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Conditional modulation of glucocorticoid receptor activities by CREB-binding protein (CBP) and p300

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Abstract

Coactivators of nuclear receptors are integral components of the signal transduction pathways of steroid hormones. Here, we show that one of the major coactivators of the glucocorticoid receptor (GR), CREB-binding protein (CBP), can also function conditionally as a negative regulator of its activities. Indeed, CBP suppressed the responsiveness of the mouse mammary tumor virus (MMTV) promoter to dexamethasone in a dose-dependent fashion in HeLa and A204 cells. Similarly, this protein suppressed the responsiveness of several glucocorticoid-responsive element (GRE)-containing synthetic promoters. Using deletion mutants of CBP, we localized the repressor effect of this protein to its N-terminal domain and showed that it was independent of the histone acetyltransferase and coactivator-binding domains but dependent upon its GR-binding domain. We also demonstrated functional differentiation between CBP and other coactivators, including SRC-1 and the CBP-related protein p300, both of which influenced GR signaling in a positive fashion. In fact, p300 completely antagonized the suppressive effects of CBP in a dose-dependent fashion, probably by competing with this protein at the level of the transcription complex. These findings suggest that CBP and p300 may function additively or antagonistically to each other depending on their relative concentrations and type of target tissue, to influence the sensitivity of tissues to glucocorticoids.

Keywords: Coactivator; SRC-1; p300; Mouse mammary tumor virus (MMTV); CREB

1. Introduction

Steroid hormones, including glucocorticoids, exert their effects on their target cells through nuclear receptors, ligand-specific and -dependent transcription factors. Binding of glucocorticoids to their receptors causes them to dissociate from a hetero-oligomer of heat shock proteins and to translocate into the nucleus, where they bind as homodimers and/or monomers, respectively, to specific DNA enhancer elements or other transcription factors, such as AP-1, NFκB and SP1 [1]. The hormone responsive element-bound receptors interact with several newly described nuclear proteins, the coactivators, which help transduce the hormonal signal to the transcription initiation complex [2]. Some coactivators have intrinsic histone acetyltransferase activity, through which they have been proposed to loosen chromatin structure and to facilitate the binding of transcription machinery components to DNA [3,4]. One of these coactivator molecules, cAMP-responsive element binding protein (CREB)-binding protein (CBP) was originally described as a coactivator of CREB [5,6]. CBP and the closely related protein p300 [7,8], may in fact serve as macromolecular docking 'platforms' for transcription factors from several signal transduction cascades, including in addition to CREB, nuclear receptors, such as the gluco-

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corticoid, progesterone and estrogen receptors, AP-1, NFκB, p53, Ras-dependent growth factor and several STATs [9–20]. Because of their central position in many signal transduction cascades, CBP/p300 have been also called co-integrators [21]. Nuclear receptor-specific coactivators were described recently; these include steroid receptor coactivator-1 (SRC-1) [22], glucocorticoid receptor interacting polypeptide-1 (GRIP-1) [23], p300/CBP/co-integrator-associated protein (p/CIP) [21], and ACTR [24], all of which also possess histone acetyltransferase activity [4,24]. Both CBP/p300 and SRC-1/GRIP-1/p/CIP/ACTR contain one or more copies of the signature motif sequence LXXLL, which is necessary for interaction with the nuclear hormone receptors [25].

By modulating nuclear receptor signal pathways, coactivators may participate in defining the sensitivity of a cell to steroid hormones either in a tissue-specific or generalized fashion. Changes in the sensitivity of tissues to steroid hormones may be important in both physiologic and pathologic conditions. In the case of glucocorticoids, tissue sensitivity change may participate in the maintenance of resting and stress-related homeostasis [26]. A change of the host tissue sensitivity to glucocorticoids, expressed either as hypersensitivity or resistance, has been encountered or inferred in several common pathologic conditions, such as, respectively, visceral obesity or hypertension, and glucocorticoid resistant asthma or other autoimmune diseases [27].

Since CBP is thought to be a crucial interaction point between nuclear receptors, such as the glucocorticoid receptor, and other signal transduction pathways, we investigated the role of this protein in altering cell sensitivity to steroid hormones on well characterized glucocorticoid-responsive reporter plasmids in two human cell lines. We report here that CBP may function not only as a coactivator of nuclear receptors but also as a corepressor, depending on its absolute or relevant to other coactivators concentrations and the cell type employed.

2. Materials and methods

2.1. Plasmids

The mouse CBP expression vector pRcRSV–CBP–HA–RK, the CREB expression vector pRcRSV–CREB341, the constitutive active form of protein kinase A (PKA) expression vector RSV–PKA and the cAMP-responsive somatostatin promoter-driven CAT reporter plasmid p(−71)SRIF–CAT were kind gifts from Dr R.H. Goodman (Oregon Health Science University, Portland, Oregon) [5]. The deletion mutant of mouse CBP expression vector pRcRSV–CBP(1–1098) was constructed by digesting pRcRSV–CBP–HA–RK with *Xba*I. This treatment resulted in deletion of the hemagglutinin epitope, which was re-introduced and amplified with the oligonucleotides, 5′ TCTAGA-TATGGCCTACCCATACGACGTGCCTGACTAC-GCCTCCTAG 3′ and 5′ TCTAGACTAGGAG-GCGTAGTCAGGCACGTCGTATGGGTAGGCCA-TA 3′, using pRcRSV–CBP–HA–RK as a template. pRcRSV–CBP(705–2441) was constructed by digesting pRcRSV–CBP–HA–RK with *Hind*III and *Apa*I. A Kozak sequence and translation start site were supplied replacing the deleted sequence with a double strand DNA oligonucleotide 5′ AGCTTCCAC-CATGGGGCC 3′ and 5′ CCATGGTGGA 3′. The sequence of the resulting product was verified by automatic sequencing. pRcRSV–CBP(Δ905–1778) was constructed by excising the *Eco*47III fragment of CBP cDNA from pRcRSV–CBP–HA–RK and allowed to religate after treating with *Exo*III, followed by blunting with the Klenow fragment of DNA polymerase. A plasmid with an inframe junction was verified by DNA sequence analysis. The human SRC-1a expression vector pCR–SRC-1a was a kind gift from Dr B.W. O'Malley (Baylor College of Medicine, Houston, TX) [4,22]. CMVβ–p300–CHA was a kind gift from Dr D.M. Livingston (Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA) [7]. pMMTV-luc was a kind gift from Dr G. Hager (National Institutes of Health, Bethesda, MD) [28], the synthetic glucocorticoid-responsive promoter-driven luciferase reporter constructs, ODLO-2 vector, MMTV-luc, TM-luc, MT-luc and TAT3-luc were kind gifts from Dr J.N. Miner (Ligand Pharmaceuticals, San Diego, CA) [29], HRE–CAT constructs, HRE14, HRE24a, HRE30, HRE40a and HRE82, contain a single, optimal, palindromic GRE separated from the TATA box by the indicated number of base pairs [30]. pRShGRα, containing a human GRα cDNA, and pRSVerbA^{-1} were generous gifts from Dr R.M. Evans (Salk Institute, La Jolla, CA). pSVLPRA, containing the full length coding region of human progesterone receptor A and HE0, containing the full length coding region of human estrogen receptor α were gifts from Dr S. Simons Jr (National Institutes of Health, Bethesda, MD) and Dr P. Chambon (College de France, Strasburg, France), respectively. ERE-tk luc containing the synthetic vitellogenin A2 ERE sequence from −336 to −310 was a gift from Dr J. Segars (National Institutes of Health, Bethesda, MD). The p53 expression vector, pRcCMV–p53 and its responsive promoter constructs, and pG13-luc were kind gifts from Dr A. J. Levine (Princeton University, Princeton, NJ) and Dr B. Vogelstein (Johns Hopkins University, Baltimore, MD), respectively. EMSV–MyoD, containing mouse MyoD cDNA, and its vector, EMSV–Scribe and 4RTK–CAT were kind gifts from Dr S.J.

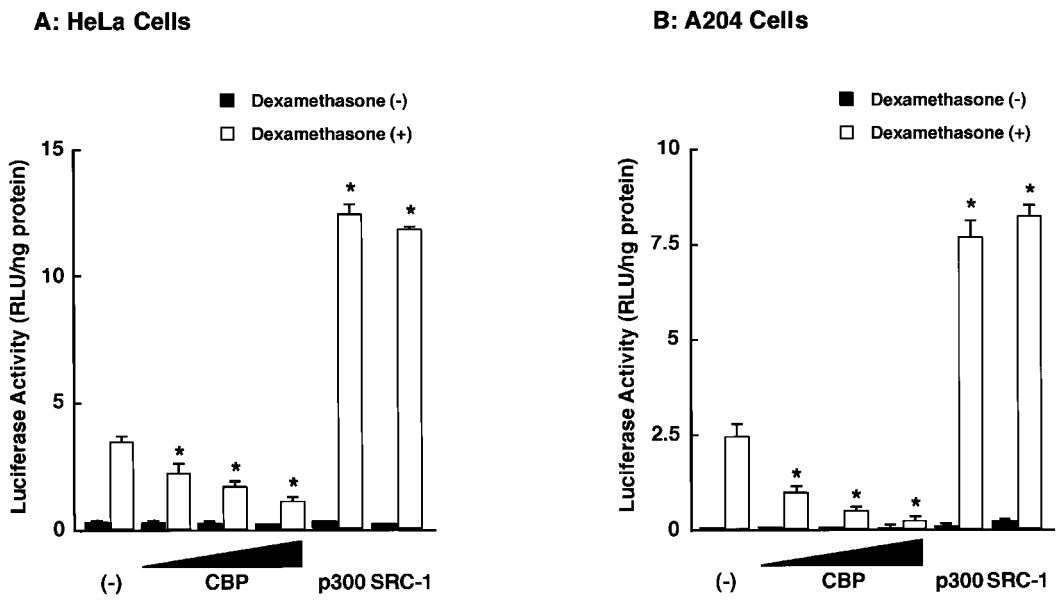

Fig. 1. The effect of CBP, p300 and SRC-1a on basal and glucocorticoid-stimulated MMTV-LTR-driven luciferase activity in HeLa and A204 cells. HeLa (A) and A204 (B) cells were transfected with graded amounts of pRcRSV–CBP–HA–RK (0.5, 1.0 and 2.0 μg/well), 2.0 μg/well of CMVβ–p300–CHA or pCR–SRC-1a and 1.5 μg/well of pMMTV-luc. Basal values were averaged and the mean value was plotted in the figure. Black and white bars show the mean ± SE values obtained in the absence or presence of 1×10^{-7} M dexamethasone, respectively. $^{*}P < 0.01$, ANOVA, followed by Student *t*-test with Bonferroni correction for comparisons with the baseline.

Tapscott (Fred Hutchingson Cancer Research Center, Seatle, WA). pSV-β-Gal was purchased from Promega (Madison, WI). pRcRSV, pRcCMV and pHook^{TM}-1 were purchased from Invitrogen (Carlsbad, CA).

2.2. Cell cultures and transfections

Human cervical carcinoma HeLa cells were a kind gift from Dr S. Simons Jr (National Institutes of Health, Bethesda, MD). Human rhabdomyosarcoma-derived A204 cells and mouse teratocarcinoma cell line F9 were purchased from the American Type Culture Collection (Rockville, MD). HeLa and F9 cells were kept in DMEM supplemented with 10% FBS containing 50 μg/ml of streptomycin and 50 units/ml of penicillin. A204 cells were cultured in DMEM/F-12 supplemented with 10% FBS containing 50 μg/ml of streptomycin and 50 units/ml of penicillin. HeLa and A204 cells or F9 cells were plated in 35 mm well plates at 3×10^5 cells/well or 1×10^6 cells/well density, respectively, and incubated for 24 h. For the experiment using HRE–CAT constructs, A204 cells were plated at 1×10^6 cells/dish density into 60 mm dish. Cells were then transfected with the appropriate plasmids by the lipofectin method. Specifically, HeLa, A204 and F9 cells were incubated with opti-MEM medium containing 5 μg/ml of lipofectin (Life Technologies, Gaithersberg, MD) and 0.5–2.0 μg/ml of DNA for 18 h. Then, the media of A204 cells were replaced with DMEM/F-12 containing 10% FBS and those of HeLa or F9 cells with DMEM containing 10% FBS and the cells were incubated for 24 h. The cells were then exposed to 1×10^{-7} M dexamethasone or vehicle for 24 h. At this time, cells were lysed in 1× cell lysis buffer (Promega) for the luciferase assay and in 1× reporter lysis buffer (Promega) for the CAT assay.

To achieve equal total DNA content, pRcRSV or pCR3.1 were used in lieu of pRcRSV–CBP–HA–RK and pRcRSV–PKA, or CMVβ–p300–CHA and pCR–SRC-1a, respectively. pRSVerbA^{-1}, pRcCMV or EMSV–Scribe were adopted for pRShGRα, pRcCMV–p53 or EMSV–MyoD, respectively, to keep the same amount of DNA content in transfection experiments.

Protein concentration of the cell lysates was determined using an assay employing bicinchoninic acid [31]. Luciferase activity or CAT activities were corrected for protein concentration. All experiments were performed in triplicate and at least twice. Data from a single representative experiment were shown in some of the figures.

2.3. Luciferase, chloramphenicol acetyltransferase (CAT) and β-galactosidase assays

For the luciferase activity assay, 350 μl of assay buffer solution consisting of 25 mM Gly–Gly, 10 mM ATP, 25 mM $MgSO_4$, and 1% Triton-X 100 (pH 8.0) were added to 50 μl of cell lysate sample. Luciferase activity was integrated for 10 s on a Monolight 2010

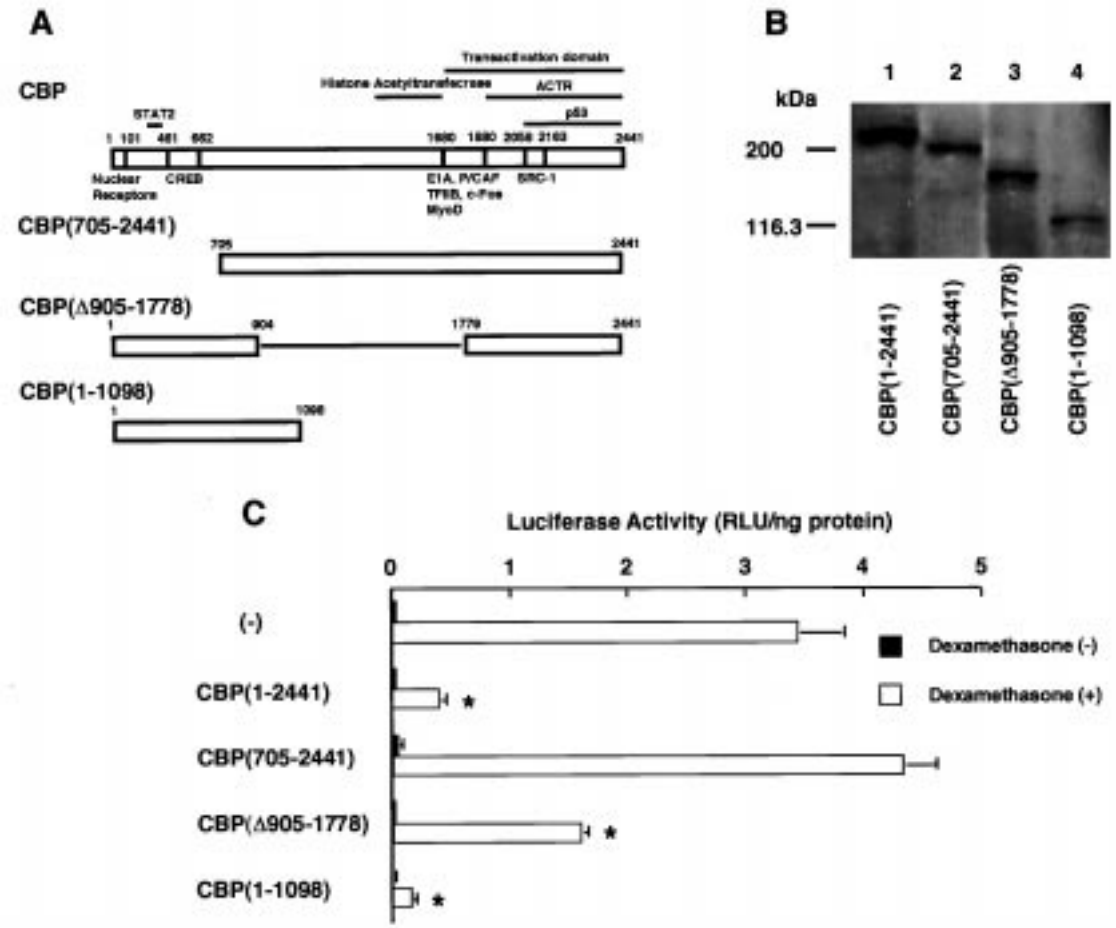

Fig. 2. The effect of CBP mutants on glucocorticoid-stimulated MMTV-LTR-driven luciferase activity in A204 cells. (A) Schematic linear diagram of CBP and its functional domains [5,6,9,12,14,36,40,41] and representation of CBP deletion mutants employed. (B) Western blot analysis of CBP and its mutants in A204 cells. A204 cells were transfected with 0.5 μg/ml of pRcRSV–CBP–HA–RK or its deletion mutants and 2.0 μg/ml of pHook^{TM}-1 expression vector. The transfection positive cells were separated by magnetic sorting. Cells were lysed and CBP–HA was separated on 6% of SDS–PAGE gel. CBP–HA was blotted on nitrocellulose membrane and detected with anti-HA-probe antibody. (C) A204 cells were transfected with 2.0 μg/well of pRcRSV: (−), pRcRSV–CBP–HA–RK: (1–2441), pRcRSV–CBP(1–1098): (1–1098), pRcRSV–CBP(705–2441): (705–2441), pRcRSV–CBP(Δ905–1778): (Δ905–1778) and 1.5 μg/well of pMMTV-luc. Black and white bars show the mean ± SE values in the absence or presence of 10^{-7} M of dexamethasone, respectively. $^{*}P < 0.01$, ANOVA, followed by Student *t*-test with Bonferroni correction for comparisons with the dexamethasone stimulated-control condition.

Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI), during addition of 100 μl of 1 mM D-luciferin sodium salt solution [32]. For the chloramphenicol acetyltransferase activity, 100 μl of cell lysate was incubated with 25 μg of *n*-butyryl co-enzyme A and 0.15 μCi of 1-dideoxy [dichloroacetyl-10-^{14}C] chloramphenicol in 125 μl of 1× reporter lysis buffer at 37°C for 4 h [33]. For the experiment using HRE–CAT constructs, cell lysates were incubated at 37°C for 16 h. Acetylated chloramphenicol was recovered from the reaction solution using ethyl acetate and separated from non-acetylated chloramphenicol by thin layer chromatography. β-Galactosidase was determined by using Galacto-Light^{TM} (Tropix, Bedford, MA), following the manufacturer's protocol on a Monolight 2010 Luminometer (Analytical Luminescence Laboratory).

2.4. Magnetic sorting and Western blot analysis

The transfection positive cells (5×10^6–1×10^7 cells) were enriched by the pHook^{TM}-1 plasmid method following the company's recommendations. Transfection-positive cells were allowed to lyse using lysis buffer [50 mM Tris–HCl (pH 7.4), 400 mM NaCl, 0.2% NP-40, Complete^{TM} tablets 1 Tab/50 ml], following the centrifuge at ×300 *g* for 5 min. Supernatants were used for Western blot analysis. Total CBP or transfected CBP were detected by specific antibodies for CBP which is raised against the epitope corresponding to amino acid sequence mapping at C terminus of human CBP also identical to corresponding mouse sequence, and anti-HA-probe antibody. (Santa Cruz Biotechnology Inc., Santa Cruz, CA.)

3. Results

We examined the consequence of overexpressing CBP on the activity of the MMTV promoter in HeLa and A204 cells. As shown in Fig. 1A and B, CBP strongly suppressed dexamethasone-stimulated MMTV promoter activity in a dose-dependent fashion in both cell lines (HeLa cells, 70%, A204 cells, 90%, respectively). For comparison, we employed the Simian virus 40, Rous sarcoma virus (RSV) and Cytomegalovirus (CMV) promoters which do not contain GREs. CBP had no or minimal effects on these promoters in HeLa or A204 cells (data not shown).

To further study the paradoxical suppressive effect of the coactivator CBP in these two cell lines, we examined the effects of the other coactivators of nuclear hormone receptors, p300 and SRC-1a, on the MMTV promoter in the same cell lines. Unlike CBP, p300 and SRC-1a enhanced dexamethasone-stimulated MMTV promoter activity in HeLa and A204 cells, as previously reported (Fig. 1) [19,22].

To investigate further the suppressive effect of CBP activity on glucocorticoid-responsive promoters, we employed several GRE-containing or -devoid promoters. The sequence of these GREs were derived either from the MMTV or the tyrosine aminotransferase (TAT) gene. CBP suppressed dexamethasone-induced activation of synthetic GRE-containing promoters in A204 cells, regardless of differences in the arrangement or origin of these GREs (OLDO-2, MMTV-luc, TM-luc, MT-luc and TAT3-luc plasmids) (data not shown).

To examine whether the distance between a GRE and the TATA box also influences the suppressive effect of CBP, we assayed a series of GRE–CAT constructs in which a single GRE was separated from the TATA box by 14–82 base pairs (HRE–CAT constructs, HRE14, HRE24a, HRE30, HRE40a and HRE82). CBP suppressed dexamethasone-induced activation of all GRE–CAT constructs employed in A204 cells, regardless of the GRE to TATA box distance (data not shown).

To map the region of CBP responsible for its repressive function, we constructed deletion mutants of the CBP expression vector. Fig. 2A shows the position of binding sites for nuclear receptors, transcription factors and coactivators, as well as histone actyltransferase and transactivation domains of the intact CBP and the linear structures of the three deletion mutants we employed. We examined the expression of these CBP deletion mutants in A204 cells by Western blot analysis using anti-HA-probe antibody and demonstrated sufficient expression in A204 cells (Fig. 2B). We also performed co-transfection experiments employing these CBP deletion mutant expression vectors with the MMTV promoter-driven luciferase plasmid in A204 cells. As shown in Fig. 2C, CBP(1–1098) showed a full suppressive effect on the activity of the MMTV promoter, similar to that of the whole molecule, while CBP(705–2441) was completely devoid of this activity. The suppressive effect of CBP(Δ905–1778) on the activity of the MMTV promoter was partial. To examine whether the N-terminus of CBP molecule suppresses the MMTV promoter by squelching the glucocorticoid receptor, we co-transfected increasing amounts of the human GRα expression vector with a constant amount of CBP expression vector and examined dexamethasone-activated MMTV promoter activity in A204 cells (Fig. 3). In this experiment, the suppressive effect of CBP on the dexamethasone-activated MMTV promoter was retained at all levels of GRα expression providing evidence against the squelching of the GR from the transcription initiation complex.

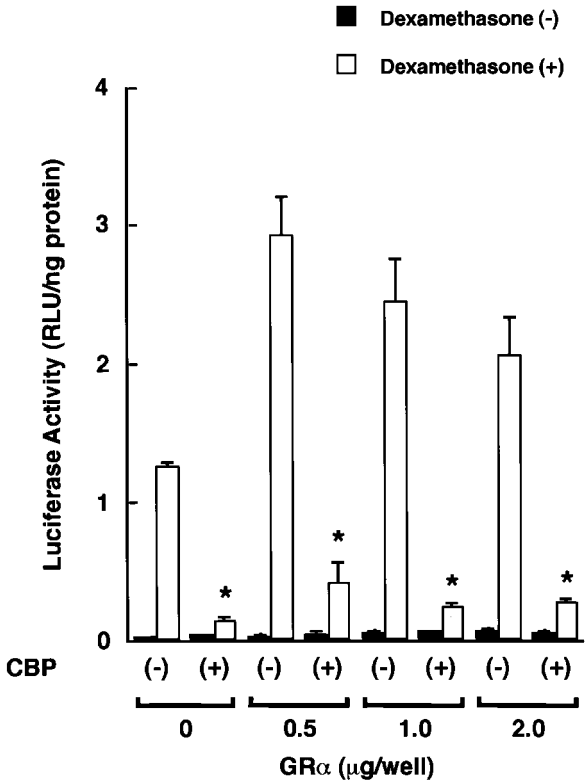

Fig. 3. The effect of overexpression of human GRα on the suppressive effect of CBP on basal and glucocorticoid-stimulated MMTV–LTR-driven luciferase activity in A204 cells. A204 cells were transfected with 2.0 μg/well of pRcRSV–CBP–HA–RK (+) or 2.0 μg/well of pRcRSV (–) and graded amounts of pRShGRα in the presence of 1.5 μg/well of pMMTV-luc. Black and white bars show the mean ± SE values obtained in the absence or presence of 1×10^{-7} M dexamethasone, respectively. $^{*}P < 0.01$, ANOVA, followed by Student *t*-test with Bonferroni correction for comparisons with the baseline.

Next, we compared the activity of CBP on the MMTV promoter in HeLa and A204 cells to that in F9 teratocarcinoma cells, since the latter cell line was

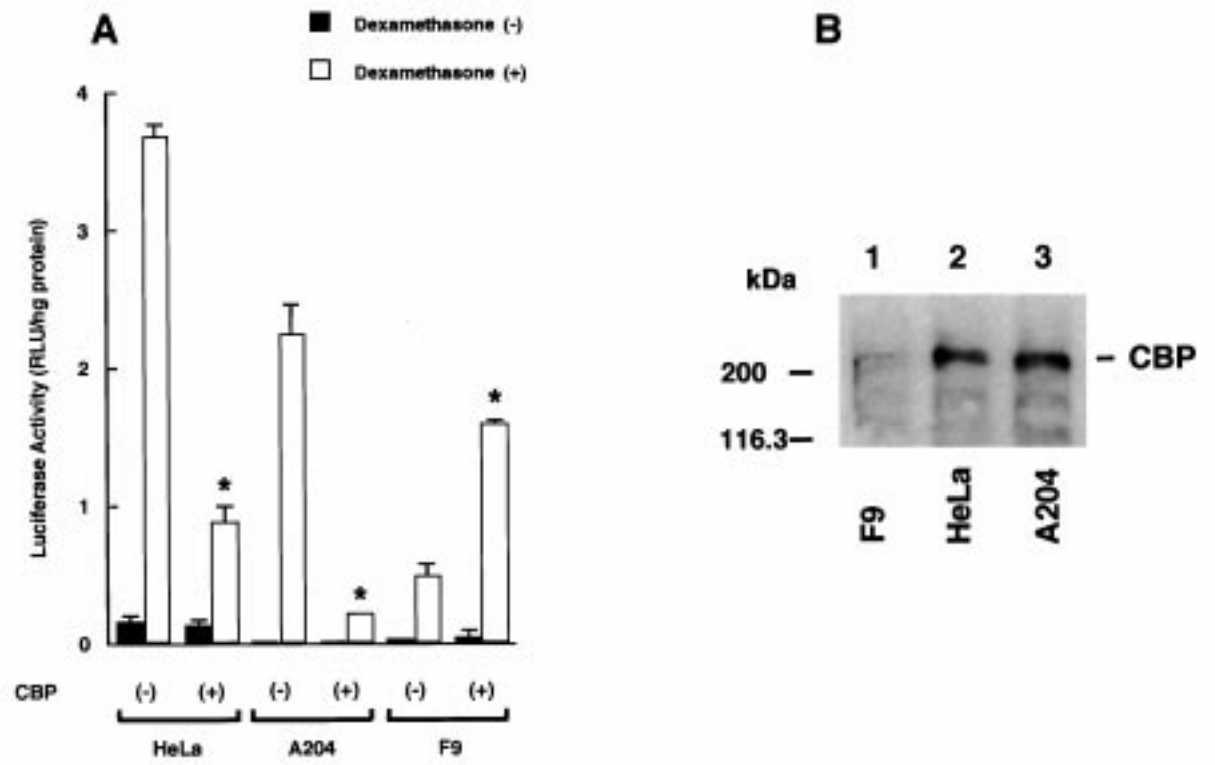

Fig. 4. Analysis of CBP activities and levels in HeLa, A204 and F9 teratocarcinoma cells. (A) The effect of CBP on basal and glucocorticoid-stimulated MMTV-LTR-driven luciferase activity in HeLa, A204 and F9 teratocarcinoma cells. HeLa, A204 and F9 teratocarcinoma cells were transfected with 2.0 μg/well of pRcRSV–CBP–HA–RK and 1.5 μg/well of pMMTV-luc. Black and white bars show the mean ± SE values obtained in the absence or presence of 1×10^{-7} M dexamethasone, respectively. $^{*}P < 0.01$, ANOVA, followed by Student *t*-test with Bonferroni correction for comparisons with the baseline. (B) Western blot analysis of CBP in HeLa, A204 and F9 teratocarcinoma cells. Cells were lysed and CBP was separated on 6% of SDS–PAGE gel; 25 μg of each cell lysate were loaded; CBP was blotted on nitrocellulose membrane and detected with specific anti-CBP antibody.

used in the original reports in which CBP was discovered to be a coactivator of CREB [5,6]. In contrast to its effects in HeLa and A204 cells, CBP enhanced the activity of dexamethasone-stimulated MMTV promoter in F9 cells (Fig. 4A). We hypothesized that the differences of CBP action in these cell lines could be due to the difference of endogenous CBP levels and examined these levels in the three cell lines (Fig. 4B). F9 cells contained only a small fraction of endogenous CBP compared to the amounts determined in HeLa and A204 cells, suggesting that endogenous CBP levels in F9 cells have submaximal coactivator effects in this cell line.

Since the high levels of endogenous CBP might be the cause of the suppressive effect of added CBP in HeLa and A204 cells, we performed cotransfection experiments using very small amounts of CBP expression vector in these cell lines. As shown in Fig. 5A and B, low levels of CBP transfection could enhance dexamethasone-activated MMTV promoter in HeLa and A204 cells, an effect that was inverted at higher doses. We monitored CBP levels in HeLa and A204 cells by Western blot during our experiment (Fig. 6A). For total CBP we employed specific anti-CBP antibody, while for transfected CBP we used the anti-HA-probe antibody. The levels of transfected CBP progressively increased with time, up to 48 h. We examined the time-course of the effect of co-transfection of CBP with the GR, PR and ER expression vectors with their responsive promoters in HeLa and A204 cells (Fig. 6B). In HeLa and A204 cells, CBP enhanced GR, PR and ER activities at 24 h (Fig. 6B-a and c), while the same protein suppressed GR, PR and ER activities at 48 h (Fig. 6B-b). In A204 cells, CBP suppressed GR and PR activities and enhanced ER activity in this period (Fig. 6B-d). To address whether overexpressed p300 could compensate for the suppressive effect of CBP, we co-transfected graded amounts of p300 with CBP and pMMTV-luc in HeLa and A204 cells (Fig. 7). In this experiment, p300 overcame the suppressive effect of CBP on GR activity. On the other hand, unlike p300, SRC-1a could not compensate for the suppression of the GR by CBP even though SRC-1a and p300 had similar potentiating activities in the absence of added CBP. The levels of transfected CBP and p300 blotted in Western blots with anti-HA antibody were roughly equal. CBP did not affect RSV or CMV promoter activities, which drive CBP or p300

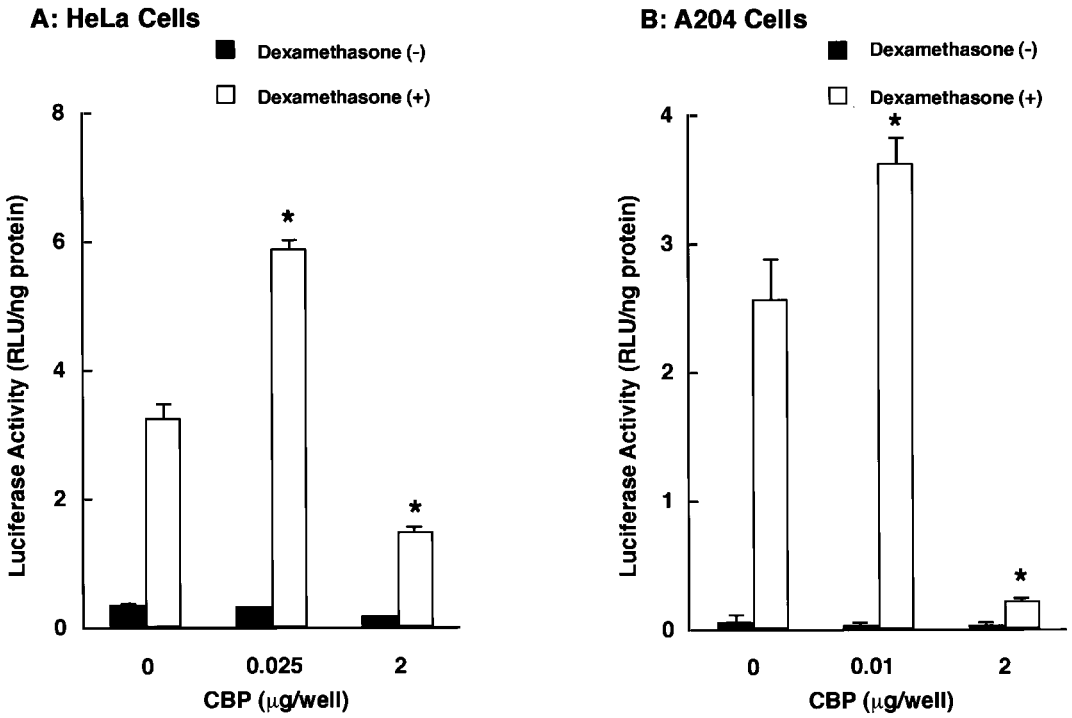

Fig. 5. The effect of small amount of CBP transfection on basal and glucocorticoid-stimulated MMTV-LTR-driven luciferase activity in HeLa and A204 cells. HeLa (A) and A204 (B) cells were transfected with indicated amount of pRcRSV–CBP–HA–RK and 1.5 μg/well of pMMTV-Luc. Black and white bars show the mean ± SE values obtained in the absence or presence of 0.5 μg/well of RSV–PKA, respectively. $^{*}P < 0.01$, ANOVA, followed by Student *t*-test with Bonferroni correction for comparisons with the baseline.

and SRC-1a expression, respectively (data not shown). Therefore, the difference of activities observed in this experiment appears to depend on qualitative rather than quantitative differences between these three molecules. As the levels of CBP appear to affect the direction of its activity on nuclear receptor- and CREB-responsive promoters, we examined the ability of CBP to influence the activities of other transcription factors which also employ CBP as a coactivator, namely p53 and MyoD. While CBP suppressed the activities of the GR and CREB on their responsive promoters in HeLa and A204 cells, it enhanced the activities of p53 and MyoD (Fig. 8), suggesting transcription factor-related specificity in its actions.

4. Discussion

CBP suppressed the activity of the glucocorticoid-responsive MMTV promoter in HeLa and A204 cells in a dose-dependent manner. On the other hand, p300 and SRC-1a enhanced GR-activated MMTV promoter activity in the same system in which CBP showed functional suppressive activity. Although CBP and p300 are highly homologous, our results showed differentiation between these two proteins, suggesting that these molecules are not functionally interchangeable, at least in the 'overexpressed' condition. Indeed, structural defects of the CBP gene alone cause the Rubinstein–Taybi syndrome and CBP and p300 cannot compensate for each other's deficiency in knockout mice [34].

To specify the mechanism of the suppressive effect of CBP, we employed GRE-driven promoters, that respond differently to distinct glucocorticoids and are variably affected by mutations of the glucocorticoid receptor [29], and promoters which have different spacing between the GRE and the TATA box [30]. CBP suppressed all of these GRE constructs suggesting that this is a GRE-dependent phenomenon, which however is not affected by the type, orientation, or spacing of the responsive element.

As mutant CBPs with altered functional domains might help to clarify the exact mechanism(s) of the CBP suppressive effect, we constructed several CBP mutants to perform the transfection experiments with the MMTV promoter. CBP(1–1098), which contains a GR binding site [9,35] but not a histone acetyltransferase activity or transactivation/coactivator-binding domains [3–5,9,24,36], still showed a full suppressive effect on this promoter. CBP(Δ905–1778), which includes the GR and transactivation/coactivator interacting sites, but not the histone acetyltransferase activity domains, showed only partial suppressive effect on the dexamethasone-activated MMTV promoter. Another mutant, CBP(705–2441), which does not have

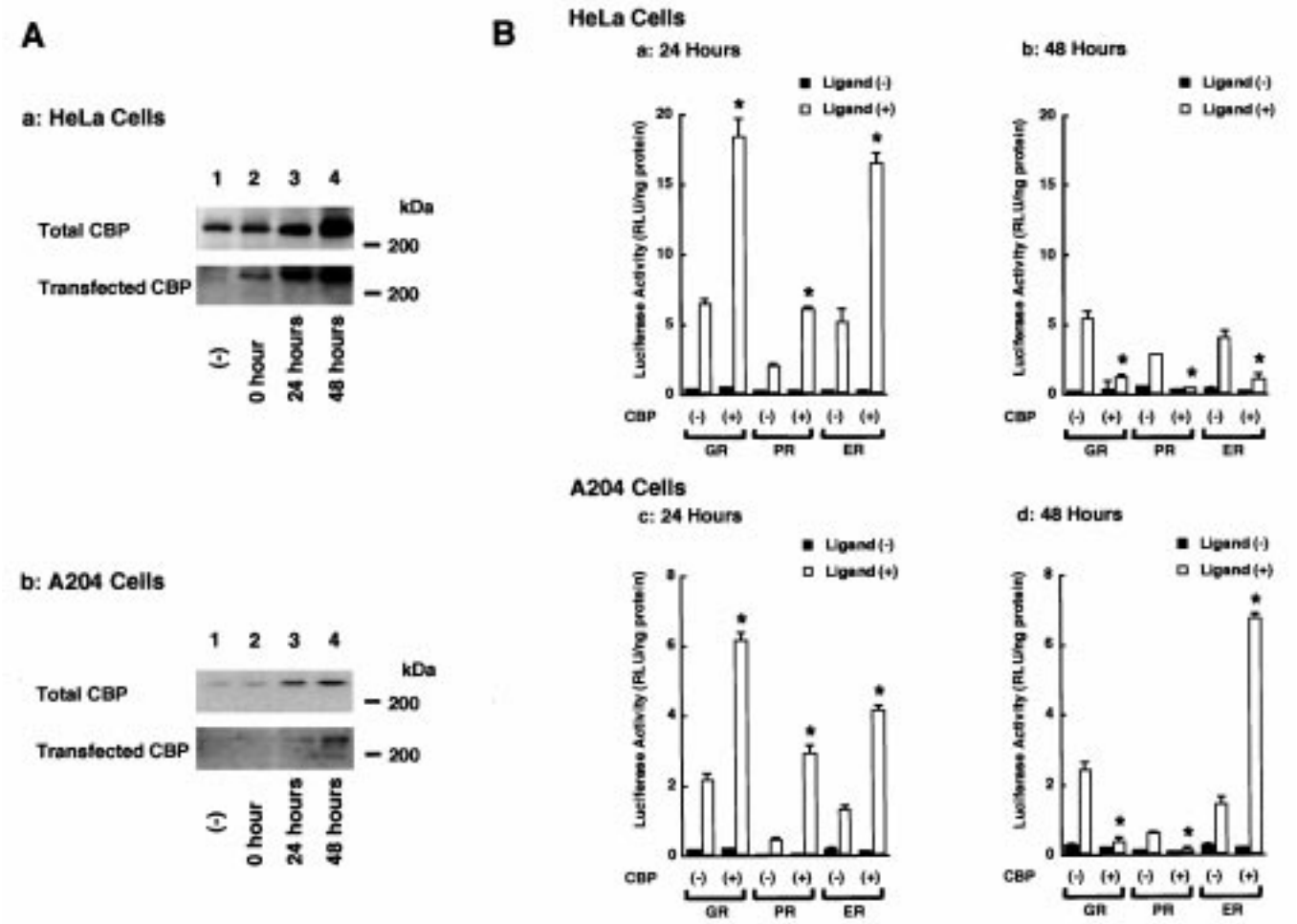

Fig. 6. (A) Change of the levels of CBP by transfection with pRcRSV–CBP–HA–RK in HeLa and A204 cells. HeLa (a) and A204 (b) cells were transfected with 2.0 μg/ml of pRcRSV–CBP–HA–RK and 2.0 μg/ml of pHook^{TM}-1 expression vector. The transfection positive cells were separated by magnetic sorting at the indicated time points and 25 μg of cell lysate was loaded and CBP was separated on 6% SDS–PAGE gel. Western blot was performed using CBP specific antibody. (B) CBP modulated the GR, PR and ER activities on their responsive promoters differently, depending on the period of incubation in HeLa and A204 cells. HeLa (a and b) and A204 (c and d) were transfected with 2.0 μg/well of pRcRSV–CBP–HA–RK (+) or 2.0 μg/well of pRcRSV (−) and 0.1 μg/well of pRShGRα, pSVLPRA or HE0 with 1.5 μg/well of pMMTV-luc (GR and PR) or ERE-tk-luc (ER) in HeLa and A204 cells. Black and white bars show the mean ± SE values obtained in the absence or presence of 1×10^{-7} M dexamethasone (GR), 1×10^{-7} M progesterone (PR) or 1×10^{-8} M estradiol (ER), respectively. For (a) and (c), ligands were added just after the replacement of transfection medium to the usual culture medium and cell lysates were harvested after 24 h of incubation. For (b) and (d), ligands were added after 24 h of replacing medium and harvested after 48 h. Phenol red-free DMEM or DMEM/F-12 supplemented with 10% charcoal-treated FBS containing 50 μg/ml of streptomycin and 50 units/ml of penicillin were used in these experiments. $^{*}P < 0.01$, ANOVA, followed by Student *t*-test with Bonferroni correction for comparisons with the baseline.

a GR-interacting domain but retains the histone acetyltransferase activity and transactivation/coactivator binding domains, lost the suppressive effect. These findings suggest that tethering of the N-terminal domain of CBP to the MMTV promoter via the GR is necessary for suppression. Therefore, while the N-terminal domain of CBP, in which the GR-interacting domain resides, is essential for CBP to show a suppressive effect on the MMTV promoter, the histone acetyltransferase activity and transactivation/coactivator-binding domains are not necessary. To rule out the possibility that ligand-activated GR was squelched by the CBP molecule, we cotransfected graded excessive amount of the GR expression vector with the CBP expression vector. The suppressive effect of CBP could still be seen in all conditions tested, suggesting that squelching of GR by CBP is not likely to be the cause of the CBP suppressive effect. CBP(Δ905–1778) and CBP(1–1098), lacking their mid portions or the C-terminal, which contains the histone acetyltransferase activity and other functions, still show suppressive effects, suggesting that the suppression observed may be caused by the disruption of the transcription complex by the N-terminal portion of CBP.

The levels of CBP in cell appear to define the direction of the effect of this protein on glucocorticoid sig-

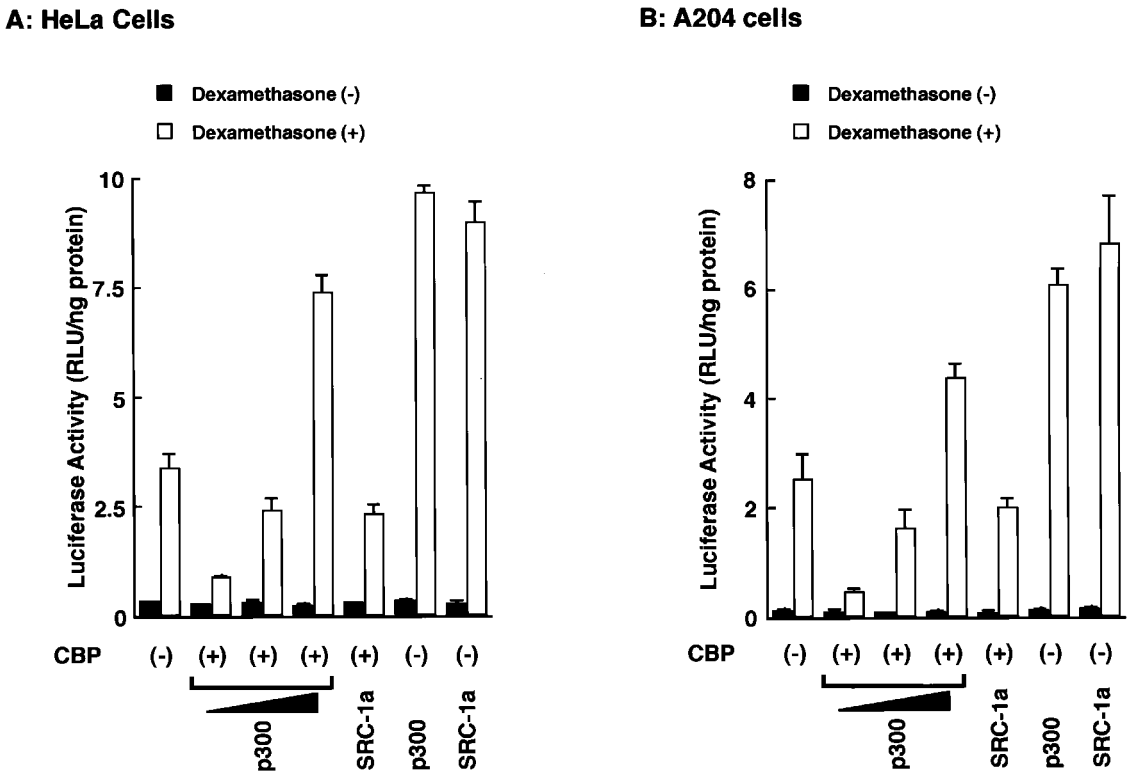

Fig. 7. The effect of p300 or SRC-1a on the CBP suppressive effect to GR transactivation in HeLa and A204 cells. HeLa (A) and A204 (B) cells were transfected with 1.0 μg/well of pRcRSV–CBP–HA–RK, graded amount of CMVβ-p300-CHA (0, 0.5, 2.0 μg/well) or 2.0 μg/well of pCR–SRC-1a and 1.5 μg/well of pMMTV-luc. In the right two lanes, 2.0 μg/well of CMV-β-p300–CHA or pCR–SRC-1a was transfected without pRcRSV–CBP–HA–RK for the comparison. Bars represent the mean ± SE values.

nal transduction. Thus, F9 teratocarcinoma cells which have inherently low levels of CBP, showed enhancement of GR activity, while HeLa and A204 cells which contain much higher levels of CBP, exhibited a suppressive response to this protein. This was further strengthened by a biphasic effect depending on the amount of CBP in the transfected cells, and by the effect of increasing the time period of expression. During the first 24 h, in which CBP was expressed at lower levels, CBP enhanced GR, PR and ER activity in HeLa and A204 cells; during the next 24 h, when CBP was expressed at higher levels, these nuclear receptor activities were mainly suppressed in the same cell lines. In these experiments, in the absence of transfected CBP, basal and ligand-activated luciferase activity levels were similar during the first and second 24 h. These data may explain the discrepancy from a previous report in which CBP acted as a coactivator of the PR and ER signal cascades in HeLa cells [33]. The difference may be due to lower levels of CBP owing to different transfection procedures, culture conditions, or different clones derived from the same original cell.

The suppressive effect of CBP on GR activity could only be seen in the 'overexpressed' condition in HeLa and A204 but not F9 cells. HeLa and A204 cells naturally express greater amounts of CBP; in these cells, low levels of CBP tranfection showed an enhancing effect on GR activity, which changed direction as the levels of transfected CBP increased. There might be one or more limiting factors conferring an enhancing effect on CBP in the transcriptional complex, which set the threshold for a change in the direction of CBP effect.

Since CBP and p300 are very close structurally and only CBP downregulated transcription in our system, we examined whether overexpressed p300 could counteract the suppressive effect of CBP, by competing with it at the level of the transcription initiation complex. Indeed, overexpression of p300 almost fully antagonized the suppressive effect of CBP on the dexamethasone-activated MMTV promoter in HeLa and A204 cells, while unlike p300, SRC-1a did not fully inhibit the CBP suppressive effect on GR activity. This is reminiscent of RIP-140, a SRC-1a-like coactivator of nuclear receptors, which suppressed nuclear receptor responsive-promoters by antagonizing SRC-1a [37]. Both CBP and p300 were reported to form complexes with TBP and competition could be at this level and at the level of nuclear receptors [38]. Similar to our findings, there is another example in nature, in which a

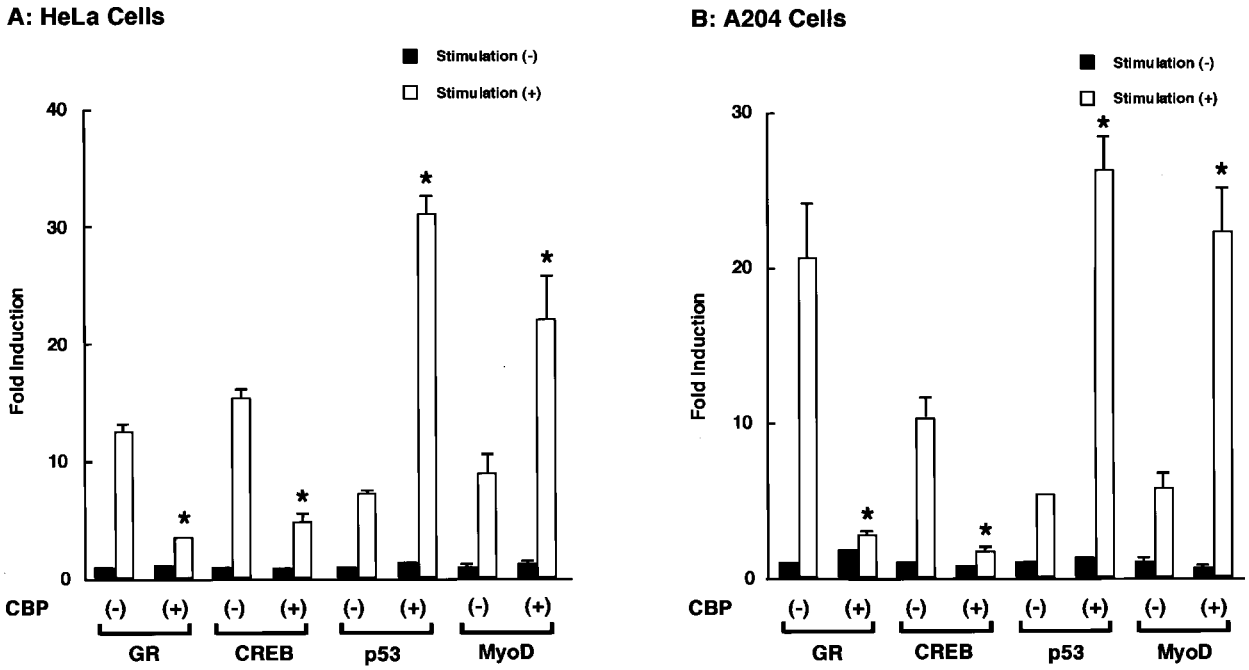

Fig. 8. The effect of CBP on GR, CREB, p53 and MyoD activities on their promoters in HeLa and A204 cells. HeLa (A) and A204 (B) cells were transfected with 2.0 μg/well of pRcRSV–CBP–HA–RK (+) or 2.0 μg/well of pRcRSV (−) and 1.5 μg/well of pMMTV-luc (GR), 0.5 μg/well of pRcRSV–CREB341 and 1,5 μg/well of p(−71)SRIF-CAT (CREB), pG13-luc (p53) or 4R–TK–CAT (MyoD). Black and white bars show the mean ± SE values obtained in the absence or presence of 1×10^{-7} M dexamethasone (GR), 0.5 μg/well of RSV–PKA (CREB), 0.2 μg/well of pRcCMV–p53 (p53) or 0.2 μg/well of EMSV–MyoD (MyoD). Black p53 and MyoD denoted bars represent controls in which 0.2 μg/well of pRcCMV or EMSV–Scribe were transfected instead of pRcCMV–p53 or EMSV–MyoD.

CBP-like molecule was found to act as a repressor in the Drosophila [39].

Finally, we showed that the suppressive effect of CBP could be seen not only in nuclear receptor activities but also in the CREB signal transduction pathway. In the same condition, however the activities of p53 and MyoD transcription factors, which bind to CBP at the C-terminus [12,40], were positively affected by the same amount of CBP expression vector. From these results, we suggest that different signal cascades may be affected differently by the same levels of CBP and that CBP and p300 may act synergistically or antagonistically to each other in some conditions. The levels of CBP and p300 in a cell might determine the sensitivity and/or responsiveness of this cell to varying stimuli transduced by different, albeit overlapping signal cascades. Each of these cascades may employ different ratios of CBP and p300 and these two molecules may compete, cooperate, or not influence each other, depending on their individual or relative levels and the cell type or tissue conditions.

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