cAMP-response-element-binding-protein-binding protein (CBP) and p300 are transcriptional co-activators of early growth response factor-1 (Egr-1)

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Egr-1 (early-growth response factor-1) is a sequence-specific transcription factor that plays a regulatory role in the expression of many genes important for cell growth, development and the pathogenesis of disease. The transcriptional co-activators CBP (cAMP-response-element-binding-protein-binding protein) and p300 interact with sequence-specific transcription factors as well as components of the basal transcription machinery to facilitate RNA polymerase II recruitment and transcriptional initiation. Here we demonstrate a unique way in which Egr-1 physically and functionally interacts with CBP/p300 to modulate gene transcription. CBP/p300 potentiated Egr-1 mediated expression of 5-lipoxygenase (5-LO) promoter–reporter constructs, and the degree of *trans*-activation was proportional to the number of Egr-1 consensus binding sites present in wild-type and naturally occurring mutants of the 5-LO promoter. The N- and C-terminal domains of CBP interact with the transcriptional activation domain of Egr-1, as demonstrated by a mammalian two-hybrid assay. Direct protein–protein interactions between CBP/p300 and Egr-1 were demonstrated by glutathione S-transferase fusion-protein binding and co-immunoprecipitation/Western-blot studies. These data suggest that CBP and p300 act as transcriptional co-activators for Egr-1-mediated gene expression and that variations between individuals in such co-activation could serve as a genetic basis for variability in gene expression.

INTRODUCTION

Early-growth response factor-1 (Egr-1, Krox-24, NGFI-A, zif268, TIS 8) is a zinc-finger protein transcription factor that binds a GC-rich consensus sequence, GCG(T/G)GGGCG, leading to changes in target-gene expression [1,2]. It is an 'immediate-early gene' product because it is rapidly and transiently induced by numerous stimuli, including growth factors, cytokines, hormones, ischaemia, ionizing radiation and mechanical injury. Mature Egr-1 protein is 533 amino acids (aa) in length and contains a strong N-terminal transcription-activation domain between aa 1 and 281 [3]. Egr-1 has been implicated in the transcriptional regulation of several genes, including platelet-derived growth factor A-chain [4,5], basic fibroblastic growth factor [6], tissue factor [7], 5-lipoxygenase (5-LO) [8,9], urokinase-type plasminogen activator [10] and luteinizing hormone receptor [11]. All these genes are similar in that their promoter regions are GC-rich and contain multiple Egr-1 consensus binding sites in tandem. In response to transcription activators such as phorbol esters, growth factors, mechanical injury or shear stress, Egr-1 expression is rapidly increased and can augment transcription by binding to these promoter regions [3–5]. This mechanism of transcriptional activation may be a generalized phenomenon responsible for the regulation of many genes [12].

cAMP-response-element-binding protein (CREB)-binding protein (CBP) and related proteins, such as p300, are transcriptional co-activators that structurally and functionally link sequence-specific transcription factors to elements of the basal transcription machinery, thereby facilitating RNA polymerase II recruitment and transcription activation [13]. CBP was identified as a co-activator that interacts with the CREB [14]. This interaction requires the cAMP-dependent protein kinase A and its phosphorylation of CREB [15]. p300 was isolated independently, based on its ability to interact with the adenovirus protein E1A [16]. CBP and p300 have been shown to interact functionally and structurally, via protein–protein interactions, with a variety of sequence-specific transcription factors, including CREB/activating transcription factor [15], c-Jun [17], c-Myb [18], YY1 [19], Myo-D [20], c-Fos [21], steroid receptors [22], signal transduction and activators of transcription (STAT) 1 and 2 [23], hypoxia inducing factor (HIF)-1α [24], serum-response-element binding protein (SREBP)-2 [25], p65 [26] and p53 [27]. CBP and p300 are also capable of binding to components of the basal transcription complex, including TATA-box binding protein (TBP) and transcription factor IIB [15,28], and appear to have histone acetylase activity by themselves or through interactions with the enzyme p300/CBP-associated factor [29]. Because CBP and p300 are present in limiting amounts and are capable of multiple interactions with transcription regulators, they may function as important integrators of diverse signal-transduction pathways at the level of gene transcription [22].

The mechanisms by which Egr-1 facilitates gene transcription are unknown. In the present study we used the 5-LO promoter as a model of inducible transcription to show that Egr-1 interacts with CBP/p300. The 5-LO promoter was particularly useful for our studies because it is responsive to Egr-1, and several naturally occurring promoter mutations have been identified in which the number of tandem Egr-1 consensus binding sites vary between three and six [8]. Using multiple parallel approaches we now show that Egr-1 physically and functionally interacts with the co-

Abbreviations used: aa, amino acids; CAT, chloramphenicol acetyltransferase; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; Egr-1, early-growth response factor-1; GST, glutathione S-transferase; HUVEC, human umbilical vein endothelial cell; 5-LO, 5-lipoxygenase; CMV, cytomegalovirus; RSV, respiratory syncytial virus.

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activators CBP and p300 to enhance Egr-1-dependent gene transcription. Our data suggest that multiple Egr-1 binding events within GC-rich promoter sequences may serve as a platform for the recruitment of CBP/p300 and may represent a generalized mechanism of gene activation.

EXPERIMENTAL

Cell culture

COS-1 cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, U.S.A.), pH 7.4, containing 50 μg/ml streptomycin, 50 units/ml penicillin and 10 % fetal calf serum at 37 °C and 5 % CO_2. At confluence, approximately every 3–4 days, cells were passaged by rinsing twice with PBS, pH 7.4, followed by a 3 min incubation at 37 °C with 0.05 % trypsin/0.02 % EDTA in Hanks' balanced salt solution (BioWhittaker, Walkersville, MD, U.S.A.) prior to resuspension in growth medium. Human umbilical vein endothelial cells (HUVECs) were isolated as described previously [30] and grown similarly to COS-1 cells except that 10 % fetal calf serum was used as a medium supplement. All experiments were completed with HUVECs between passage 2 and 6. For Egr-1 induction, 1 μg of PMA (Sigma, St. Louis, MO, U.S.A.) dissolved in ethanol was added to each 100 mm² plate containing 10 ml of growth medium for 1 h. Proteins were metabolically labelled by incubating cells in methionine-free medium 1 h prior to the addition of [^{35}S]methionine (1 mCi/10 ml of medium) and PMA.

Plasmid construction

pCAT5LO reporter plasmids were constructed by placing approximately 280 bp of the GC-rich wild-type [WT(5)] or naturally occurring mutant promoter regions [M(3), M(4)] upstream of the chloramphenicol acetyltransferase (CAT) gene in a pCAT®-basic vector (Promega, Madison, WI, U.S.A.). The pCAT5LO-WT(5) promoter contains five tandem Egr-1 consensus sites. The mutant promoters designated pCAT5LO-M(4) and pCAT5LO-M(3) have lost one or two Egr-1 binding sites and therefore contain four and three tandem Egr-1 sites, respectively. Promoter regions were cut enzymically from the p5LO–CAT constructs, as described in detail in In et al. [8], using *Hin*dIII and *Xba*I and ligated into the pCAT®-basic polylinker using standard methods. Cytomegalovirus (CMV)-based Egr-1 (pCMVEgr-1) and Sp1 (pCMVSp1) expression vectors were provided by V. P. Sukhatme (Beth Israel Hospital, Boston, MA, U.S.A.) and R. Tjian (University of California, Berkeley, CA, U.S.A.), respectively. Full-length respiratory syncytial virus (RSV)-based p300 and CBP expression vectors were provided by R. Eckner and D. Livingston (Dana-Farber Cancer Institute, Boston, MA, U.S.A.) and R. Goodman (Oregon Health Science University, Portland, OR, U.S.A.), respectively. Constructs pMCBP (aa 1–771, aa 706–1069, aa 1068–1459, aa 1451–1891), VP16Egr-1 (aa 1–281, aa 250–450, aa 420–533) and VP16p65 (aa 286–551) were prepared as described using the Mammalian Matchmaker two-hybrid assay kit (Clontech Laboratories, Palo Alto, CA, U.S.A.). Correct orientation was confirmed by restriction digest, utilizing sites within the polylinker and the insert to release the appropriate-size fragments. All constructs were sequenced using a standard dideoxy method [31].

Transient transfection/CAT assays

COS cells were transfected at 30 % confluence with 1–10 μg of caesium chloride-purified plasmid by a modification of a calcium phosphate-precipitation protocol [32]. The cells were generally co-transfected with 2 μg of pTKGH (Nichols Institute Diagnostics, San Juan Capistrano, CA, U.S.A.) to correct for transfection efficiency. At the time of harvest, the conditioned medium was sampled and assayed for human growth hormone by radioimmunoassay (Nichols Institute Diagnostics). CAT assays were performed as described in [32].

Glutathione S-transferase (GST) binding assay

Fragments of CBP were subcloned in-frame into a pGEX expression vector (Pharmacia, Piscataway, NJ, U.S.A.). GST-fusion proteins were expressed in *Escherichia coli* DH5α cells according to the manufacturer's instructions. Approximately 4 μg of fusion protein was bound to glutathione–agarose, washed four times in binding buffer (125 mM potassium acetate/25 mM Hepes, pH 7.4/2 mM EDTA/5 mM dithiothreitol/0.1 % Nonidet P-40/0.5 mM PMSF/1 μg/ml leupeptin/1 μg/ml aprotinin/1.5 μg/ml pepstatin A/0.2 mM levamisole/10 mM β-glycerophosphate/0.5 mM benzamidine) and incubated with 200 μl of PMA-treated HUVEC nuclear extract (10 μg protein/μl) for 4 h. The bound proteins were washed four times, eluted with 25 μl of SDS sample buffer (75 mM Tris/HCl, pH 6.8/5 % glycerol/1 % SDS/4 % 2-mercaptoethanol/0.01 % Bromophenol Blue) and boiled for 5 min before separation by SDS/PAGE (8 % gel). Eluted proteins were subject to Western-blot analysis as described below.

Immunoprecipitation/Western-blot analysis

Whole-cell extracts were prepared from HUVECs by mechanical disruption in 700 μl of lysis buffer (100 mM Tris/HCl, pH 7.6/100 mM NaCl/5 mM EDTA/0.5 % Triton X-100/0.5 mM PMSF/1 μg/ml leupeptin/1 μg/ml aprotinin/1.5 μg/ml pepstatin A/0.2 mM levamisole/10 mM β-glycerophosphate/0.5 mM benzamidine) per 100-mm² plate of confluent cells. Particulate matter was removed by centrifugation at 10000 ***g*** for 20 min. Supernatants were incubated with 10 μg/ml rabbit anti-Egr1, anti-p300, anti-CBP (all from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or non-immune IgG (Caltag, Burlingame, CA, U.S.A.) for 4 h at 4 °C with gentle agitation. The antibodies were bound to 50 μl of an antibody affinity gel containing goat antibody to rabbit IgG (ICN Pharmaceuticals, Aurora, OH, U.S.A.) or, in the case of ^{35}S-labelled proteins, 50 μl of protein A–agarose beads (Pierce, Rockford, IL, U.S.A.). The affinity gel was washed four times with 1 ml of lysis buffer and resuspended in SDS sample buffer. The eluted proteins were boiled for 3 min and separated by SDS/PAGE (6–10 %). Gels containing radiolabelled proteins were dried and exposed to film. Non-labelled proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, U.S.A.), blocked with 5 % non-fat dried milk in TBST buffer (20 mM Tris/HCl, pH 7.6/137 mM NaCl/0.5 % Tween 20), and incubated with anti-Egr-1, anti-p300 or anti-CBP antibodies for 1 h at room temperature. Blots were washed three times in TBST buffer, incubated with a donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington, IL, U.S.A.), and washed three times in TBST. Bound proteins were visualized using the ECL chemiluminescence reagent (Amersham) followed by autoradiography for 30 s–60 min.

Statistical analysis

Computations were performed using StatView® 4.5 software (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). Once differences among transfection groups were detected by ANOVA, *post hoc*

comparisons were made using the Scheffe's procedure. A linear-regression model was used to establish a significant correlation between the number of Egr-1 consensus sites and reporter activity. For all experiments, $n \geqslant 3$. Results are expressed as means $\pm$ S.D. and considered statistically significant at the $P < 0.05$ level.

RESULTS

CBP/p300 increases Egr-1-dependent transcription

The 5-LO promoter was used as a model of Egr-1-inducible gene transcription to study the effect of CBP/p300. The 5-LO promoter–reporter construct pCAT5LO-WT(5) contains a region of the wild-type core promoter between -294 and -15 bp relative to the transcription-initiation site upstream of the CAT gene. This region is GC-rich and contains five tandem Egr-1 consensus binding sites overlapping five tandem Sp1 consensus binding sites. When pCAT5LO-WT(5) was co-transfected in COS cells with a CMV-based expression construct over-expressing Egr-1 (pCMVEgr-1), reporter activity increased approximately 4-fold ($P < 0.001$) (Table 1a). Similarly, over-expression of Sp1 (pCMVSP1) increased reporter activity approximately 2-fold. When CBP was added to the system by co-transfection with an RSV-based expression construct (pRSVCBP), as well as pCMVEgr-1, reporter activity increased approximately 11-fold ($P < 0.001$). In contrast, when CBP was co-transfected with Sp1, no increase in reporter activity was observed, suggesting that CBP does not interact with Sp1. pRSVCBP without pCMVEgr-1 had little effect on 5-LO reporter-construct activity, suggesting that it was the combination of Egr-1 and CBP that had the most intensive effect on *trans*-activation. Western-blot analysis of nuclear extracts showed that over-expression of CBP did not change Egr-1 levels, and that changes in Egr-1 with CBP over-expression were not responsible for augmented transcription (see Table 1, insert). These data are consistent with the idea that the transcriptional co-activator CBP is present in COS cells in limiting amounts and that its over-expression increases Egr-1-dependent 5-LO reporter transcription but not Sp1-dependent 5-LO reporter transcription.

The GC-rich region of the 5-LO promoter contains naturally occurring mutations that consist of additions or deletions of tandem Egr-1 consensus binding sites. The wild-type promoter contains five tandem Egr-1 consensus sites and the reporter construct containing its promoter is designated 5LOpCAT-WT(5). The mutant promoter–reporter constructs designated 5LOpCAT-M(4) and 5LOpCAT-M(3) have lost one and two Egr-1 binding sites, and therefore contain four and three tandem Egr-1 sites, respectively. For constructs 5LOpCAT-M(3), 5LOpCAT-M(4) and 5LOpCAT-WT(5), co-transfection of COS cells with reporter construct plus the CBP expression construct, pRSVCBP, resulted in increased CAT activity, $P < 0.01$ (Table 1b). The backbone reporter construct pCAT®-basic contains no Egr-1 binding site and was not significantly responsive to CBP. Moreover, linear-regression analysis indicated that the degree of *trans*-activation was proportional to the number of tandem Egr-1 consensus binding sites within the promoter ($R^2 = 0.62$,

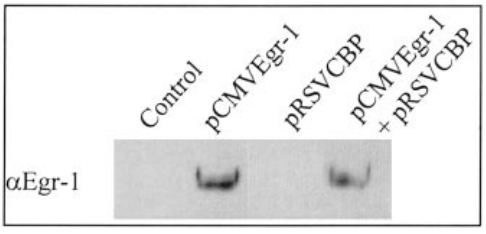
Table 1 (a) CBP increases Egr-1-mediated transcription of a 5-LO promoter–reporter construct and (b) comparison of naturally occurring 5-LO mutants shows that the degree of CBP reporter activation is proportional to the number of Egr-1 consensus binding sites

(a) CAT activity of COS cells transfected with pCAT5LO-WT(5) (10 μg), with or without co-transfection of Egr-1 (1 μg), Sp1 (1 μg) or CBP (10 μg) expression plasmids was measured after 24 h. Total DNA was kept constant by using empty vectors pCR-RSV and pCR3(CMV). CAT activity (c.p.m.) for 3 experiments is expressed as means $\pm$ S.D. Insert shows Egr-1 quantification by Western-blot analysis of COS nuclear extracts. (**b**) 5-LO reporter plasmid (10 μg) was transfected alone or with 5 μg of pRSVCBP and harvested 24 h later for CAT assays. CAT activity for 3 experiments is expressed as means $\pm$ S.D.

(a)

pCAT5LO WT(5) (μg)...	10	10	10	10	10	10
pCMVEgr-1 (μg)...	–	1	–	–	1	–
pCMVSp1 (μg)...	–	–	1	–	–	1
pRSVCBP (μg)...	–	–	–	10	10	10
CAT activity	740 $\pm$ 125	3130 $\pm$ 88*	1402 $\pm$ 255	1699 $\pm$ 255	8122 $\pm$ 303†	1135 $\pm$ 8

* Greater than pCAT5LO-WT(5) alone, $P < 0.001$.
† Greater than all other transfections, $P < 0.001$.



(b)

	5LOpCAT-0		5LOpCAT-M(3)		5LOpCAT-M(4)		5LOpCAT-WT	
pRSVCBP (μg)...	–	5	–	5	–	5	–	5
CAT activity	486 $\pm$ 84	500 $\pm$ 35	942 $\pm$ 142	1771 $\pm$ 248	1407 $\pm$ 95	5778 $\pm$ 835	2893 $\pm$ 423	12773 $\pm$ 3949

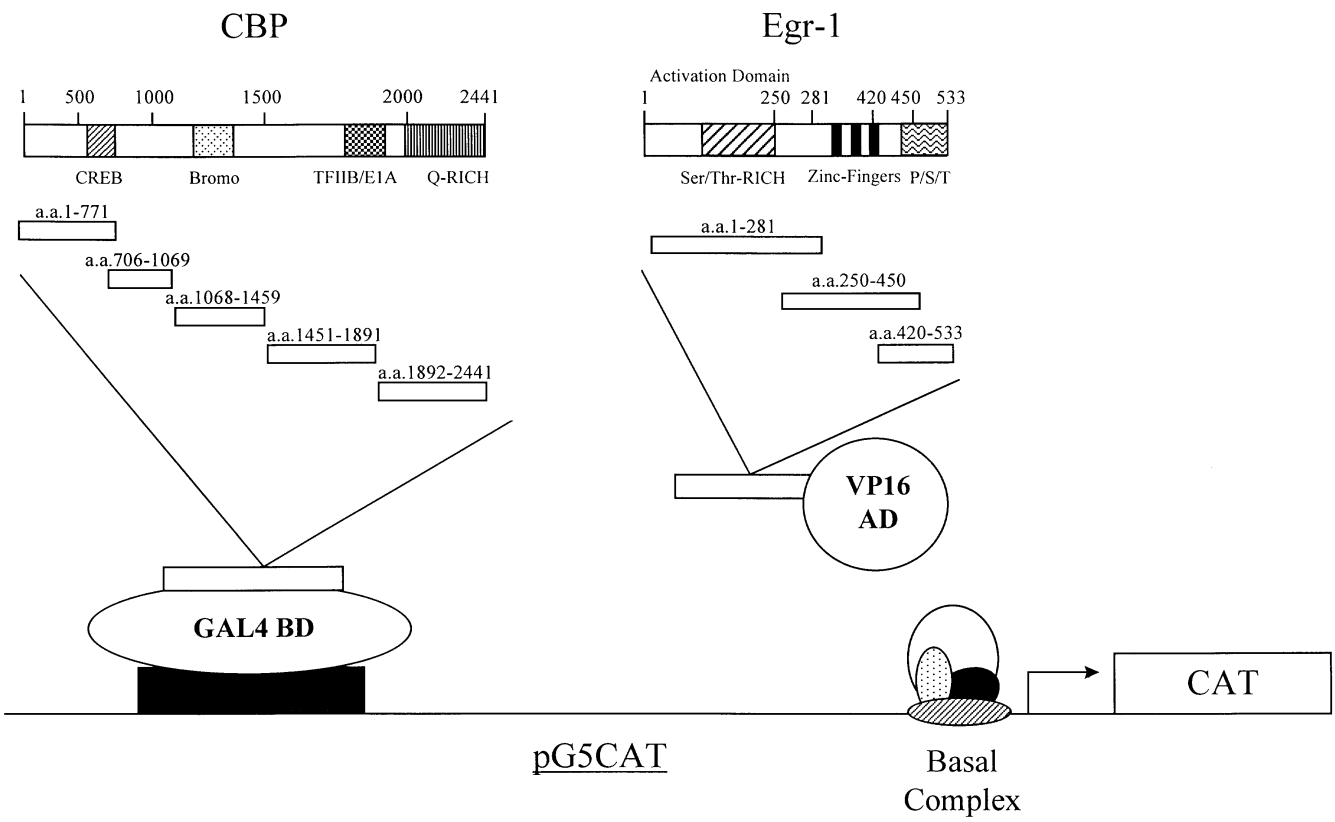


Figure 1 CBP and Egr-1 interact physically, *in vivo*, in a mammalian two-hybrid system

The structure of the GAL4–CBP and VP16–Egr-1 constructs are shown. Five overlapping CBP protein fragments consisting of aa 1–771, aa 706–1069, aa 1068–1459, aa 1451–1891 and aa 1892–2441 were fused to the DNA-binding protein GAL4. Three overlapping Egr-1 protein fragments consisting of aa 1–281, aa 250–450 and aa 420–533 were fused to the transcriptional-activation domain (AD) VP16. TFIIB, transcription factor IIB; EIA, adenovirus EIA protein; Q-rich, glutamine rich; P/S/T, proline/serine/threonine.

Table 2 (a) COS cells were transfected with 2 μg of expression constructs and 10 μg of pG5CAT reporter construct, and relative CAT activity was measured after 24 h, and (b) two-hybrid assay comparing p65 and Egr-1 interactions with CBP

(**a**) Values are expressed as the means ± S.D. of 3 experiments. (**b**) p65 is known to interact with the N- and C-terminal regions of CBP. Each indicated expression construct (2 μg) was transfected into COS cells along with 10 μg of pG5CAT. Cells were harvested after 24 h and CAT activity (c.p.m.) was measured. CAT activity is expressed as the means ± S.D. of 3 experiments.

(a)

	pMCBP 1–771	pMCBP 706–1069	pMCBP 1068–1459	pMCBP 11451–1891	pMCBP 1892–2441	No pMCBP
No VP16Egr	13982 ± 3539	368 ± 97	280 ± 50	308 ± 35	7515 ± 1038	350 ± 50
VP16Egr 1–281	28686 ± 4917*	400 ± 180	326 ± 78	329 ± 83	30342 ± 2342*	286 ± 77
VP16Egr 250–450	16532 ± 953	307 ± 33	248 ± 38	266 ± 69	12999 ± 3234	311 ± 49
VP16Egr 420–533	11845 ± 2366	415 ± 77	317 ± 48	318 ± 32	10294 ± 1508	401 ± 83

* Greater than all other values, $P \leqslant 0.01$, except each other, $P > 0.99$.

(b)

VP16Egr 1–281 (μg) ...	–	2	–	–	2	–	–	2	–
VP16Egr 286–551 (μg) ...	–	–	2	–	–	2	–	–	2
pMCBP 1–771 (μg) ...	–	–	–	2	2	2	–	–	–
pMCBP 1892–2441 (μg) ...	–	–	–	–	–	–	2	2	2
CAT activity	468 ± 58	241 ± 32	259 ± 17	452 ± 11	1280 ± 39	4886 ± 178	740 ± 138	2786 ± 78	4345 ± 98

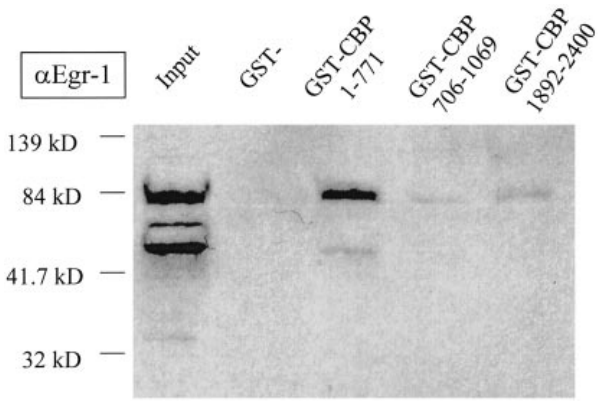


Figure 2 ***In vitro*** **binding of Egr-1 to a CBP–GST fusion protein**

Three overlapping fragments of CBP were fused to GST and linked to glutathione–agarose beads to form an affinity matrix. PMA-treated HUVEC extract contained Egr-1, as shown in the input lane. Approximately 200 μl (10 μg protein/μl) of PMA-treated HUVEC nuclear extract was incubated with each matrix for 4 h. After washing, the bound proteins were eluted and detected by Western-blot analysis. Molecular-mass (kD) markers are indicated.

$P = 0.002$). These studies also suggest that Egr-1 and CBP/p300 can interact functionally to augment 5-LO transcription.

Physical interactions between CBP/p300 and Egr-1

Three approaches were used to demonstrate that Egr-1 and CBP/p300 bind physically through protein–protein interactions: (i) a mammalian two-hybrid system; (ii) GST-fusion protein interactions; and (iii) co-immunoprecipitation/Western-blot analysis.

CBP/p300 interacts with Egr-1 in a two-hybrid system

For the two-hybrid studies, we generated a series of five overlapping CBP protein fragments (namely aa 1–771, aa 706–1069, aa 1068–1459, aa 1451–1891 and aa 1892–2441; Figure 1) that were fused to the DNA-binding protein GAL4. Similarly, three overlapping Egr-1 protein fragments were fused to the transcriptional-activation domain VP16 (aa 1–281, aa 250–450 and aa 420–533; Figure 1). The domains of CBP and Egr-1 capable of physically interacting were identified by using the mammalian two-hybrid system in COS cells. All permutations of the fusion-protein expression constructs were co-transfected along with a reporter construct containing a GAL4 consensus binding site (pG5CAT). When transfected alone, the N- and C-terminal portions of CBP (aa 1–771 and aa 1892–2441) were capable of *trans*-activation in this system (Table 2a). These findings are in agreement with reports suggesting that CBP/p300 may function as a transcriptional activator when bound to DNA through a heterologous DNA-binding domain [16]. The highest levels of CAT activity were obtained when the N-terminal region of Egr-1 (aa 1–281) was co-transfected with either the N-terminal region of CBP (aa 1–771) or the C-terminal region of CBP (aa 1892–2441) (Table 2a). Both CAT values are significantly greater than any other, $P \leqslant 0.01$, but not different from each other, $P > 0.99$. These data suggest that the N-terminal region of Egr-1, containing the *trans*-activation domain, was capable of interacting with the N- and C-termini of CBP.

To better assess the relative strength of these interactions we directly compared Egr-1 with the nuclear factor-κB component

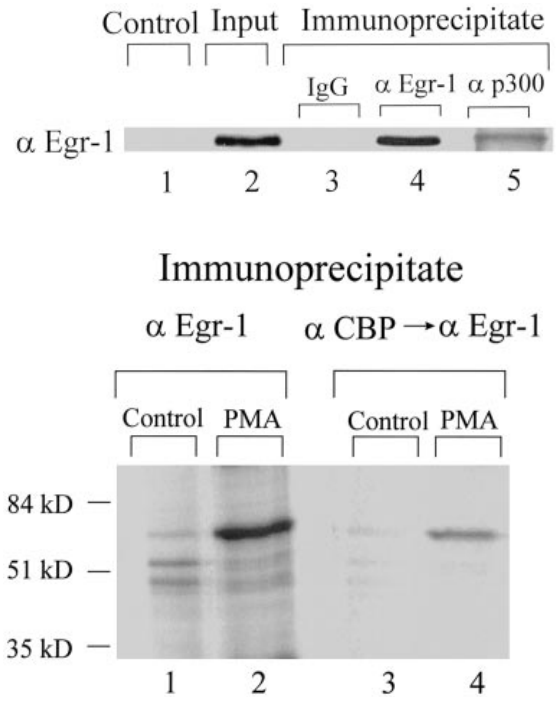


Figure 3 Co-immunoprecipitation of Egr-1 and CBP/p300

(Top panel) PMA treatment of HUVECs induces Egr-1 synthesis, as detected by Western-blot analysis (lane 2). No Egr-1 is detected in extract from non-PMA-treated (Control) cells (lane 1). Approximately 700 μl (5 μg of protein/μl) of Egr-1-containing extract was incubated with 50 μl of an antibody affinity gel for 4 h at 4 °C with gentle agitation. After extensive washing, retained proteins were detected by Western-blot analysis. Both anti-Egr-1 and anti-p300 antibodies retained Egr-1 (lanes 4 and 5). Identical quantities of non-immune IgG did not retain Egr-1 (lane 3). (Lower panel) Similar findings were obtained using [^{35}S]methionine-labelled proteins. After extensive washings, retained proteins were eluted, separated by SDS/PAGE and detected by autoradiography. Anti-Egr-1 and anti-CBP antibodies were both capable of retaining PMA-induced Egr-1 (lanes 2 and 4). Molecular-mass (kD) markers are indicated.

p65(RelA) in our two-hybrid system. CBP and p300 are established transcriptional co-activators of p65 [26]. Protein–protein interactions were confirmed using the two-hybrid assay and suggest that the C-terminal *trans*-activation region of p65, aa 286–551, interacts strongly with CBP [26]. In addition, as with Egr-1, p65 can interact with both the N- and C-terminal regions of CBP. When the C-terminal region of p65 (aa 286–551) was co-transfected with either the N-terminal region of CBP (aa 1–771) or the C-terminal region of CBP (aa 1892–2441), highest CAT activity was obtained (Table 2b). Although statistically significant ($P < 0.001$), these values were not much greater than those obtained with the N-terminal region of Egr-1 (aa 1–281): four times greater for the N-terminal interaction and less than twice as great for the C-terminal interaction. This direct comparison suggests that the interaction between Egr-1 and CBP is significant.

Egr-1 binds to a CBP/p300–GST-fusion protein

Protein–protein interactions between Egr-1 and CBP were detected, *in vitro*, using GST-fusion proteins. Three CBP fragments, the N-terminal 1–771 aa, the C-terminal 1892–2400 aa and the central 706–1069 aa, were fused to GST and linked to glutathione–agarose beads to form an affinity matrix. Egr-1 was strongly bound to the N-terminal fragment and only weakly associated with the C-terminal region (Figure 2). Minimal binding

to the central region of CBP was detected. These findings are consistent with the two-hybrid system and suggest that Egr-1 is capable of protein–protein interactions with CBP involving the N-terminal region and possibly the C-terminal region of CBP.

Egr-1 binds CBP/p300 as detected by co-immunoprecipitation

To further support our hypothesis that Egr-1 and CBP/p300 bind in a more physiologically relevant environment, the two proteins were co-immunoprecipitated from PMA-treated HUVEC extract. When HUVECs were treated with PMA, Egr-1 was induced as detected by Western-blot analysis of whole-cell extracts (Figure 3, top panel, compare lanes 1 and 2). Levels of CBP/p300 did not change after PMA treatment (results not shown). When the cell extract was incubated with a matrix containing Egr-1 or p300 antibodies, the matrix was capable of retaining Egr-1 (Figure 3, top panel, lanes 4 and 5). In contrast, a matrix containing non-immune IgG was unable to retain Egr-1, suggesting that the interaction was specific (Figure 3, top panel, lane 3). Conversely, it was also possible to co-immunoprecipitate p300 with antibodies to Egr-1 (results not shown).

As a complementary approach, we radiolabelled proteins with [^{35}S]methionine, subjected samples to sequential immunoprecipitations and detected retained proteins by autoradiography. When extracts of PMA-treated cells were added to protein A–agarose beads with bound anti-Egr-1 antibodies, Egr-1 was detected (Figure 3, lower panel, lane 2), whereas extracts from non-PMA-treated cells did not contain any detectable Egr-1 (Figure 3, lower panel, lane 1). Immunoprecipitation with anti-CBP antibodies prior to Egr-1 immunoprecipitation also caused retention of Egr-1 (Figure 3, lower panel, lane 4). Non-PMA-treated cell extract immunoprecipitated with anti-CBP antibodies prior to Egr-1 immunoprecipitation did not contain detectable Egr-1 (Figure 3, lower panel, lane 3).

Collectively, these studies demonstrate both functional and physical interactions between Egr-1 and CPB/p300.

DISCUSSION

The transcriptional co-activators CBP and p300 functionally link sequence-specific transcription factors with the basal transcription apparatus to regulate the recruitment of RNA polymerase II and the initiation of gene transcription. In this study, we add Egr-1 to the growing list of sequence-specific transcription factors that function via a CBP/p300-dependent pathway. We have shown that CBP/p300 is a limiting component of Egr-1-mediated transcription and that its over-expression increases transcription proportional to the number of Egr-1 consensus binding sites. In contrast, Sp1 does not appear to interact with CBP/p300. We have also used several complementary, techniques, *in vivo* and *in vitro*, to demonstrate that the N-terminal region of Egr-1 binds to the N- and C-terminal regions of CPB/p300 via protein–protein interactions.

Many genes have GC-rich promoter sequences that contain multiple overlapping Egr-1/Sp1 sites. For example, the wild-type 5-LO gene contains five Egr-1 sites overlapping with five Sp1 sites, the platelet-derived growth factor A-chain gene contains three Egr-1 sites overlapping with two Sp1 sites, the tissue-factor promoter has two Sp1 sites overlapping with one Egr-1 site, and the transforming growth factor-β promoter has one Egr-1 site overlapping with one Sp1 site. Under quiescent conditions Sp1 may occupy these promoter regions and mediate basal levels of transcription. In response to several types of inducible stimuli, Egr-1 increases and displaces Sp1 from the promoter. This displacement phenomenon is associated with higher levels of transcription and may represent a common mechanism of inducible gene expression [12]. In this study, we have used the wild-type and naturally occurring mutant forms of the 5-LO promoter as a model of Egr-1-inducible gene transcription.

5-LO is a calcium-, ATP- and non-haem iron-requiring enzyme that catalyses the two-step lipoxygenation of arachidonic acid to form leukotriene A_4. It is expressed primarily in leucocytes and is essential for the formation of all leukotrienes (for review, see [33]). 5-LO gene expression is modulated by a number of mechanisms, including transcriptional controls that involve Egr-1. The wild-type 5-LO promoter has a unique GC-rich sequence, located between 176 and 147 bp upstream of the ATG translation start site, which contains five tandem Egr-1 consensus binding sites overlapping five tandem Sp1 sites [34]. Egr-1 binds specifically to this region and its over-expression augments the activity of 5-LO promoter–reporter constructs [8,9]. Likewise, Sp1 binds and activates this region of the 5-LO promoter, although it does not interact with CBP, consistent with previous reports [26].

The 5-LO gene is quite novel in that it is the only gene in the GenBank database that contains five Sp1 and five Egr-l binding motifs in tandem. The promoter is also unique among these genes because several naturally occurring mutations have been described that contain additions or deletions of these Egr-1 binding sites. In particular, we have discovered a series of mutations in asthmatic patients in which there are deletions of one or two, or addition of one, Egr-1/Sp1 binding motifs (i.e. GGGCGG) [8]. These promoter mutations alter Egr-1 binding events, as detected by electrophoretic mobility-shift assay, and promoter function as measured by reporter-construct activity. Moreover, the *trans*-activation potential of Egr-1 is directly proportional to the number of tandem Egr-1 consensus binding sites. The mechanisms by which single or multiple tandem Egr-1 sites potentiate *trans*-activation are unknown.

It has been widely observed that the introduction of a single transcription-factor binding site upstream of a minimal promoter results in relatively little transcription, whereas multiple binding sites for the same factor result in high levels of transcription. Our finding that CBP/p300 increases the transcriptional response from multimerized Egr-1 sites in the 5-LO promoter may provide some mechanistic insights into this response. A co-activator would not be expected to be able to interact with two molecules of the same transcription factor through a single interaction domain simultaneously. However, we have shown through studies of protein fragments that the activation region of Egr-1 makes at least two distinct contacts with CBP/p300, one at the N-terminal and another at the C-terminal region of the molecule. The increased transcriptional response associated with multimerization of sites may result from the ability of Egr-1 to simultaneously interact with two or more regions of a single CBP/p300 molecule, or with more than one protein in the co-activator complex. Appropriately spaced Egr-1 binding sites would be expected to maximize these interactions and be more effective in the recruitment of the co-activator complex, resulting in higher levels of transcription. By changing the number or helical phasing of the Egr-1 sites, the naturally occurring mutations in the 5-LO promoter may alter the effectiveness of CBP recruitment and diminish or enhance the expression of the gene.

CBP may also play a role in the functional interactions between Egr-1 and other transcriptional activators. Egr-1 and the nuclear factor-κB component p65 can interact synergistically in the context of the p105 promoter [35], and both are required for maximal expression of the intercellular adhesion molecule-1 [36], tumour necrosis factor-α [37] and interleukin-2 [38]

promoters. Comparable with our findings with Egr-1, the p65 component also binds to both the N- and C-terminal regions of CBP [26]. Although the regions bound by these activators are similar, these CBP binding sites may not overlap, permitting simultaneous occupancy. Because the transcription factors can independently recruit CBP to a promoter, simultaneous activation of these factors could lead to more efficient recruitment of CBP and synergistic transcriptional responses. It is interesting to speculate that negative regulators of Egr-1, such as nerve-growth-factor-induced binding protein (NAB)-1 [39] and NAB-2 [40], may disrupt the interaction between the Egr-1 and CBP and decrease transcription of target genes.

CBP and p300 function as integrators of diverse signal pathways. Because CBP and p300 seem to be limiting components of cells, transcription factors activated by one signal pathway may interfere with one another by competing for CBP/p300. In this way expression of Egr-1-dependent genes may antagonize other signal-dependent activators. Competition for CBP/p300 may allow cross-talk between different signalling systems important in regulating responses to growth factors and stress signals.

The authors wish to thank K. Case, W. Atkinson, L. Khachigian, M. Frosch, M. Reid, M. Gerritsen, T. Bigby, A. Neish and M. Gimbrone Jr. for their assistance and invaluable suggestions, and R. Eckner, D. Livingston, R. Goodman and V. P. Sukhatme for providing expression constructs. This work was supported in part by HL09613 to E. S. S., PO1 HL36110 to J. M. D. and HL35716, HL45462 and PO1 HL36028 to T. C.

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Received 22 June 1998/19 August 1998; accepted 8 September 1998