulins were prepared by the same method from sera of uninjected rabbits. Affinity purification of antibodies against α-gal-CREB fusion protein was prepared in Escherichia coli in the method from sera of uninjected rabbits. Affinity purification of antibodies was done by methods described by us (5) using β-gal-CREB bound covalently to CNBr-activated Sepharose 4B (Pharmacia).

RESULTS
Effect of dexamethasone on C6 glioma cell CRE-binding protein expression
Initial evidence that dexamethasone affected glioma cell CREB levels and/or CRE-binding activity came from Southwestern blotting assays. The assays were carried out with nuclear extracts prepared from unstimulated and dexamethasone-stimulated C6 glioma cells. CRE-binding activity was evaluated using a 32P-labeled, double-stranded 40-bp oligonucleotide containing 3 tandem repeats of the CREB binding site 5'-TGACGTCA-3'. The autoradiographs of the blot are shown in Fig. 1. Even though there is a great number of proteins present in the gel as seen by Coomassie Blue staining (not shown), only a limited number of proteins bound to the 32P-labeled CRE-trioctamer probe among them the 47-kDa CRE-binding protein (Fig. 1A). After probing with a 32P-labeled CRE-trioctamer in which the wild-type CRE sequence had been mutated to 5'-TAACGTGA-3', no binding of the mutant probe to the 47-kDa CRE-binding protein was observed (Fig. 1B). Probing of the blot with the 32P-labeled wild-type CRE-trioctamer fragment in the presence of a 100-fold molar excess of non-radioactive CRE-trioctamer resulted in a successful competition, and no binding of the 32P-labeled probe to the 47-kDa protein was observed (Fig. 1C) establishing this protein specifically as a CRE-binding protein. Dexamethasone had a marked stimulatory effect on the binding activity/level of the nuclear 47-kDa CRE-binding protein 24 and 48 hours after stimulation.

In view of findings that phosphorylation of CREB can change its binding affinity for CRE (20), the results of the Southwestern assay may be interpreted to result from an altered

Figure 1. Southwestern blot analysis of the CRE-binding activity of nuclear extracts from rat C6 glioma cells treated with dexamethasone. Nuclear protein extracts (150 μg of protein/lane) from dexamethasone-treated C6 glioma cells were separated electrophoretically on 8% SDS-polyacrylamide gels, renatured, and transferred by electroblotting to nitrocellulose. The filters were probed with 0.1 mg/ml of 32P-labeled (about 2.5×10⁶ cpm) wild-type CRE trioctamer oligonucleotide of the sequence: 5'-GAT CCA GTG ACG TCA GAG TGA CTG CAG AGT GAC GTC ATA G-3' (panel A); or with a 32P-labeled 34-bp mutant CRE trioctamer: 5'-AGT AAC GTG AGA GTA ACG TAA CGT GAT A-3' (panel B). Panel C shows an autoradiograph where the filter was probed as in panel A but in the presence of a 100-fold molar excess of the non-radioactive CRE trioctamer probe as competitor. The position of the 47-kDa CREB is indicated on the left of the figure.
The apparent molecular weight of the protein is indicated. The 5W lane contains 120 ng of purified fusion protein.

The 47-kDa CRE-binding protein is detected. No additional bands were seen on the blot. Densitometric scanning of the blot indicates an approximate 20-fold higher CRE level 52 hours after dexamethasone stimulation. These results clearly indicate that dexamethasone stimulation of rat C6 glioma cells increases CREB protein levels which, in part, may be responsible for the increased CRE-binding activity seen by Southwestern blotting.

**Dexamethasone regulation of steady state CREB mRNA expression**

In view of the (unexplored) possibility that the antibody against \( \beta \)-gal-CREB-fusion protein may react with various CREB isoforms which cannot be separated and distinguished by SDS-polyacrylamide gel electrophoresis, we attempted to distinguish between \( \Delta \) and \( \alpha \)CREB mRNAs based on their selective sequence differences (10). To that purpose we prepared an oligonucleotide probe which is specific for the \( \alpha \)-trans-activating region of \( \alpha \)CREB and is not present in \( \Delta \)CREB (10). Since there is sufficient sequence homology between \( \alpha \) and \( \Delta \)CREB, the \( \Delta \)CREB cDNA probe will hybridize with both \( \alpha \) and \( \Delta \)CREB mRNAs, whereas the oligonucleotide corresponding to the \( \alpha \)-trans-activating region will hybridize with \( \alpha \)CREB only. By sequential probing of the same Northern blot filter with \( \alpha \)CREB oligonucleotide and then with \( \Delta \)CREB cDNA, we were able to demonstrate differences in the molecular weight of their cognate mRNAs. At least five bands of mRNA reacted with the \( \Delta \)CREB probe with apparent molecular sizes of about 7.2, 6.8, 5.1, 2.1, and 1.3 kb (Fig. 3A). Of these, the prominent 7.2-kb species represents \( \Delta \)CREB mRNA in agreement with previously reported data (14). On the basis of their specific hybridization with the \( \alpha \)CREB oligonucleotide probe, the 5.1- and 1.3-kb species can be identified as \( \alpha \)CREB forms (Fig. 3B). The nature of the 6.8- and 2.1-kb mRNAs is unknown. It is conceivable that one (or both) of them represents different splice forms (21,22) or the recently reported CREM mRNA which exhibits considerable sequence similarity with \( \Delta \)CREB (23).

Treatment of glioma cells with dexamethasone for various time periods significantly increased the cellular level of \( \Delta \)CREB mRNA (7.2 kb), the 6.8-kb mRNA (see Fig. 3A) as well as the \( \alpha \)CREB mRNA forms (5.1, 1.3, and conceivably 2.1 kb) (Fig. 3B) although with a slightly different induction kinetics. Maximal levels of the \( \alpha \)CREB mRNA forms were seen 36 hours after the induction, whereas \( \Delta \)CREB (7.2 kb) and the 6.8-kb mRNAs reached peak levels 48 hours after dexamethasone addition.

**Mechanistic analysis of dexamethasone-induced CREB mRNAs**

Nuclear run-off assays were performed to determine whether transcriptional mechanisms are involved in the regulation of CREB isoforms by dexamethasone. As control, we studied the transcription of \( \alpha \)-tubulin before and after dexamethasone stimulation. There was detectable \( \alpha \)-tubulin transcription which was unchanged by dexamethasone (Fig. 4). Within 18 hours, dexamethasone treatment increased the rate of transcription of both the \( \alpha \) and \( \Delta \)CREB mRNAs, reaching maximal rates 32 hours after stimulation (Fig. 4). Densitometric scanning indicated

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**Figure 2. Immunodetection of CREB in nuclear extracts of glioma cells by immunoblotting.** C6 glioma cells were stimulated with dexamethasone for the indicated time periods. Nuclear protein extracts (200 \( \mu \)g of protein/lane) were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with antiserum against purified \( \beta \)-gal-CREB fusion protein as described under 'Materials and Methods'. The Std lane contains 120 ng of purified \( \beta \)-gal-CREB fusion protein. The apparent molecular weight of the protein is indicated.

**Figure 3.** Northern blot analysis of rat C6 glioma cell poly(A)\(^\text{+}\)RNA for CREB mRNA sequences. Confluent C6 glioma cells were stimulated with dexamethasone for the time periods indicated and poly(A)\(^\text{+}\)RNA was isolated. The RNA was subjected to electrophoresis on a 1.2% agarose/ formaldehyde gel. After transfer to nitrocellulose, CREB mRNA sequences were detected by hybridization to a \( ^{32} \)P-labeled 1100-bp \( \Delta \)CREB cDNA (panel A), or to a \( ^{32} \)P-labeled oligonucleotide consisting of the \( \alpha \)CREB \( \text{trans} \)-activating region (panel B) (10). The indicated molecular weights (in kb) of the mRNA species were estimated using RNA standards. The exposure time of the autoradiograph shown in panel B was four times longer than that required for panel A.

**Figure 4.** Analysis of CREB gene transcription in rat C6 glioma cells treated with dexamethasone. Rat C6 glioma cells were treated with dexamethasone for the indicated time periods. Nuclei were isolated, and newly elongated \( ^{35} \)P-labeled RNA transcripts were hybridized to slot-blotted rat \( \alpha \)-tubulin DNA, \( \Delta \)CREB cDNA, and catenated \( \alpha \)CREB oligonucleotide (5 \( \mu \)g each). The figure shown is representative of two separate experiments.
an approximate 25-fold increase of total CREB isoforms (measured with the ΔCREB probe) and a 10-fold increase of the αCREB forms. It should be kept in mind that these transcription assays measure either the combined total of all CREB isoforms including the α-forms (with the ΔCREB probe) or the combined total of the α-sequence-containing CREB isoforms (with the αCREB oligonucleotide probe). Taken together, these data indicate that several CREB isoforms are regulated, at least in part, at the transcriptional level.

**DISCUSSION**

Recent studies have shown that CRE-binding proteins are important modulators of the actions of cAMP at the gene level. Cloning of the cDNAs encoding the CRE-binding proteins has identified a complex superfamily of transcriptional trans-activator proteins whose regulation for the most part is unknown, although one recognized feature of their regulation is the modulation of their binding and/or transcriptional activity through phosphorylative modification by cAMP-dependent protein kinase and/or diacyl glycerol-dependent protein kinase C (9). In the present studies, we demonstrated that the synthetic glucocorticoid dexamethasone increases the intracellular level of several CREB isoform mRNAs in rat C6 glioma cells. This results in an increase of at least the ΔCREB protein isoform and possibly other isoforms which can not be investigated at this time. To explore this possibility, the molecular characteristics of isoforms, whose existence can be predicted on the basis of the observed multiplicity of CREB mRNAs, must be known and specific antisera need to be generated.

We do not know the molecular events involved in this response to a glucocorticoid, although it is clear from the nuclear run-off data that the regulation is due, at least in part, to an alteration in the rate of transcription. Whether or not the stimulation of CREB transcription is the consequence of a glucocorticoid/receptor interaction with a putative glucocorticoid responsive element in the CREB gene(s) promoter remains to be determined. However, the relatively delayed onset of the increased transcriptional rate argues for a more indirect action of dexamethasone on CREB transcription. To our knowledge, this is the first demonstration that glucocorticoid regulation of CREB isoforms occurs at the level of mRNA abundance. Increased mRNA levels may reflect changes in mRNA synthesis, mRNA stability, or both. Additional detailed studies are necessary after successful cloning of the CREB gene(s) to determine the molecular basis of the induction mechanism.

The possible physiological implications of glucocorticoid induction of CREB remain to be elucidated, particularly in view of the induction of not only one but several isoforms of CREB whose in vivo interactions and functions are only partially understood at this time. Increased expression of CREB forms may contribute in an as yet unknown mechanism to cAMP-modulated gene regulation. For instance, several reports have demonstrated increased reporter gene transcription after cotransfection and expression of CREB cDNA (10,11,14). On the other hand, recent studies in our laboratory concerned with the cAMP-regulation of the LDH A subunit gene have shown a marked reduction in the degree of forskolin stimulation of LDH A subunit mRNA levels, when C6 glioma cells had simultaneously been stimulated with dexamethasone under conditions producing increased levels of CREB mRNA. These findings suggest that the effect of the glucocorticoid is to counteract the induction of LDH by cAMP probably at the transcriptional level. A possible mechanism for this down-regulatory action of glucocorticoid is suggested by reports that the glucocorticoid receptor exerts its negative effect on transcription by competition with CREB for the CRE (24–27). Another significant observation in this connection is the ability of glucocorticoids to down-regulate the state of phosphorylation of specific proteins (28). This seemingly contradictory action, e.g. increasing CREB levels while possibly decreasing the state of phosphorylation and, therefore, transcriptional activity of CREB, raises the possibility that glucocorticoid, in addition to other factors, may play a bifunctional role in determining the intracellular pool of transcriptionally active CREB. This question is presently under investigation in our laboratory.

**ACKNOWLEDGEMENTS**

This work was supported in part by grants from the National Institutes of Health, and by the Research and Education Fund, Northwestern University. We thank Youhan Jiang for his help in preparing the antibody.

**REFERENCES**