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Specificity of Transcriptional Regulation by the Zinc Finger Transcription Factors Sp1, Sp3, and Egr-1

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Abstract The transcription factors Sp1, Sp3, and Egr-1 bind with their zinc finger DNA-binding domains to GC-rich sequences in the regulatory regions of their target genes. The similarity of the DNA-binding sites of Sp1, Sp3, and Egr-1 has triggered the hypothesis that they compete for the same DNA-binding site. We have investigated the specificity of transcriptional regulation by Sp1, Sp3, and Egr-1 using dominant-negative mutants that block the DNA-binding site of Sp1, Sp3, or Egr-1, respectively. The results show that constitutive transcription of Sp1 regulated reporter genes, containing Sp1 sites derived from the *aldolase C* and *p21*$^{WAF1/Cip1}$ genes, or the long terminal repeat of HIV-1, was impaired by dominant-negative mutants of Sp1 and Sp3, but not by a dominant-negative Egr-1. Transcription mediated by Egr-1 was induced by transfection of expression vectors encoding wild-type or mutated Egr-1 or by stimulation of the extracellular signal-regulated protein kinase pathway via an inducible B-Raf-estrogen receptor fusion protein. In all cases transcription of Egr-1-regulated reporter genes, containing Egr-1 binding sites derived from the Egr-1 or the *synapsin I* gene was impaired by a dominant-negative Egr-1, but not by dominant-negative Sp1 or Sp3 mutants. These results show that there are genuine Sp1/Sp3 or Egr-1 controlled genes showing no cross-regulation of Sp1/Sp3 and Egr-1 through the same DNA-binding site. This does not exclude the existence of composite Sp1/Sp3/Egr-1 binding sites, where competition for a common DNA-binding site occurs. J. Cell. Biochem. 94: 153–167, 2005.

Key words: Sp1; Sp3; Egr-1; p21$^{WAF1/Cip1}$; angiotensin-converting enzyme

The transcription factor Sp1 was originally discovered as a DNA-binding protein that interacts with multiple 5′-GGGCGG-3′ sequences (GC boxes) of the SV40 early promoter [Kadonaga et al., 1987]. Sp1 is ubiquitously expressed and single or multiple Sp1 binding sites are found in many cellular and viral promoters. The transcription factor Sp3 shares several features with Sp1, including the ubiquitous expression, the glutamine and serine/threonine rich-domains and the zinc finger DNA-binding region. The zinc finger domains of Sp1 and Sp3 show 90% homology and the specificity and affinity of Sp1 and Sp3 to bind to the GC box is very similar [Hagen et al., 1992; Kingsley and Winoto, 1992]. Sp1 and Sp3 recognize the classical GC-rich sequence 5′-GGGCGG-3′. In contrast to the constitutive expression of Sp1 and Sp3, synthesis of the zinc finger transcription factor Egr-1, also known as zif268, NGFI-A, Krox24, and TIS8 [Lim et al., 1987; Milbrandt, 1987; Christy et al., 1988; Lemaire et al., 1988; Sukhatme et al., 1988], is negligible in unstimulated cells. However, a variety of environmental signals including growth factors, hormones, and neurotransmitters induce a robust and transient expression of Egr-1 [reviewed by Thiel and Cibelli, 2002], showing that Egr-1 couples extracellular signals to long-term responses by altering Egr-1 target gene transcription. The DNA-binding domain of Egr-1 contains three zinc finger motifs. The structure of a complex formed between

Abbreviations used: ACE, angiotensin-converting enzyme; Egr-1, early growth response 1; GST, glutathione S-transferase; 4OHT, 4-hydroxytamoxifen.

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these three zinc fingers and its cognate DNA-binding site has been solved [Pavletich and Pabo, 1991]. Egr-1 preferentially binds to the GC-rich sequence 5′-GCGGGGGCG-3′ [Christy and Nathans, 1989; Cao et al., 1993].

The transcription factors of the Sp1 family, including Sp1 and Sp3, compete for DNA-binding to the GC-box, due to the homology of their DNA-binding domains. A comparison of Sp1 and Egr-1 binding specificities to DNA reveals that the DNA-binding sites of Egr-1 (GCG GGG GCG = A B A) and Sp1 (GGG GCG GGG = B A B) are similar and appear as a rearrangement of one another (A B A vs. B A B) [Kriwacki et al., 1992]. In addition, the free solution structures of the Cys_2-His_2-zinc finger domains 2 and 3 of Sp1 are very similar to those of Egr-1 [Narayan et al., 1997], further suggesting that Sp1 and Egr-1 have similar DNA-binding specificities. These facts provoked the hypothesis that Egr-1 competes with Sp1 and members of the Sp1 family of transcription factors for DNA-binding [Huang et al., 1997] and, in fact, a competition between Sp1 and Egr-1 has been demonstrated for the gene encoding the platelet-derived growth factor (PDGF) A chain. In cultured quiescent vascular endothelial cells, a GC-rich genetic element within the PDGF A chain promoter is occupied by Sp1. Stimulation of cells with phorbol ester, however, triggers the biosynthesis of Egr-1 that subsequently displaces Sp1 from the GC-rich sequence motif [Khachigian et al., 1995]. Overlapping Sp1/Egr-1 binding sites have also been described in the regulatory region of the genes encoding PDGF B chain, adenosine deaminase, tissue factor, thrombospondin 1, monoamine oxidase B, ABCA2 transporter, and β_1-adrenergic receptor [Ackerman et al., 1991; Shingu and Bornstein, 1994; Cui et al., 1996; Khachigian et al., 1996; Bahouth et al., 2002; Davis et al., 2003].

In this study, we have used dominant-negative mutants of Sp1, Sp3, or Egr-1 to investigate the specificity of transcriptional activation by Sp1, Sp3, and Egr-1. These mutants contain an intact DNA-binding region but lack any transcriptional activation function. Thus, putative Sp1, Sp3, or Egr-1 DNA-binding sites are blocked by these mutants. Given the fact that Sp1, Sp1 related transcription factors, and Egr-1 have been shown to compete for DNA-binding, the dominant-negative mutants solely copies nature's method of competition for a common DNA-binding site. Moreover, compensation due to the activity of related and potentially redundant proteins of the Sp1 or Egr-1 families of transcription factors that occurs readily in the case of transgenic mice models, was eliminated by this approach. The results show that while Sp1 and Sp3 generally compete for the same DNA-binding site, Egr-1 has a distinct sequence requirement to bind and activate Egr-1 responsive target genes. This study also demonstrates that dominant-negative Sp1, Sp3, and Egr-1 proteins are valuable tools to investigate the impact of Sp1, Sp3, and Egr-1 in transcriptional regulation of putative Sp1, Sp3, and/or Egr-1 target genes in living cells.

MATERIALS AND METHODS

Reporter Constructs

Plasmids pHIVTATAluc, pEBS1^{4}luc, and pACE230 luc have been described [Thiel et al., 2000; Cibelli et al., 2002; Day et al., 2004]. The minimal Egr-1-responsive reporter plasmid pEBS2^{4}luc containing four binding sites for Egr-1 derived from the human synapsin I promoter was constructed by subcloning a SacI/SalI fragment derived from plasmid pEBS2^{4}O-VEC [Thiel et al., 1994] into the SacI/XhoI digested plasmid pHIVTATAluc. The minimal Sp1-responsive reporter plasmid pAldGCB^{4}luc contains four binding sites for Sp1 derived from the aldolase C promoter. This plasmid was generated by subcloning a filled-in XbaI/SacI fragment derived from plasmid pGCB^{4}CAT [Cibelli et al., 1996] into pHIVTATAluc. The transcription units present in the reporter plasmids pEBS1^{4}luc, pEBS2^{4}luc, and pAldGC-B^{4}luc contain a minimal promoter consisting of the human immunodeficiency virus TATA box and the adenovirus major late promoter initiator element. Plasmid pGL3-HIV-1 LTR that directs luciferase transcription under the control of the HIV long terminal repeat (sequence from -120 to $+83$) was a kind gift of Jakob Troppmair, Julius-Maximilians-University, Würzburg, Germany. The p21$^{WAF1/Cip1}$ promoter/luciferase reporter plasmid p21Pluc [Datto et al., 1995] was kindly provided by Xiao-Fan Wang, Cell and Molecular Biology Program, Duke University (Durham, NC).

Expression Vectors

The expression vectors pEBGN and pEBGN-Sp1 encoding glutathione S-transferase (GST)

and a fusion protein consisting of GST and the zinc finger DNA-binding domain of Sp1, respectively, have been described [Petersohn and Thiel, 1996; Thiel and Cibelli, 1999]. Similarly, expression vectors encoding GST-Sp3 or GST-Egr-1 fusion proteins were generated. The cDNA encoding human Sp3 was purchased from ATCC (ATCC # 95505, accession number M97191). To construct an expression vector encoding a GST-Sp3 fusion protein, we cloned a KpnI fragment encoding the C-terminal DNA-binding domain of Sp3, into KpnI cut pEBGN. The GST-Sp3 fusion protein contains amino acids 485–653 of Sp3, using the assignment of Kingsley and Winoto [1992]. The expression vector pEBGN-Egr-1, encoding a GST-Egr-1 fusion protein, was generated by inserting a blunt-ended BglII/SmaI fragment of the murine Egr-1 cDNA into the filled-in NotI site of pEBGN. The GST-Egr-1 fusion protein contains amino acids 322–533 of Egr-1, encompassing the zinc finger DNA-binding site. The expression vector of murine Egr-1, pCMVEgr-1, formerly termed pCMVzif, has been described [Thiel et al., 1994]. An expression vector encoding a CREB2/Egr-1 fusion protein (C2/Egr-1) was made by insertion of a filled-in BglII/SmaI fragment derived from the murine Egr-1 cDNA into EcoRV cut pCMV-FLAG-C2N [Steinmüller and Thiel, 2003]. The CREB2/Egr-1 fusion protein encodes amino acids 1–187 from CREB2 fused to amino acids 322–533 of Egr-1. In addition, a triple FLAG tag (sequence MDYKDHDGDYKDHDIDYKDDDDK) was present on the N-terminus. Expression vectors pRSVβ and pSV40lacZ, encoding β-galactosidase under the control of the Rous sarcoma virus long terminal repeat or the SV40 promoter, have been described [Jüngling et al., 1994; Schoch et al., 2001].

Cell Culture, Transfections, and Reporter Gene Assays

The human 293T embryonal kidney cells were cultured and transfected as described [Kaufmann et al., 2001]. The amounts of expression vectors transfected are indicated in the figure legends. The luciferase reporter plasmids and the internal reference plasmid pRSVβ or pSV40LacZ were transfected into cells grown on 60 mm plates. Twenty-four hours post-transfection, the serum concentration was lowered to 0.05% and the cells were incubated for further 24 h. Lysates were prepared using cell culture lysis buffer (Promega) and β-galactosidase and luciferase activities were measured [Thiel et al., 2000]. Each experiment was repeated at least two times with consistent results. Stimulation of 293TΔB-Raf:ER cells with 4-hydroxytamoxifen (4OHT, used at a concentration of 200 nM with ethanol as solvent) was done for 24 h or as indicated in the figure legend.

Retroviral Gene Transfer

Plasmid pBabepuro3ΔB-Raf:ER, encoding an activated form of the protein kinase B-Raf as a fusion protein with the hormone binding domain of the murine estrogen receptor (ER^{TM}), was kindly provided by Martin McMahon, Cancer Research Institute and Department of Cellular and Molecular Pharmacology, UCSF, San Francisco, CA. The packaging cell line Phoenix-Ampho was obtained from Gary Nolan, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA. Cells were transfected with retroviral vectors using the calcium coprecipitation procedure. Retroviral infection of 293T cells were performed as described [Rössler et al., 2004]. 293T cells were selected with 0.6 μg puromycin/ml. Mass pools of stable transfectants were selected and used for all experiments in order to eliminate the possibility of specific clonal effects.

RNase Protection Assay

The template for human Egr-1 cRNA synthesis (plasmid pT7-hEgr1-1) has been described [Kaufmann and Thiel, 2001]. Plasmid T3hp21-1 was generated by subcloning of a EheI/Bsp120I fragment from plasmid pCEP-WAF1 [El-Deiry et al., 1993] into pBluescriptKSII. The plasmid was used to synthesize a riboprobe specific for human $p21^{WAF1/Cip1}$. The $p21^{WAF1/Cip1}$ expression vector pCEP-WAF1 was a kind gift of Bert Vogelstein, Johns Hopkins University, Baltimore, MD. Plasmid pSP6-G3PDH used for the synthesis of a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cRNA probe was purchased from Ambion. 293Tpac and 293TΔB-Raf:ER cells were seeded at a density of 1.5×10^6 cells/100 mm plate and incubated in medium containing 10% serum overnight. The serum concentration was reduced to 0.05% and the cells were cultivated for another 24 h. Cells were stimulated with 4OHT (200 nM) for 5 or 8 h, washed with cold phosphate-buffered saline (PBS, 170 mM NaCl, 33 mM KCl, 40 mM

Na_2HPO_4, 18 mM KH_2PO_4, pH 7.2) and harvested. Cytoplasmic RNA was prepared and analyzed by RNase protection mapping as described [Thiel et al., 2000]. Thirty-microgram cytoplasmic RNA was used for the analysis of human Egr-1, and 2.5 μg RNA for the detection of GAPDH mRNA. Hybridization of Egr-1, $p21^{WAF1/Cip1}$, or GAPDH mRNAs with these riboprobes protected fragments of 210, 250, or 417 nucleotides, respectively, from RNase digestion.

Western Blots

Nuclear extracts were prepared as described [Kaufmann and Thiel, 2002]. To analyze Egr-1 biosynthesis, 20 μg of nuclear proteins were separated by SDS–PAGE and the blots were incubated with an antibody directed against human Egr-1 (Santa Cruz, Heidelberg, Germany, #sc-110). Immunoreactive bands were detected using the ECL plus system (Amersham).

RESULTS

Modular Structure of Dominant-Negative Sp1, Sp3, and Egr-1 Mutants

Sp1 is constitutively expressed and is responsible for the constitutive transcriptional activity of many genes. An analysis of Sp1 mediated gene transcription requires therefore a loss-of function instead of a gain-of function approach. Some years ago, we designed a dominant-negative Sp1 mutant [Petersohn and Thiel, 1996], consisting of glutathione S-transferase fused to the DNA-binding domain of Sp1. Both domains were separated by a nuclear localization sequence to ensure nuclear targeting. This mutant, which blocks the Sp1 DNA-binding site, has been successfully used in several studies addressing Sp1-mediated gene transcription [Petersohn and Thiel, 1996; Lietz et al., 2003] and biological functions of Sp1 [Kavurma et al., 2001; Kavurma and Khachigian, 2003]. To extend these studies and to compare the transcriptional targets of Sp1 with those of the zinc finger transcription factors Sp3 and Egr-1, we designed dominant-negative mutants for Sp3 and Egr-1. Accordingly, these mutants lack the N-terminal activation domains. Instead, glutathione S-transferase was fused to the DNA-binding domain of Sp3 and Egr-1, respectively. The modular structure of the GST-Sp1, GST-Sp3, and GST-Egr-1 proteins is depicted in Figure 1A.

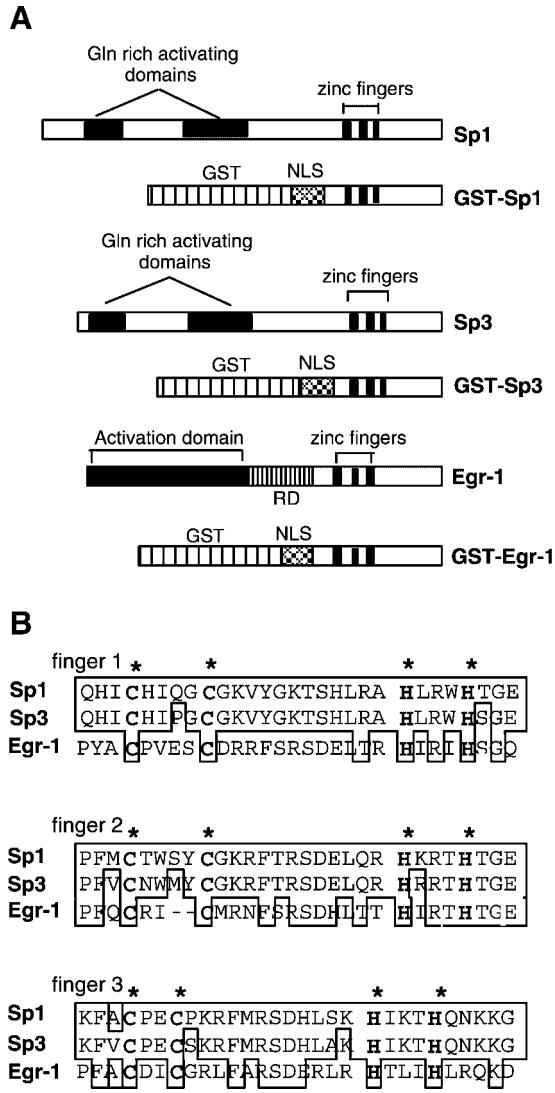
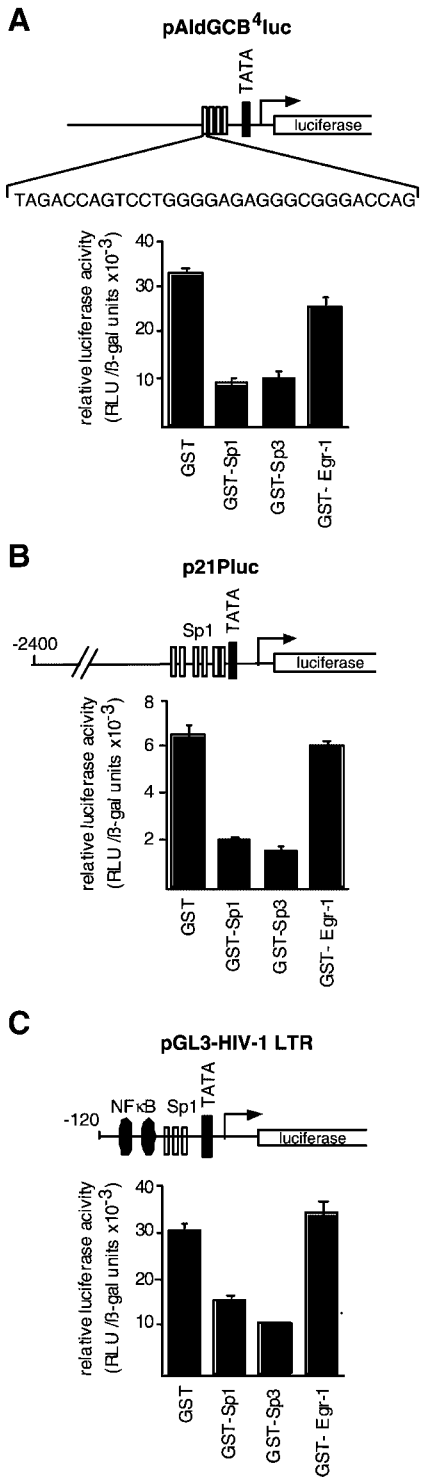


Fig. 1. Modular structure of Sp1, Sp3, Egr-1 and the dominant-negative mutants GST-Sp1, GST-Sp3, and GST-Egr-1. **A**: Schematic representation of the modular structure of Sp1, Sp3, Egr-1 and the dominant-negative mutants GST-Sp1, GST-Sp3, and GST-Egr-1. The zinc finger DNA-binding domains are indicated by black bars. Sp1 and Sp3 both contain a bipartite glutamine-rich activation domain on the N-terminus. The Egr-1 protein contains an extented transcriptional activation domain at the N-terminus and an inhibitory domain (repression domain, RD) between the activation and DNA-binding domains that functions as a binding site for the transcriptional corepressor proteins NAB1 and NAB2. The mutants GST-Sp1, GST-Sp3, and GST-Egr-1 consist of an amino-terminal glutathione-S-transferase, a nuclear localization signal derived from the SV40 large T antigen, and the zinc finger domains of Sp1, Sp3, and Egr-1, respectively, on the C-termini. **B**: Protein sequence alignment of the zinc finger domains of Sp1, Sp3, and Egr-1. The cysteine and histidine residues required for zinc coordination are indicated by stars.

Figure 1B shows the primary structure of the three Cys_2-His_2-zinc finger domains of Sp1, Sp3, and Egr-1. Very obvious is the high homology between the zinc finger domains of Sp1 and Sp3, in line with the similar binding affinities of Sp1 and Sp3 to the classical GC-rich sequence 5′-GGGCGG-3′ that have been previously reported [Hagen et al., 1992; Kingsley and Winoto, 1992]. The primary structure of the zinc finger domains of Egr-1 differ substantially from those of Sp1 and Sp3. However, the free solution structures of the zinc finger domains 2 and 3 of Sp1 and Egr-1 are very similar [Narayan et al., 1997] suggesting that both proteins may still recognize a similar DNA sequence.



Biological Activity of Dominant-Negative Sp1, Sp3, and Egr-1 Mutants Assayed by Constitutively Active Reporter Genes

The *aldolase C* gene contains a GC-rich genetic element that functions as a binding site for Sp1. A reporter gene under control of this element was shown to be constitutively expressed, due to the constitutive expression of Sp1. In contrast, Egr-1 was unable to bind to this element and to transactivate a reporter gene under control of this GC box [Cibelli et al., 1996]. We constructed a reporter plasmid that contained four copies of this motif, a TATA box, and the luciferase open reading frame, as shown in Figure 2A (plasmid pAldGCB^{4}luc). In addition, we tested luciferase reporter genes controlled by either the p21 promoter (plasmid p21Pluc) or a truncated HIV-1 long terminal repeat (plasmid pGL3-HIV-1 LTR), both containing multiple Sp1 binding sites. Human 293T cells were transfected with one of the luciferase reporter plasmids and an expression vector encoding GST-Sp1, GST-Sp3, or GST-Egr-1, respectively. As a control, an expression vector encoding a nuclear-targeted glutathione S-transferase (GST) was used. In addition, we transfected plasmid pRSVβ, encoding β-galactosidase under the control of the Rous sarcoma virus

Fig. 2. Dominant-negative mutants of Sp1 and Sp3 block the constitutive transcriptional activity mediated by the aldolase C-derived GC-rich box, the p21$^{WAF1/Cip1}$ promoter or the HIV-1 LTR. The reporter plasmid pAldGCB^{4}luc (**A**) contains the coding region for luciferase, a TATA box, and four copies of a GC-rich motif derived from the *aldolase C* gene. In the transcription units present in the reporter plasmids p21Pluc (**B**) and pGL3-HIV-1 LTR (**C**) luciferase expression is controlled by the *p21$^{WAF1/Cip1}$* gene promoter (B) or a portion of the LTR of HIV-1 (C), respectively. One of the reporter plasmids pAldGCB^{4}luc, p21Pluc, or pGL3-HIV-1 LTR (0.5 μg/plate) was transfected into 293T cells together with the pRSVβ internal standard plasmid and expression vectors encoding either GST, GST-Sp1, GST-Sp3, or GST-Egr-1 (250 ng/plate). Luciferase activities were normalized for transfection efficiency by dividing luciferase light units by β-galactosidase activities. At least four experiments were performed and the mean ± SD is depicted.

long-terminal repeat, to correct for variations in transfection efficiencies. The results show that the dominant-negative Sp1 and Sp3 mutants decreased the constitutive transcriptional activity of the aldolase C derived GC-rich box, the p21 promoter and the HIV-1 LTR (Fig. 2A–C), indicating that both Sp1 and Sp3 may be responsible for the constitutive transcriptional activity of these genes. Thus, a functional interference of Sp1 and Sp3 through the same DNA-binding occurred, due to the highly conserved DNA-binding domains. In contrast, the dominant-negative GST-Egr-1 was unable to block the constitutive transcriptional activity of the aldolase C derived GC-rich box, the $p21^{WAF1/Cip1}$ promoter and the HIV-1 LTR, indicating that Sp1/Sp3 and Egr-1 differ in their transcriptional targets.

Activation of Egr-1-Mediated Gene Transcription Via Expression of Egr-1 or the Egr-1 Mutant C2/Egr-1

The biological activity of Egr-1 is mainly regulated by the stimulus-induced biosynthesis of Egr-1. In order to increase the intracellular Egr-1 concentration, we used an expression vector encoding Egr-1 (plasmid pCMVEgr-1). Egr-1 is negatively regulated by the co-repressor proteins NAB1 and NAB2, and also by phosphorylation, although this has not been clearly elucidated. We therefore decided to design an Egr-1 mutant that contains a constitutive activation domain derived from CREB2, fused to the DNA-binding domain of Egr-1. This mutant lacks the inhibitory domain of Egr-1, making repression by NAB1 or NAB2 impossible. The modular structure of this C2/Egr-1 mutant is depicted in Figure 3A, together with the domain structure of Egr-1 and CREB2. To test the transcriptional activation by Egr-1, transfection experiments were performed using the reporter plasmids pEBS1^{4}luc and pEBS2^{4}luc containing luciferase as a reporter gene. Immediately upstream of the TATA box, four binding sites for Egr-1, derived from the Egr-1 promoter (plasmid pEBS1^{4}luc) or the human synapsin I promoter (plasmid pEBS2^{4}luc) were present (Fig. 3B). One of the reporter plasmids was transfected into 293T cells together with the "empty" expression vector pCMV5 (denoted "–") or expression vectors encoding Egr-1 (pCMVEgr-1) or C2/Egr-1 (pCMVFLAG-C2/Egr-1) (denoted "+"). As a reference plasmid pRSVβ was transfected. The results of the transfection experiments are depicted in Figure 3C. Expression of wild-type Egr-1 induced reporter gene transcription on the order of five fold (reporter pEBS1^{4}luc) or eight fold (reporter pEBS2^{4}luc), indicating that both reporters were activatable by increased cellular concentrations of Egr-1. The Egr-1 mutant C2/Egr-1 was strikingly more active in inducing transcription from Egr-1-responsive reporters. Expression of C2/Egr-1 in 293T cells induced reporter gene transcription on the order of 60-fold (reporter pEBS1^{4}luc) or 90-fold (reporter pEBS2^{4}luc). In contrast to the Egr-1-responsive reporters, the transcriptional activity of the Sp1/Sp3 regulated reporters, pAldGCB^{4}luc, plasmid p21Pluc, or pGL3-HIV-1 LTR, was not enhanced, following expression of Egr-1 or C2/Egr-1 (data not shown). Competition experiments with GST-Sp1, GST-Sp3, and GST-Egr-1 revealed that solely GST-Egr-1 showed a significant downregulation of reporter gene transcription, induced by Egr-1 or C2/Egr-1 (Fig. 3D). Thus, while dominant-negative mutants of Sp1 and Sp3, but not of Egr-1 were able to block the constitutive transcriptional activity from reporter plasmids under the control of the aldolase C derived GC-rich box, the $p21^{WAF1/Cip1}$ promoter and the HIV-1 LTR, only transcription of the Egr-1-responsive reporter plasmids pEBS1^{4}luc and pEBS2^{4}luc were blocked by the dominant-negative GST-Egr-1, but not by GST-Sp1 or GST-Sp3. These data indicate that Egr-1 and Sp1/Sp3 function via distinct genetic elements.

Induction of Egr-1 Biosynthesis and Transcription of Egr-1-Responsive Reporter Genes by a ΔB-Raf-Estrogen Receptor Fusion Protein

The biosynthesis of Egr-1 is induced in different cell types by the activation of the classic Ras-Raf-MEK-ERK signaling pathway [Kaufmann and Thiel, 2001, 2002]. Accordingly, expression of a constitutively active mitogen-activated protein kinase kinase, the kinase responsible for the phosphorylation and activation of ERK, strongly stimulates Egr-1 promoter activity [Kaufmann et al., 2001]. To specifically activate the ERK pathway in 293T cells, we generated 293T cells expressing a ΔB-Raf-estrogen receptor fusion protein using retroviral gene transfer (293TΔB-Raf:ER cells). As a control, 293T cells were infected with a recombinant retrovirus encoding puromycin acetyltransferase (293Tpac cells). The modular

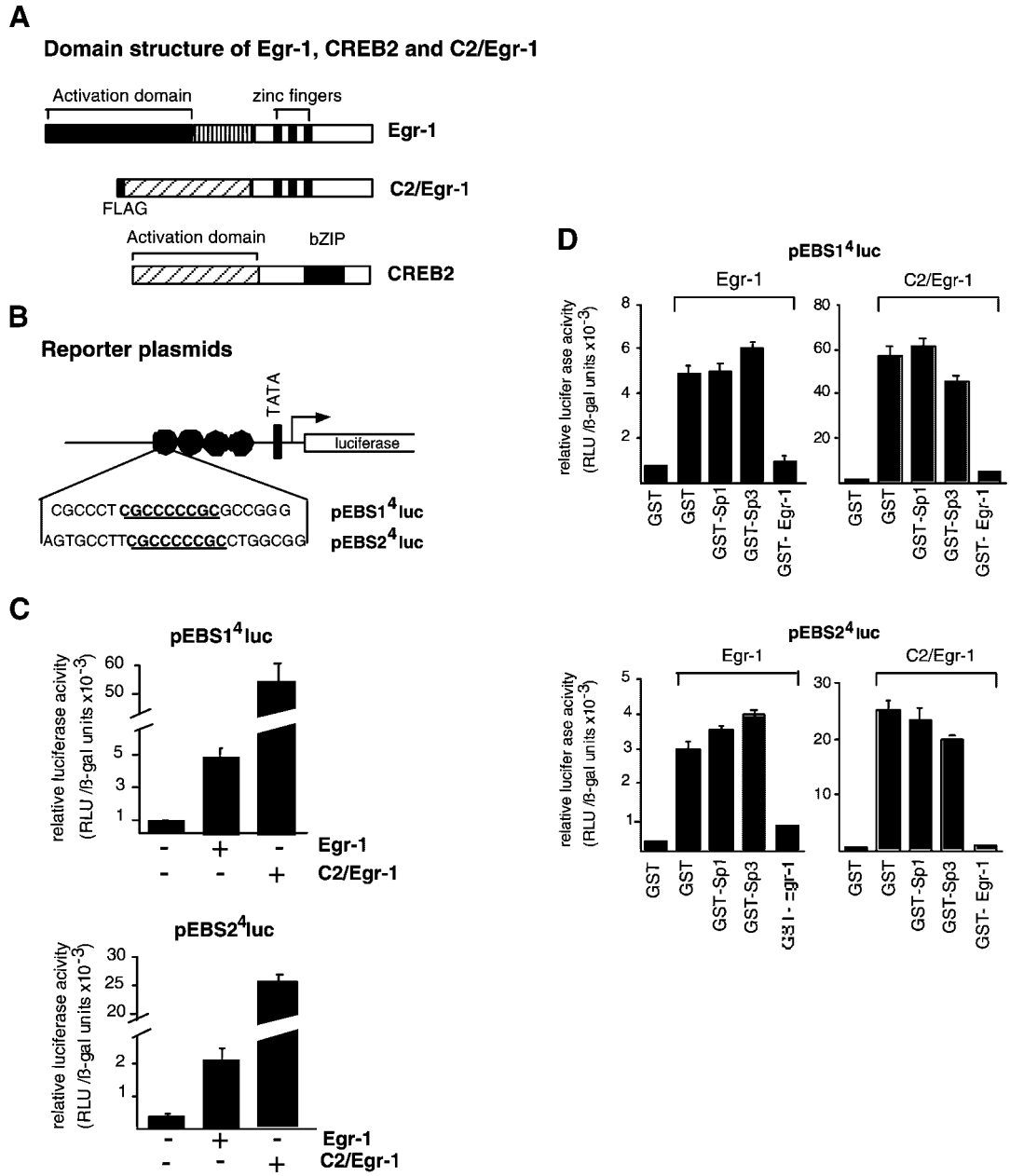


Fig. 3. Activation of Egr-1-responsive reporter gene transcription by overexpression of Egr-1 and C2/Egr-1 in 293T cells. **A**: Schematic representation of the modular structure of Egr-1, the Egr-1 mutant C2/Egr-1, and CREB2. The DNA-binding domains of Egr-1 and C2/Egr-1 (zinc fingers) are indicated as well as the basic region leucine zipper domain (bZIP) of CREB2. The chimeric C2/Egr-1 protein consists of the constitutively active transcriptional activation domain of CREB2 and the C-terminal region of Egr-1 that is responsible for DNA-binding. **B**: The reporter plasmids pEBS1^{4}luc and pEBS2^{4}luc contain the luciferase reporter gene, a minimal promoter consisting of the human immunodeficiency virus TATA box and the adenovirus major late promoter initiator element. Immediately upstream of the TATA box are four binding sites for Egr-1 derived from the Egr-1 promoter (plasmid pEBS1^{4}luc) or the synapsin I promoter (plasmid pEBS2^{4}luc). **C**: One of the reporter plasmids pEBS1^{4}luc or pEBS2^{4}luc (0.5 μg/plate) was transfected into 293T cells together with the pRSVβ internal standard plasmid and expression vectors encoding Egr-1 or the CREB2/Egr-1 mutant C2/Egr-1 (50 ng/plate). **D**: pEBS1^{4}luc and pEBS2^{4}luc reporter gene transcription induced by the expression of Egr-1 or C2/Egr-1 was challenged by the expression of the dominant-negative mutants GST-Sp1, GST-Sp3, or GST-Egr-1 (250 ng plasmid/plate) as indicated. Relative luciferase activities were determined by measuring β-galactosidase and luciferase activities of the transfected cells. At least four experiments were performed and the mean ± SD is depicted.

structure of the B-Raf protein kinase is depicted in Figure 4A. B-Raf contains three domains termed CR1, CR2, and CR3. CR1 is a cysteine-rich region and functions as binding site for activated Ras-GTP at the cell membrane. CR2 is rich in serine and threonine residues and negatively regulates the biological activity of the catalytic domain, perhaps via direct protein–protein interaction with the kinase domain. CR3 encompasses the protein kinase domain. Expression of this catalytic domain of B-Raf as a fusion protein with the ligand binding domain of the murine estrogen receptor (ER) keeps the protein kinase in an inactive state in the absence of hormone, but allows activation of the mutant B-Raf:ER fusion protein by the addition of hormone [Picard, 1993]. The use of the estrogen receptor mutant termed $ER^{Tamoxifen\ Mutant}$ allowed us to use the synthetic ligand, 4-hydroxytamoxifen (4OHT), for induction. The encoded ligand binding domain of the estrogen receptor contained a glycine residue at position 525, instead of an arginine. As a result, the receptor is largely insensitive to 17β-estradiol, but is readily activatable by 4OHT [Littlewood et al., 1995]. To study the effect of ΔB-Raf:ER on Egr-1 biosynthesis, we incubated 293TΔB-Raf:ER cells with 4OHT. Nuclear extracts were prepared and analyzed for Egr-1 synthesis. Figure 4B shows that the biosynthesis of Egr-1 is induced in 4OHT stimulated 293TΔB-Raf:ER cells. In contrast to the transient and robust synthesis of Egr-1 following growth factor stimulation [Kaufmann and Thiel, 2002; Rössler and Thiel, 2004], we observed a delayed but sustained synthesis in 4OHT treated 293TΔB-Raf:ER cells, reaching highest levels of Egr-1 24 h after stimulation (Fig. 4B). To analyze the effect of ΔB-Raf:ER activation on Egr-1 mediated gene

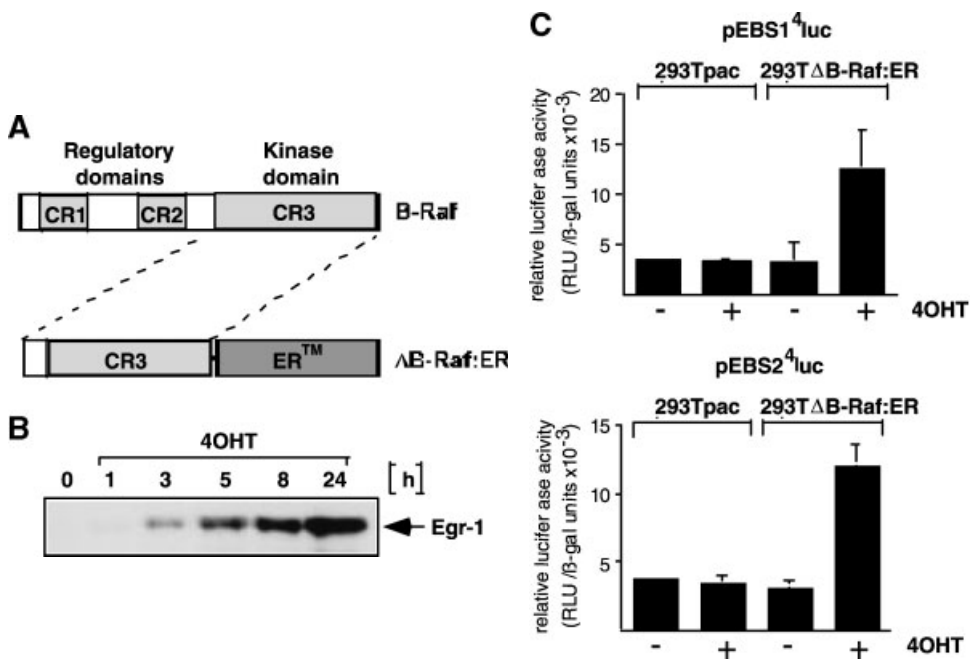


Fig. 4. Activation of Egr-1-mediated gene transcription by ΔB-Raf:ER, a conditionally active form of B-Raf protein kinase. **A**: Modular structure of B-Raf and ΔB-Raf:ER. The functional domains of B-Raf (CR3, CR2, and CR1) are depicted. Fusion of the catalytic CR3 domain to the hormone binding domain of the estrogen receptor generates the ΔB-Raf:ER fusion protein. **B**: 293TΔB-Raf:ER cells were serum-starved for 24 h, and then treated with 4OHT (200 nM). Nuclear extracts were prepared from cells incubated with 4OHT for different time points and subjected to Western blot analysis. The blot was incubated with an antibody directed against Egr-1. **C**: 293Tpac cells (left side) or 293TΔB-Raf:ER cells (right side) were transfected with one of the reporter plasmids pEBS1^{4}luc or pEBS2^{4}luc, and the internal standard plasmid pSV40lacZ. The serum concentration was lowered from 10% to 0.05% and the cells were incubated for 24 h. Stimulation of the cells with 4OHT (200 nM) was performed for 24 h. **D**: Similarly, 293TΔB-Raf:ER cells were transfected with the reporter plasmids pAldGCB^{4}luc, p21Pluc, or pGL3-HIV-1 LTR, in the presence (+) or absence (–) of 4OHT (200 nM). **E**: pEBS1^{4}luc or pEBS2^{4}luc reporter gene transcription induced by 4OHT in 293TΔB-Raf:ER cells was challenged by the expression of the dominant-negative mutants GST-Sp1, GST-Sp3, or GST-Egr-1 (250 ng expression vector/plate) as indicated. As a control, an expression vector encoding GST was transfected. Relative luciferase activities were determined by measuring β-galactosidase and luciferase activities of the transfected cells. At least four experiments were performed and the mean ± SD is depicted.

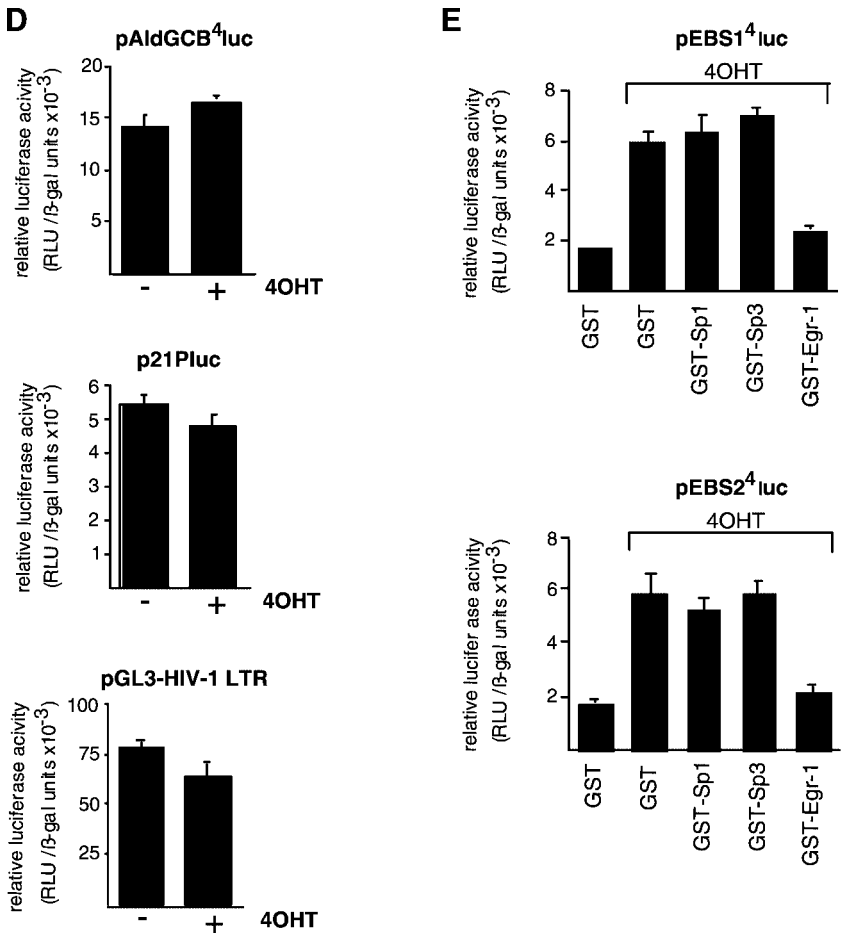


Fig. 4. (*Continued*)

transcription, we transfected 293Tpac and 293TΔB-Raf:ER cells with the reporter plasmids pEBS1^{4}luc, or pEBS2^{4}luc. Figure 4C reveals that 4OHT enhanced transcription of the Egr-1-responsive reporter genes following activation of the ΔB-Raf:ER fusion protein by 4OHT. In contrast, 4OHT had no effect on reporter gene transcription in 293Tpac cells lacking ΔB-Raf:ER. Likewise, no transcriptional activation was observed following transfection of the Sp1/Sp3-responsive reporter plasmids pAldGCB^{4}luc, p21Pluc, or pGL3-HIV-1 LTR into 293T-ΔB-Raf:ER cells, followed by stimulation with 4OHT (Fig. 4D). Thus, sustained activation of the ERK signaling pathway has no impact in Sp1/Sp3 regulated gene transcription. To test the functional relevance of Egr-1 in ΔB-Raf:ER induced transcriptional activation of pEBS1^{4}luc or pEBS2^{4}luc reporter gene transcription, we performed competition experiments. 293TΔB-Raf:ER cells were transfected with the indicated reporter plasmids and expression vectors encoding GST, GST-Sp1, GST-Sp3, and GST-Egr-1, respectively. The transfected cells were serum-starved for 24 h and then stimulated with 4OHT for 24 h. The results depicted in Figure 4E show that only the dominant-negative GST-Egr-1 interfered with ΔB-Raf:ER induced activation of reporter gene transcription. Expression of GST, or the fusion proteins GST-Sp1 or GST-Sp3 in the nucleus did not impair ΔB-Raf:ER-mediated transcription of Egr-1-responsive reporter genes, indicating that Sp1 and Sp3 are not capable to block the Egr-1 DNA-binding present in these reporter genes.

Lack of Correlation Between Egr-1 Biosynthesis and *p21$^{WAF1/Cip1}$* Gene Transcription in Stimulated 293TΔB-Raf:ER Cells

The regulation of the *p21$^{WAF1/Cip1}$* gene by Sp1 and Sp3 has been well documented [Prowse et al., 1997; Pagliuca et al., 2000; Koutsodontis et al., 2002] and these data are in perfect

agreement with our results showing that dominant-negative mutants of Sp1 and Sp3 blocked constitutive $p21^{WAF1/Cip1}$ promoter activity. However, a recent report proposed that the $p21^{WAF1/Cip1}$ gene is also regulated by Egr-1 [Ragione et al., 2003]. We have been unable to detect elevated $p21^{WAF1/Cip1}$ promoter activities, using different strategies to increase the Egr-1 concentration in the cells. To directly test a correlation between Egr-1 synthesis and $p21^{WAF1/Cip1}$ gene transcription, we performed RNase protection mapping. 293Tpac and 293TΔB-Raf:ER cells were stimulated with 4OHT for 5 or 8 h. The cytoplasmic RNA was prepared and hybridized to highly specific cRNAs for Egr-1, $p21^{WAF1/Cip1}$, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

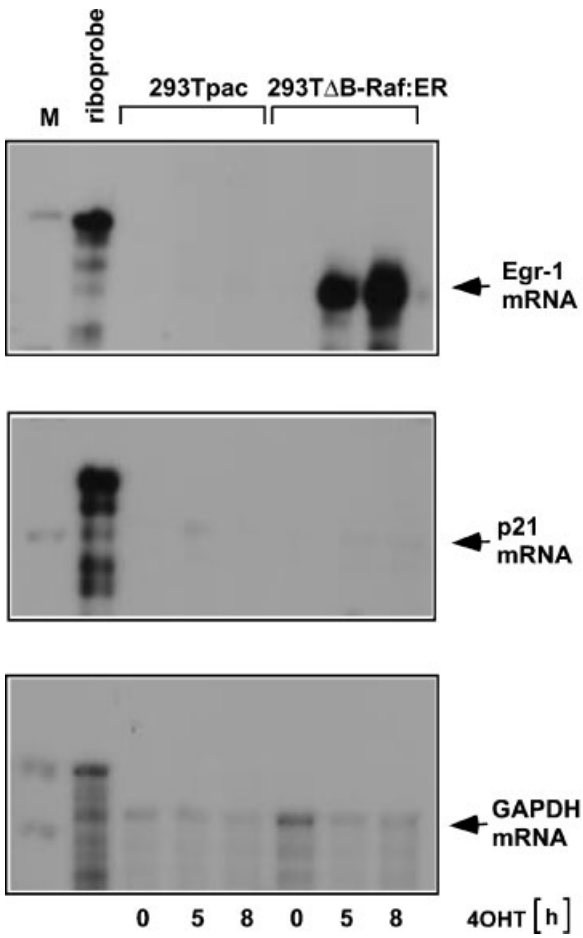
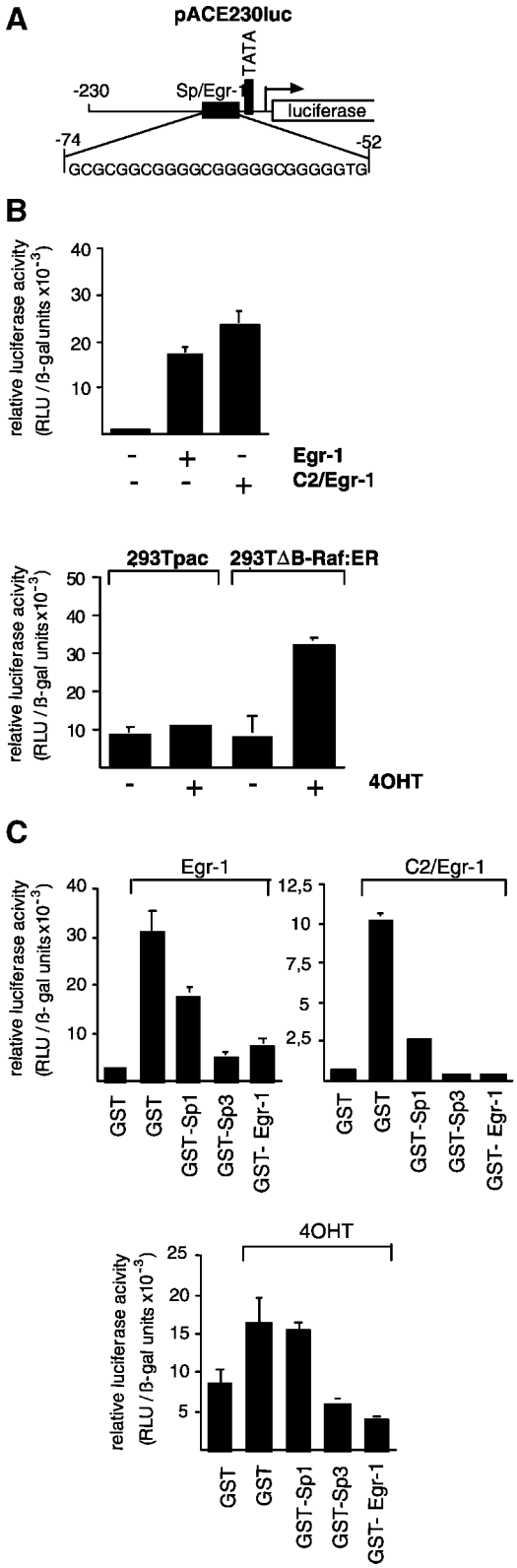


Fig. 5. $p21^{WAF1/Cip1}$ gene expression is not modulated by Egr-1. 293Tpac and 293ΔB-Raf:ER cells were stimulated with 4OHT (200 nM) for 5 or 8 h. Cytoplasmic RNA was isolated and analyzed by RNase protection mapping using cRNAs specific for Egr-1, $p21^{WAF1/Cip1}$ and GAPDH. An aliquot of the undigested riboprobes is shown. Size marker *Msp*I–cut PUC19 are shown in **lane** M.

The results show that the levels of Egr-1 mRNA are strikingly increased following stimulation of 293TΔB-Raf:ER cells with 4OHT (Fig. 5). In addition, we already showed that Egr-1 is transcriptionally active under these circumstances (Fig. 4C). However, $p21^{WAF1/Cip1}$ mRNA was hardly detectable and there was no significant difference between $p21^{WAF1/Cip1}$ mRNA levels in 293Tpac or 293TΔB-Raf:ER cells. In contrast, Egr-1 was not expressed in resting or stimulated 293Tpac cells. These results confirm the data obtained in the analysis of the $p21^{WAF1/Cip1}$ promoter and indicate that the $p21^{WAF1/Cip1}$ gene is not transactivated by high concentrations of Egr-1 in 293TΔB-Raf:ER cells.

The Overlapping Sp3/Egr-1 Site Within the Angiotensin-Converting Enzyme Promoter

So far, we have reported on gene promoters responsive to either Sp1/Sp3 or Egr-1. However, these results do not exclude the existence of composite Sp1/Sp3/Egr-1 binding sites, where competition for a common DNA-binding site occurs. In fact, we recently reported that hepatocyte growth factor or phorbol ester stimulation of the human angiotensin-converting enzyme gene promoter involved a composite Sp3/Egr-1 binding [Day et al., 2004]. We therefore used the ACE promoter in this study as an example for a composite Sp3/Egr-1 binding site. The reporter gene pACE230luc is depicted in Figure 6A. Transfection experiments of 293T cells revealed that Egr-1 and the Egr-1 mutant C2/Egr-1 transactivated the angiotensin-converting enzyme gene promoter/luciferase reporter gene consisting of the proximal portion of the ACE promoter fused to the luciferase open reading frame (Fig. 6B top panel). Moreover, stimulation of Egr-1 biosynthesis in 4OHT treated 293TΔB-Raf:ER cells led to an upregulation of ACE promoter activity (Fig. 6B, bottom panel), thus confirming previous observations that the *ACE* gene is a target for Egr-1 [Day et al., 2004]. Competition experiments revealed that GST-Sp1, GST-Sp3, and GST-Egr-1 counteracted transcriptional upregulation of the ACE promoter by overexpression of Egr-1 or C2/Egr-1 (Fig. 6C, top panel). The fact that dominant-negative Sp1 or Sp3 impaired Egr-1 mediated gene transcription indicates a competition between Sp1, Sp3, and Egr-1 for binding to the ACE promoter. Interestingly, GST-Sp1 was less active than GST-Sp3 to compete with-Egr-1, suggesting that the GC-rich sequence



of the ACE promoter functions as a binding site for Sp3 rather than for Sp1. This assumption was supported by the next experiment. 293TΔB-Raf:ER cells were transfected with the ACE promoter/luciferase plasmid pACE230luc and expression vectors encoding GST, GST-Sp1, GST-Sp3, or GST-Egr-1. Cells were stimulated with 4OHT to induce the Raf-MEK-ERK signaling pathway leading to the synthesis of Egr-1. Figure 6C (bottom panel) shows that expression of GST-Sp3 and GST-Egr-1 completely abrogated 4OHT induced reporter gene transcription. In contrast, GST-Sp1 did not compete, supporting the view that the GC-rich region of the ACE promoter contains rather an overlapping Sp3/Egr-1 binding site than a composite Sp1/Sp3/Egr-1 site. Collectively, the data show that Egr-1 transactivates the *ACE* gene, using transfection of Egr-1 expression vectors or stimulation of the extracellular signal-regulated protein kinase pathway via an inducible B-Raf-estrogen receptor fusion protein (ΔB-Raf:ER) as the trigger to activate Egr-1 synthesis. Competition experiments revealed that a dominant-negative Egr-1 abrogated Egr-1-induced upregulation of the ACE promoter. In addition, in 293T cells a dominant-negative Sp3 was also able to compete with wild-type Egr-1 for binding to the DNA.

DISCUSSION

A large number of mammalian genes are regulated by proteins of the Sp1 family of transcription factors. The prototype member of

Fig. 6. The angiotensin-converting enzyme gene promoter contains an overlapping Sp3/Egr-1 binding site. **A**: Reporter plasmid pACE230luc. The sequence of the composite Sp3/Egr-1 site is depicted. **B**: The ACE promoter/luciferase reporter gene is transactivated by Egr-1. Egr-1 synthesis was induced by transfection of 293T cells with expression vectors encoding Egr-1 or C2/Egr-1 (0.5 μg/plate) (top), by stimulation of 293TΔB-Raf:ER cells with 4OHT (bottom). Relative luciferase activities were determined by measuring β-galactosidase and luciferase activities of the transfected cells. **C**: Competition experiments with dominant negative Sp1, Sp3, and Egr-1. 293T cells (top) and 293TΔB-Raf:ER (bottom) were transfected with the reporter plasmid pACE230luc (0.5 μg/plate) together with the pSV40lacZ internal standard plasmid and expression vectors encoding either GST, GST-Sp1, GST-Sp3, or GST-Egr-1 as indicated. We used 250 ng/plate of expression vectors encoding one of the GST fusion proteins to compete with Egr-1 synthesized as a result of 4OHT. 2 μg/plate of expression vectors encoding one of the GST fusion proteins were required to compete with Egr-1 or C2/Egr-1 synthesized in 293T cells as a result of transfection of expression vectors. Luciferase activities were normalized for transfection efficiency by dividing luciferase light units by β-galactosidase activities.

this protein family is the ubiquitously expressed Sp1 protein. A variety of signaling cascades induced by hormones, cytokines, neurotransmitters, or cytotoxic chemicals converge on the synthesis of Egr-1, thus making it very interesting to identify those Egr-1 target genes that continue the initial signaling cascades and are most likely responsible for the effects of Egr-1 in the control of cellular growth, differentiation, and death [Thiel and Cibelli, 2002]. The hypothesis that Egr-1 and Sp1 compete for a similar GC-rich DNA-binding site suggests that many Sp1 regulated genes are putative targets for Egr-1. The objective of this study was to analyze the transcriptional regulation of Sp1, Sp3, and Egr-1 on selected Sp1 or Egr-1-regulated transcription units using the expression of dominant-negative mutants. We chose to measure transcriptional activation instead of DNA-binding, because although DNA-binding is necessary and required for a subsequent transcriptional activation by Sp1, Sp3, or Egr-1, an enhanced binding activity of a transcription factor to DNA, monitored by an in vitro binding assay, does not necessarily prove an enhanced transcriptional activation potential of this protein. We showed, for example, by in vitro electrophoretic mobility-shift assay that Egr-1 binds specifically to a GC-rich sequence of the synaptobrevin II promoter. However, in intact cells, Sp1 blocks this site and Egr-1 has no access to the regulatory region of the *synaptobrevin II* gene for activation of transcription [Petersohn and Thiel, 1996].

As Sp1-responsive targets, we chose the aldolase C and $p21^{WAF1/Cip1}$ promoters and the LTR from HIV-1. For a GC-rich region of the *aldolase C* gene, we showed by in vitro DNA-binding experiments as well as by transient transfections that Sp1 interacts with this site [Cibelli et al., 1996]. Here, we confirmed these data and showed in addition that constitutive transcription of a reporter gene having four copies of this GC-rich motif from the aldolase C gene in its regulatory region is also impaired by a dominant-negative Sp3. Likewise, the regulation of a reporter gene controlled by a proximal portion of the HIV-1 LTR was controlled by Sp1 and Sp3, but not by Egr-1. Several studies have shown that the gene encoding the cyclin-dependent protein kinase inhibitor $p21^{WAF1/Cip1}$ is positively regulated by Sp1 and Sp3 [Prowse et al., 1997; Pagliuca et al., 2000]. In fact, a recent analysis revealed that Sp1 and Sp3 bound with high affinity to five of the six GC-rich motifs of the proximal $p21^{WAF1/Cip1}$ promoter [Koutsodontis et al., 2002]. Accordingly, we found that dominant-negative mutants of Sp1 and Sp3 blocked constitutive $p21^{WAF1/Cip1}$ promoter activity. Recently, a report claimed that the $p21^{WAF1/Cip1}$ gene is regulated by Egr-1, following treatment of erythroleukemic cells with resveratrol [Ragione et al., 2003]. We have been unable to detect elevated $p21^{WAF1/Cip1}$ promoter activities, following several distinct strategies to increase the Egr-1 concentrations in the cells. Moreover, a direct comparison between the Egr-1 and $p21^{WAF1/Cip1}$ mRNA levels present in resting or stimulated 293Tpac and 293TΔB-Raf:ER cells confirmed the results obtained in the analysis of the $p21^{WAF1/Cip1}$ promoter and showed that $p21^{WAF1/Cip1}$ gene expression is not modulated by Egr-1.

As Egr-1-responsive targets, we used reporter genes containing Egr-1 binding sites derived from the Egr-1 and synapsin I promoters. Egr-1 transactivates reporter genes having one of these genetic elements in the regulatory region [Thiel et al., 1994, 2000; Cibelli et al., 2002]. Egr-1 immunoreactivity is hardly detectable in unstimulated cells. Accordingly, the basal activity of the Egr-1-responsive transcription units present in plasmids pEBS1^{4}luc and pEBS2^{4}luc was low. To achieve induction of Egr-1 gene transcription, we adopted two strategies, first, the overexpression of wild-type and chimeric Egr-1 via transient transfection of expression vectors, and second, the stimulation of engineering 293T cells expressing a 4OHT inducible ΔB-Raf:ER fusion protein. The activation of the ERK signaling pathway via stimulation of the chimeric ΔB-Raf:ER protein triggered a sustained synthesis of Egr-1. Transcription of Egr-1-sensitive reporter genes was activated in all cases, and repressed by a dominant-negative Egr-1 mutant. The fact that the chimeric CREB2/Egr-1 mutant displayed a higher transcriptional activity may be explained by the strength of the CREB2-derived activation domain in comparison to the Egr-1 activation domain. Alternatively, the lack of NAB1/NAB2 binding sites in C2/Egr-1 may avoid repressive constraints due to residual NAB1/2 expression. The lack of transcriptional repression by dominant-negative Sp1 or Sp3 mutants indicates that these transcription factors are unable to bind to the Egr-1 cognate sites present in the reporter plasmids pEBS1^{4}luc and pEBS2^{4}luc.

Recently, criteria have been proposed for the identification of Egr-1 target genes [Adamson and Mercola, 2001]. These "levels of certainty" include a correlation of Egr-1 expression with the indicated gene (level 1) using Egr-1 inducible signal molecules or Egr-1 expression vectors, the in vitro identification of Egr-1 binding to the promoter of the gene (level 2), and the verification of Egr-1 binding by chromatin immunoprecipitation. We propose to add, as a further criterion, the inhibition of stimulus-induced target gene transcription or target gene promoter activity by a dominant-negative Egr-1. The additional expression of the dominant-negative Sp1 and Sp3 mutants may also help to distinguish between target genes regulated solely by Egr-1 or target genes having composite Sp1/Egr-1 or Sp3/Egr-1 motifs. Recently, we successfully used this approach to show that the hepatocyte growth factor induced stimulation of the angiotensin converting enzyme gene transcription in pulmonary artery endothelial cells is regulated by an overlapping Sp3/Egr-1 motif [Day et al., 2004]. Here, we tested transactivation of an ACE promoter/reporter gene by Egr-1 and confirmed that ACE promoter activity is upregulated by Egr-1. A dominant-negative Egr-1 mutant blocked this upregulation. Competition experiments involving dominant-negative Sp1 and Sp3 showed that Sp3 competes with Egr-1 for a common binding site. The dominant-negative Sp1 provided little if any impairment indicating that the GC-rich sequence of the ACE promoter binds rather Sp3 than Sp1. Taken together, the analysis of the ACE promoter demonstrated the value of dominant-negative Sp1, Sp3, and Egr-1 proteins to unravel the impact of a particular transcription factor in gene transcription.

In summary, we have shown that distinct Sp1/Sp3 and Egr-1 binding sites exist and that no competition for binding to these sites between Sp1/Sp3 and Egr-1 occurs. However, some genes contain overlapping Sp1/Egr-1 or Sp3/Egr-1 binding sites, and the actual concentration of the different zinc finger proteins decides, which discrete sets of genes are turned on or off. This study shows furthermore that the dominant-negative Sp1, Sp3, and Egr-1 mutants are remarkable tools for the study of Sp1/Sp3 or Egr-1-regulated transcription and the analysis of putative Sp1/Egr-1 composite sites in living cells.

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REFERENCES

Ackerman SL, Minden AG, Williams GT, Bobonis C, Yeung C-Y. 1991. Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. Proc Natl Acad Sci USA 88:7523–7527.

Adamson ED, Mercola D. 2001. Egr1 transcription factor: Multiple roles in prostate tumor cell growth and survival. Tumor Biol 23:93–102.

Bahouth SW, Beauchamp MJ, Vu KN. 2002. Reciprocal regulation of β_1-adrenergic receptor gene transcription by Sp1 and early growth response gene 1: Induction of EGR-1 inhibits the expression of the β_1-adrenergic receptor gene. Mol Pharmacol 61:379–390.

Cao X, Mahendran R, Guy GR, Tan YH. 1993. Detection and characterization of cellular Egr-1 binding to its recognition site. J Biol Chem 268:16949–16957.

Christy B, Nathans D. 1989. DNA-binding site of the growth factor-inducible protein Zif268. Proc Natl Acad Sci USA 86:8737–8741.

Christy B, Lau LF, Nathans D. 1988. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc Natl Acad Sci USA 85:7857–7861.

Cibelli G, Schoch S, Pajunk H, Brand I, Thiel G. 1996. A (G + C)-rich motif in the aldolase C promoter functions as a constitutive transcriptional enhancer element. Eur J Biochem 237:311–317.

Cibelli G, Policastro V, Rössler O, Thiel G. 2002. Nitric oxide-induced programmed cell death in human neuroblastoma cells is accompanied by the synthesis of Egr-1, a zinc finger transcription factor. J Neurosci Res 67:450–460.

Cui M-Z, Parry GCN, Oeth P, Larson H, Smith M, Huang R-P, Adamson ED, Mackman N. 1996. Transcriptional regulation of the tissue factor gene in human epithelial cells is mediated by Sp1 and Egr-1. J Biol Chem 271: 2731–2739.

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang X-F. 1995. Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci USA 92:5545–5549.

Davis W, Jr., Chen ZJ, Ile KE, Tew KD. 2003. Reciprocal regulation of expression of the human adenosine 5′-triphosphate binding casette, sub-family A, transporter 2 (ABCA2) promoter by early growth response-1 (EGR-1) and Sp-family transcription factors. Nucl Acids Res 31: 1097–1107.

Day RM, Thiel G, Lum J, Chévere RD, Yang Y, Stevens J, Silbert L, Fanburg BL. 2004. Hepatocyte growth factor regulates angiotensin converting enzyme expression. J Biol Chem 279:8792–8801.

El-Deiry WS, Tokino T, Velculesco VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer EW, Kinzler KW, Vogelstein B. 1993. WAF1, a potential mediator of p53 tumor suppression. Cell 75:817–825.

Hagen G, Müller S, Beato M, Suske G. 1992. Cloning by recognition site screening of two novel GT box binding proteins: A family of Sp1 related genes. Nucl Acids Res 20:5519–5525.

Huang R-P, Fan Y, Ni Z, Mercola D, Adamson ED. 1997. Reciprocal modulation between Sp1 and Egr-1. J Cell Biochem 66:489–499.

Jüngling S, Cibelli G, Czardybon M, Gerdes H-H, Thiel G. 1994. Differential regulation of chromogranin B and synapsin I gene promoter activity by cyclic AMP and cyclic AMP-dependent protein kinase. Eur J Biochem 226:925–935.

Kadonaga JT, Carner KR, Marsiarz FR, Tjian R. 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51: 1079–1090.

Kaufmann K, Thiel G. 2001. Epidermal growth factor and platelet-derived growth factor induce expression of Egr-1, a zinc finger transcription factor, in human malignant glioma cells. J Neurol Sci 189:83–91.

Kaufmann K, Thiel G. 2002. Epidermal growth factor and thrombin induced proliferation of immortalized human keratinocytes is coupled to the synthesis of Egr-1, a zinc finger transcriptional regulator. J Cell Biochem 85:381–391.

Kaufmann K, Bach K, Thiel G. 2001. Extracellular signal-regulated protein kinases Erk1/Erk2 stimulate expression and biological activity of the transcriptional regulator Egr-1. Biol Chem 382:1077–1081.

Kavurma MM, Khachigian LM. 2003. Sp1 inhibits proliferation and induces apoptosis in vascular smooth muscle cells by repressing $p21^{WAF1/Cip1}$ transcription and cyclin D1-Cdk4- $p21^{WAF1/Cip1}$ complex formation. J Biol Chem 278:32537–32543.

Kavurma MM, Santiago FS, Bonfoco E, Khachigian LM. 2001. Sp1 phosphorylation regulates apoptosis via extracellular FasL-Fas engagement. J Biol Chem 276: 4964–4971.

Khachigian LM, Williams AJ, Collins T. 1995. Interplay of Sp1 and Egr-1 in the proximal platelet-derived growth factor A—chain promoter in cultured vascular endothelial cells. J Biol Chem 270:27679–27686.

Khachigian LM, Lindner V, Williams AJ, Collins T. 1996. Egr-1-induced endothelial gene expression: A common theme in vascular injury. Science 271:1427–1431.

Kingsley C, Winoto A. 1992. Cloning of GT box-binding proteins: A novel Sp1 multigene family regulating T-cell receptor gene expression. Mol Cell Biol 12:4251–4261.

Koutsodontis G, Moustakas A, Kardassis D. 2002. The role of Sp1 family members, the proximal GC-rich motifs, and the upstream enhancer region in the regulation of the human cell cycle inhibitor $p21^{WAF-1/Cip1}$ gene promoter. Biochemistry 41:12771–12784.

Kriwacki RW, Schultz SC, Steitz TA, Cardonna JP. 1992. Sequence-specific recognition of DNA by zinc-finger peptides derived from the transcription factor Sp1. Proc Natl Acad Sci USA 89:9759–9763.

Lemaire P, Revelant O, Bravo R, Charnay P. 1988. Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc Natl Acad Sci USA 85:4691–4695.

Lietz M, Hohl M, Thiel G. 2003. RE-1 silencing transcription factor (REST) regulates human synaptophysin gene transcription through an intronic sequence-specific DNA-binding site. Eur J Biochem 270:2–9.

Lim RW, Varnum BC, Herschman HR. 1987. Cloning of tetradecanoyl phorbol ester-induced 'primary response' sequences and their expression in density-arrested Swiss 3T3 cells and a TPA non-proliferative variant. Oncogene 1:263–270.

Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI. 1995. A modified estrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucl Acids Res 23:1686–1690.

Milbrandt J. 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 238:797–799.

Narayan VA, Kriwacki RW, Cardonna JP. 1997. Structures of zinc finger domains from transcription factor Sp1. Insights into sequence-specific protein-DNA recognition. J Biol Chem 272:7801–7809.

Pagliuca A, Gallo P, Lania L. 2000. Differential role for Sp1/Sp3 transcription factors in the regulation of the promoter activity of multiple cyclin-dependent kinase inhibitor genes. J Cell Biochem 76:360–367.

Pavletich NP, Pabo CO. 1991. Zinc finger-DNA recognition: Crystal structure of a zif268-DNA complex at 2.1Å. Science 252:809–817.

Petersohn D, Thiel G. 1996. Role of zinc finger proteins Sp1 and zif268/egr-1 in transcriptional regulation of the human synaptobrevin II gene. Eur J Biochem 239:827–834.

Picard D. 1993. Steroid-binding domains for regulating the function of heterologous proteins in cis. Trends Cell Biol 3:278–280.

Prowse DM, Bolgan L, Molnár Á, Dotto GP. 1997. Involvement of the Sp3 transcription factor in induction of $p21^{Cip1/WAF1}$ in keratinocyte differentiation. J Biol Chem 272:1308–1314.

Ragione FD, Cucciolla V, Criniti V, Indaco S, Borriello A, Zappia V. 2003. $p21^{Cip1}$ gene is modulated by Egr1. J Biol Chem 278:23360–23368.

Rössler OG, Thiel G. 2004. Brain-derived neurotrophic factor, epidermal growth factor, or A-Raf induced growth of HaCaT keratinocytes requires extracellular signal-regulated kinase. Am J Physiol–Cell Physiol 286:C1118–C1129.

Rössler OG, Giehl KM, Thiel G. 2004. Neuroprotection of immortalized hippocampal neurons by brain-derived neurotrophic factor and Raf-1 protein kinase: Role of extracellular signal-regulated protein kinase and phosphatidylinositol 3-kinase. J Neurochem 88:1240–1252.

Schoch S, Cibelli G, Magin A, Steinmüller L, Thiel G. 2001. Modular structure cAMP response element binding protein 2 (CREB2). Neurochem Int 38:601–608.

Shingu T, Bornstein P. 1994. Overlapping Egr-1 and Sp1 sites function in the regulation of transcription of the mouse thrombospondin 1 gene. J Biol Chem 269:32551–32557.

Steinmüller L, Thiel G. 2003. Regulation of gene transcription by a constitutively active mutant of activating transcription factor 2 (ATF2). Biol Chem 384:667–672.

Sukhatme VP, Cao X, Chang LC, Tsai-Morris C-H, Stamenkovich D, Ferreira PCP, Cohen DR, Edwards SA, Shows TB, Curran T, Le Beau MM, Adamson ED. 1988. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell 53:37–43.

Thiel G, Cibelli G. 1999. Corticotropin-releasing factor and vasoactive intestinal polypeptide activate gene transcription through the cAMP signaling pathway in a catecholaminergic immortalized neuron. Neurochem Int 34: 183–191.

Thiel G, Cibelli G. 2002. Regulation of life and death by the zinc finger transcription factor Egr- 1. J Cell Physiol 193: 287–292.

Thiel G, Schoch S, Petersohn D. 1994. Regulation of synapsin I gene expression by the zinc finger transcription factor zif268/egr-1. J Biol Chem 269:15294–15301.

Thiel G, Kaufmann K, Magin A, Lietz M, Bach K, Cramer M. 2000. The human transcriptional repressor protein NAB1: Expression and biological activity. Biochim Biophys Acta 1493:289–301.