ARTICLE
Journal of Cellular Biochemistry 114:681–696 (2013)

Journal of Cellular Biochemistry

Regulation of Immediate-Early Gene Transcription Following Activation of $G\alpha_q$-Coupled Designer Receptors

Anke Kaufmann, Anja Keim, and Gerald Thiel*

Department of Medical Biochemistry and Molecular Biology, University of Saarland Medical Center, D-66421 Homburg, Germany

ABSTRACT

G-protein coupled designer receptors that are specifically activated by designer drugs have been developed. Here, we have analyzed the regulation of gene transcription following activation of $G\alpha_q$-coupled designer receptor ($R\alpha_q$). Stimulation of human embryonic kidney (HEK) 293 cells expressing $R\alpha_q$ with clozapine-N-oxide (CNO), a pharmacologically inert compound, induced the expression of biologically active Egr-1, a zinc finger transcription factor. Expression of a dominant-negative mutant of the ternary complex factor (TCF) Elk-1, a key transcriptional regulator of serum response element (SRE)-driven gene transcription, prevented Egr-1 expression. Stimulation of $R\alpha_q$ with CNO increased the transcriptional activation potential of Elk-1 and enhanced transcription of an SRE regulated reporter gene. In addition, AP-1 transcriptional activity was significantly elevated. AP-1 activity was controlled by TCFs and c-Jun in cells expressing an activated $G\alpha_q$-coupled designer receptor. CNO stimulation did not increase Egr-1 and AP-1 activity in neuroblastoma cells expressing endogenous M3 muscarinic acetylcholine receptors, indicating that CNO did not function as a ligand for these receptors. $R\alpha_q$ stimulation also increased the transcriptional activation potential of CREB and cAMP response controlled gene transcription. Pharmacological and genetic experiments revealed that the protein kinases Raf and ERK were essential to connect $R\alpha_q$ stimulation with enhanced Egr-1 and AP-1 controlled transcription. In contrast, MAP kinase phosphatase-1 functioned as a nuclear shut-off device of stimulus-transcription coupling. The fact that $R\alpha_q$ stimulation activates the transcription factors Egr-1, Elk-1, AP-1, and CREB indicates that regulation of gene transcription is an integral part of $G\alpha_q$-coupled receptor signaling. J. Cell. Biochem. 114: 681–696, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: G-PROTEIN COUPLED RECEPTOR; DESIGNER RECEPTOR; SIGNALING; TRANSCRIPTION; LENTIVIRUS

G-protein coupled receptors (GPCRs) are the largest group of plasma membrane receptors in nature, with at least 799 full-length GPCRs in humans [Lagerström and Schiöth, 2008]. GPCR is one of the principal targets of drugs in pharmacology. In particular, many clinically important drugs target class I GPCRs, the largest subfamily of this receptor type, including adrenergic, cannabinoid, dopamine, muscarinic, opioid, serotonin, and somatostatin receptors [Lagerström and Schiöth, 2008]. However, the complexity and diversity of GPCRs makes it difficult to manipulate the signaling pathway of a specific GPCR by natural or pharmacological ligands [Hermans, 2003]. Distinct G-protein coupling specificities and alternative splicing increase the complexity of GPCR signaling, generating multiple isoforms of the same receptor with distinct biochemical properties. Multiple receptors for a single ligand are often coexpressed in tissue preparations, making it difficult to interpret the responses to GPCR stimulation, that may be due either to the activation of several receptor subtypes, or due to the complexity of signaling pathways following activation of a single receptor subtype.

To circumvent the problem of GPCR complexity, G-protein coupled designer receptors have been developed that are unresponsive to endogenous ligands, but can be activated by otherwise pharmacologically inert compounds. These receptors have been termed designer receptors exclusively activated by designer drugs

Abbreviations: AP-1, activator protein-1; bZIP, basic region leucine zipper; CCh, carbachol; CNO, clozapine-N-oxide; CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; GPCR, G-protein coupled receptor; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factors; TRE, 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: University of Saarland.

*Correspondence to: Prof. Gerald Thiel, PhD, Department of Medical Biochemistry and Molecular Biology, University of Saarland Medical Center, D-66421 Homburg, Germany E-mail: gerald.thiel@uniklinik-saarland.de

Manuscript Received: 18 June 2012; Manuscript Accepted: 24 September 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 11 October 2012

DOI 10.1002/jcb.24410 • © 2012 Wiley Periodicals, Inc.

[Conklin et al., 2008; Pei et al., 2008]. The lack of constitutive activity of these designer receptors allows their use for in vitro and in vivo studies of signal transduction. Studies performed with transgenic mice demonstrated the power of analyzing designer receptor induced signaling. Stimulation of $G\alpha_q$ coupled designer receptors in β-cells of the pancreas enhanced insulin release, improved glucose tolerance in obese, insulin-resistant mice, and enhanced β-cell mass [Guettier et al., 2009]. Peripheral administration of the designer ligand clozapine-N-oxide (CNO) to transgenic mice expressing a $G\alpha_q$-coupled designer receptor in neurons showed a selective activation of hippocampal neurons expressing the designer receptor. Designer receptor activation could be correlated with behavioral changes of neuronal activation, including locomotion, and limbic seizures [Alexander et al., 2009].

Recently, we showed that stimulation of $G\alpha_q$-coupled muscarinic acetylcholine receptors, gonadotropin-releasing hormone receptors, and AT_1 angiotensin II receptors activates expression of the zinc finger transcription factor Egr-1 [Mayer et al., 2008; Rössler et al., 2008; Thiel and Rössler, 2011], and thus triggers a signaling cascade that ultimately leads to transcriptional changes. Here, we have analyzed the impact of $G\alpha_q$-coupled designer receptor ($R\alpha_q$) stimulation on immediate-early gene transcription. The results of this study show that the stimulation of $R\alpha_q$ activates transcription controlled by the transcription factors Egr-1, Elk-1, AP-1, and CREB. In addition, the role of protein kinases Raf and ERK and protein phosphatase MKP-1 on stimulus-transcription coupling was assessed.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Human embryonic kidney (HEK)293 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. Human SH-SY5Y neuroblastoma cells were cultured as previously described [Cibelli et al., 2002; Keim et al., 2012b]. HEK293ΔBRaf:ER cells expressing a conditionally active mutant of BRaf were previously described [Al-Sarraj et al., 2005]. The cells were cultured in the presence of 0.6 μg/ml puromycin. HEK293 and HEK293ΔBRaf:ER cells were incubated for 24 h in DMEM containing 0.05% fetal bovine serum. Stimulation was performed in medium containing 0.05% fetal bovine serum with 1 μM clozapine-N-oxide (CNO, Enzo Life sciences, # NS-105-0005), dissolved in ethanol. 4-Hydroxytamoxifen (4OHT, Sigma # H7904) was dissolved in ethanol and used at a concentration of 200 or 250 nM for 24 h. The MAP kinase kinase inhibitor PD98059 was purchased from Axxora (Lauser, Switzerland, Cat. no. 385-023) dissolved in DMSO and used at a concentration of 50 μM.

LENTIVIRAL GENE TRANSFER

All lentiviral transfer vectors used in this study are based on plasmids pFUW or pFUWG [Lois et al., 2002]. The transgenes were expressed under the control of the human ubiquitin-C promoter. The lentiviral transfer vectors pFUW-FLAG-REST/Elk-1ΔC, pFUW-mycDA-Raf1, pFUW-MKP-1, pFUWc-JunΔN, pFUW-GAL4-Elk-1, pFUW-GAL4-Sp1, and pFUW-GAL4-CREB have been described elsewhere [Rössler et al., 2008; Mayer et al., 2009; Rössler and Thiel, 2009; Müller et al., 2010; Thiel and Rössler, 2011; Ekici et al., 2012]. Plasmid pHA-$R\alpha_q$ [Guettier et al., 2009] was a kind gift of Jürgen Wess, Laboratory of Bioorganic Chemistry, NIH, Bethesda, USA. The coding region was excised with *Bam*HI and cloned into the *Bam*HI site of plasmid pFUW. Plasmid pEBGN-CREB, encoding amino acids 235–326 of CREB as a fusion protein with *Schistosoma japonicum* glutathione S-transferase (GST) has been described elsewhere [Thiel et al., 2005]. The plasmid was cut with XbaI. The fragment was filled-in with the Klenow fragment of DNA polymerase I and cloned into the Hpa I site of pFUW, generating the lentiviral transfer vector pFUW-GST-CREB. The viral particles were produced as previously described [Keim et al., 2012a].

REPORTER ASSAYS

The lentiviral transfer vectors pFWEgr-1.2luc, pFWEgr-1SREluc, pFWEBS1^4luc, pFWColl-luc, pFWColl-lucΔTRE, pFWc-Fos-luc, pFWc-FosCRE^4luc, and pFWUAS^{5}Sp1^2luc have been described elsewhere [Rössler et al., 2008; Rössler and Thiel, 2009; Müller et al., 2010; Thiel and Rössler, 2011; Ekici et al., 2012). HEK293 cells were infected with recombinant lentiviruses encoding promoter/luciferase reporter genes. In addition, cells were infected with a lentivirus encoding $R\alpha_q$. Cells were stimulated with CNO (1 μM) for 24 h. Cell extracts were prepared using reporter lysis buffer (Promega, Mannheim, Germany) and analyzed for luciferase activities as described [Thiel et al., 2000]. Luciferase activity was normalized to the protein concentration.

WESTERN BLOTS

Whole cell extracts and nuclear extracts were prepared as described [Kaufmann and Thiel, 2002]. Of nuclear proteins, 10–30 μg were separated by SDS–PAGE and the blots were incubated with antibodies directed against Egr-1 (Santa Cruz, Heidelberg, Germany, # sc-189), c-Fos (Santa Cruz, # sc-52), c-Jun (Santa Cruz, # sc-1694), or the phosphorylated form of c-Jun (Upstate Biotechnology, Lake Placid, NY, # 06-659). An antibody directed against HDAC1 (Santa Cruz, # sc-81598) was used as a loading control as described recently [Spohn et al., 2010; Mayer et al., 2011]. To detect FLAG-tagged proteins, we used the M2 monoclonal antibody directed against the FLAG epitope (Sigma-Aldrich, Steinheim, Germany, # F3165), at 1:3,000 dilution in TBS. Antibodies against the myc epitope were prepared from CRL-1729 hybridomas, purchased from ATCC. Immunoreactive bands were detected via enhanced chemiluminescence as described [Spohn et al., 2010; Mayer et al., 2011].

RESULTS

BIOSYNTHESIS OF EGR-1 FOLLOWING STIMULATION OF $G\alpha_Q$-COUPLED DESIGNER RECEPTORS WITH THE DESIGNER DRUG CNO

Designer GPCRs have been developed that specifically activate distinct G-protein induced signaling cascades. Here, we have analyzed signaling mediated by stimulation of a $G\alpha_q$-coupled designer receptor termed $R\alpha_q$. The backbone of $R\alpha_q$ is the rat type 3 muscarinic acetylcholine receptor, a prototype class 1 GPCR [Wess et al., 2008]. Binding of acetylcholine to the designer receptor was

prevented by introducing two point mutations (Y148C and A238G) within the third and fifth transmembrane domain of the type 3 muscarinic acetylcholine receptor [Guettier et al., 2009]. The $R\alpha_q$ designer receptor can be specifically activated by CNO, a metabolite of the antipsychotic agent clozapine that is otherwise pharmacologically inert for wild-type muscarinic acetylcholine receptors. Recently, we showed that stimulation of $G\alpha_q$-coupled M3 acetylcholine receptors, gonadotropin-releasing hormone receptors, or AT_1 angiotensin II receptors induces the biosynthesis of the zinc finger transcription factor Egr-1 [Mayer et al., 2008; Rössler et al., 2008; Thiel and Rössler, 2011]. Thus, we assessed whether stimulation of $G\alpha_q$-coupled designer receptors ($R\alpha_q$) with CNO leads to an upregulation of Egr-1 expression as well. HEK 293 cells were serum-starved for 24 h. Stimulation was performed with medium containing 1 μM CNO. The cells were harvested 1, 3, and 5 h after stimulation, nuclear extracts were prepared and Egr-1 expression was analyzed via immunoblotting. Egr-1 immunoreactivity was not detectable in the absence of stimulation, indicating that the $R\alpha_q$ designer receptor lacks detectable constitutive activity. Stimulation with CNO strikingly increased the biosynthesis of Egr-1 with a peak expression occurring 1 h after stimulation (Fig. 1A).

THE NEWLY SYNTHESIZED EGR-1 IS BIOLOGICALLY ACTIVE

The ability of Egr-1 to activate transcription depends upon the concentrations of the Egr-1 negative cofactors NAB1 and NAB2. These proteins bind to Egr-1 and block transcriptional activation via Egr-1 [Russo et al., 1995; Svaren et al., 1996; Thiel et al., 2000]. Thus, elevated Egr-1 protein levels do not automatically indicate an increased transcription of Egr-1 target genes. We determined the transcription of an Egr-1-responsive target gene in HEK293 cells expressing stimulated $G\alpha_q$-coupled designer receptors. To detect Egr-1 activity, a chromosomally embedded Egr-1-responsive luciferase reporter gene was used. A schematic depiction of the integrated provirus is shown in Figure 1B. The results show that the stimulation of HEK293 cells expressing $R\alpha_q$ with CNO significantly increased the transcription of the Egr-1-responsive reporter gene (Fig. 1C), indicating that biologically active Egr-1 was synthesized. To demonstrate the specificity of CNO stimulation of $R\alpha_q$, we infected SH-SY5Y neuroblastoma cells with a lentivirus encoding the Egr-1-responsive $EBS1^4luc$ reporter gene. SH-SY5Y cells express functional $G\alpha_q$-coupled M3 muscarinic acetylcholine receptors [Rössler et al., 2008]. Cells were stimulated with carbachol and CNO, respectively. Figure 1D shows that carbachol treatment leads to an upregulation of Egr-1 activity in SH-SY5Y cells, while stimulation of the cells with CNO had no effect. These data indicate that CNO failed to activate endogenous M3 muscarinic acetylcholine receptors.

ENHANCED EGR-1 PROMOTER ACTIVITY FOLLOWING STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS

The expression data of Egr-1, shown in Figure 1A, were complemented by promoter studies. We inserted an Egr-1 promoter/luciferase reporter gene into the chromatin of HEK293 cells using lentiviral gene transfer. The provirus encodes an Egr-1 promoter/luciferase reporter gene that contains 490 nucleotides of the human Egr-1 gene 5′ upstream region together with 235 nucleotides of the 5′-nontranslated region (Fig. 1E). HEK293 cells were infected with recombinant lentiviruses encoding the Egr-1 promoter/luciferase reporter gene. In addition, cells were infected with a lentivirus encoding $R\alpha_q$. The stimulation of the cells with CNO increased transcription of the Egr-1 promoter/reporter gene (Fig. 1F). In contrast, stimulation of SH-SY5Y neuroblastoma cells with CNO failed to induced transcription from an integrated Egr-1 promoter/luciferase reporter gene, while stimulation with carbachol significantly induced reporter gene transcription (Fig. 1G).

STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS INDUCE TRANSCRIPTION VIA THE SERUM RESPONSE ELEMENT (SRE)

The most important genetic elements within the Egr-1 promoter are five SREs that function as binding sites for the serum response factor (SRF) and ternary complex factors (TCFs). TCFs are proteins that contact DNA and also bind to SRF. To assess the involvement of SREs in $R\alpha_q$-mediated stimulus-transcription coupling, we analyzed transcription of a reporter gene that was under the control of the proximal SREs derived from the Egr-1 promoter (Fig. 1H). The analysis showed that stimulation of $R\alpha_q$ with the designer ligand CNO increased transcription of this SRE-controlled transcription unit (Fig. 1I).

STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS ENHANCES THE TRANSCRIPTIONAL ACTIVATION POTENTIAL OF THE TCF ELK-1

Elk-1 has a modular structure. The DNA binding domain of Elk-1 is localized on the N-terminus while the transcriptional activation domain is localized on the C terminus. A regulatory domain lies within this transcriptional activation domain encompassing the key phosphoacceptor sites S383 and S389. The B domain is required for the formation of the ternary Elk-1-SRF complex. A fusion protein consisting of the GAL4 DNA-binding domain fused to the activation domain of Elk-1 was expressed in HEK293 cells. As a control, we expressed a GAL4-Sp1 fusion protein (Fig. 2A). Since GAL4 does not bind to any known mammalian gene promoter element, interference by other transcriptional regulatory proteins was avoided. To measure the biological activities of the GAL4-Elk-1 and GAL4-Sp1 fusion proteins we implanted a GAL4 responsive reporter gene into the chromatin of HEK293 cells to ensure that the reporter gene is packed into an ordered nucleosomal structure. Figure 2B shows a schematic depiction of the integrated provirus, encoding the GAL4-responsive luciferase reporter gene. HEK293 cells were infected with a lentivirus encoding the luciferase reporter together with a lentivirus that encoded either the GAL4-Elk-1 or the GAL4-Sp1 fusion protein. In addition, cells were infected with a lentivirus encoding $R\alpha_q$. The results, shown in Figure 2C (left panel), reveal that the transcriptional activation potential of Elk-1 was significantly elevated in cells expressing an activated $R\alpha_q$-coupled designer receptor. In contrast, the transcriptional activation potential of the GAL4-Sp1 fusion protein was not changed in $R\alpha_q$ expressing cells that were stimulated with CNO (Fig. 2C, right panel).

SUPPRESSION OF TCF ACTIVITY BLOCKS THE UPREGULATION OF EGR-1 EXPRESSION FOLLOWING STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS

We directly assessed the impact of TCF activation on the regulation of Egr-1 gene transcription in $R\alpha_q$ expressing HEK293 cells that had been stimulated with CNO. To overcome the problem associated with redundancy of functions between the TCFs [Cesari et al., 2004], we expressed a dominant-negative mutant of Elk-1, termed REST/Elk-1ΔC (Fig. 3A). This mutant binds to the Elk-1 cognate site and to the SRF and thus blocks the access of wild-type Elk-1 to DNA. Expression of the REST/Elk-1ΔC mutant was verified in HEK293 cells infected with a REST/Elk-1ΔC encoding lentivirus (Fig. 3B). Expression of REST/Elk-1ΔC blocked the induction of Egr-1 biosynthesis in HEK293 cells expressing a stimulated $G\alpha_q$-coupled

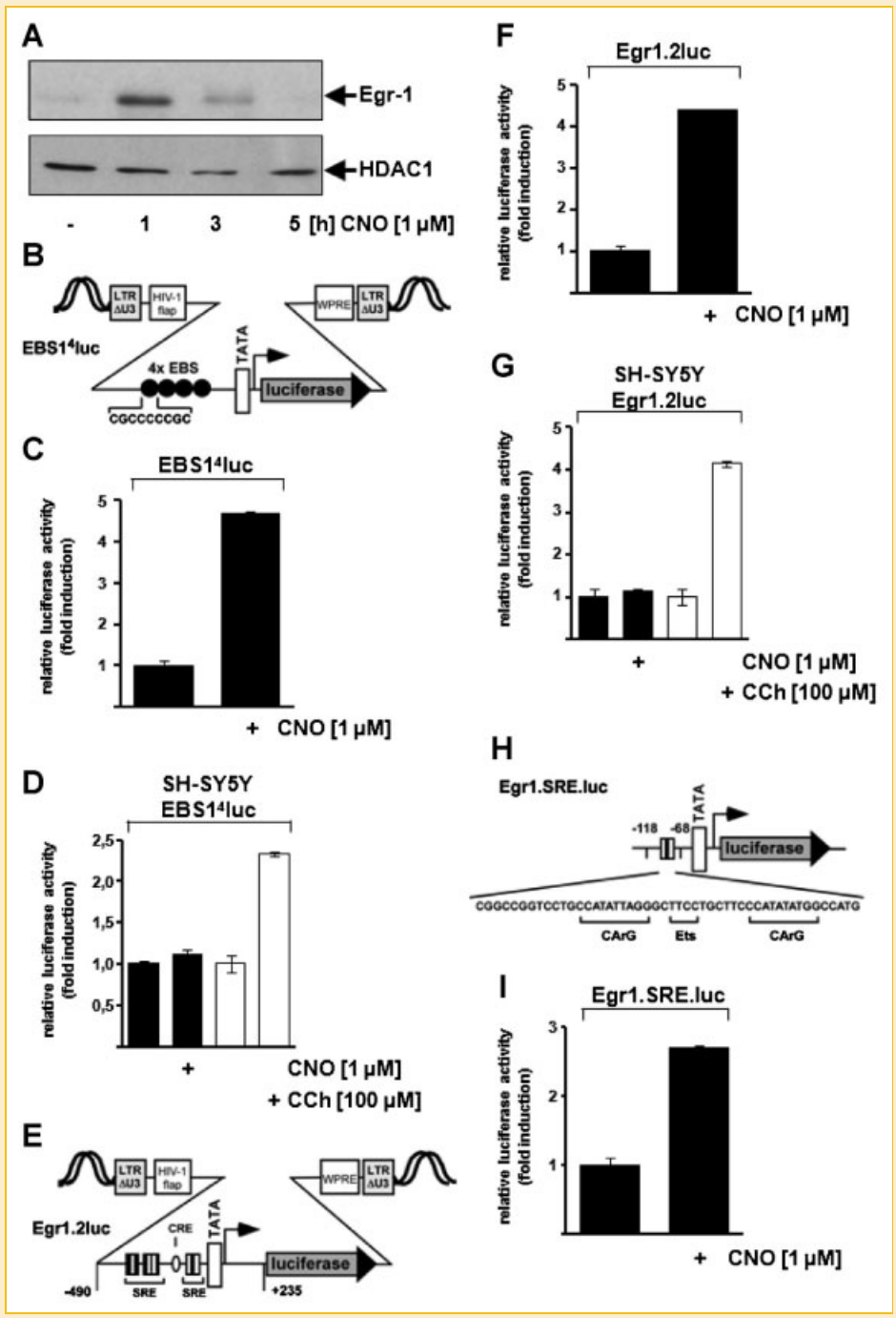

Fig. 1.

designer receptor (Fig. 3C). Thus, TCF activation is essential for inducing Egr-1 transcription in HEK293 cells expressing an activated $G\alpha_q$-coupled designer receptor.

SUPPRESSION OF TCF ACTIVITY BLOCKS TRANSCRIPTION OF A CHROMATIN-EMBEDDED EGR-1 PROMOTER/LUCIFERASE REPORTER GENE FOLLOWING STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS WITH A DESIGNER DRUG

We directly assessed the effect of REST/Elk-1ΔC on gene transcription. HEK293 cells were infected with a lentivirus encoding the luciferase reporter gene under control of the Egr-1 promoter (Egr1.2.luc in Fig. 1E) and with a lentivirus encoding REST/Elk-1ΔC. In addition, cells were infected with a lentivirus encoding $R\alpha_q$. The results show that expression of REST/Elk-1ΔC reduced the upregulation of reporter gene transcription following stimulation of the cells with CNO (Fig. 3D). The relative luciferase values, depicted in the figure, reveal that expression of REST/Elk-1ΔC reduced the transcriptional activity of the reporter gene under basal as well as under stimulated conditions.

SUPPRESSION OF TCF ACTIVITY BLOCKS THE UPREGULATION OF EGR-1 ACTIVITY AS A RESULT OF $G\alpha_q$-COUPLED DESIGNER RECEPTOR STIMULATION

We have shown that stimulation of $G\alpha_q$-coupled designer receptors with CNO induces an upregulation of Egr-1 activity in the cells, as measured with an Egr-1 responsive reporter gene. Figure 3E shows that stimulation of $G\alpha_q$-coupled designer receptors did not induce an upregulation of Egr-1 activity in HEK293 cells expressing the dominant-negative mutant of Elk-1, REST/Elk-1ΔC. Thus, TCF activity is essential to stimulate the transcriptional activity of Egr-1 as a result of $G\alpha_q$-coupled receptor stimulation.

SUPPRESSION OF TCF ACTIVITY BLOCKS THE TRANSCRIPTION OF AN ELK-1-REGULATED REPORTER GENE FOLLOWING STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS

The previous results showed that TCFs are required to couple $G\alpha_q$-coupled receptor stimulation with enhanced transcription of the SRE-regulated Egr-1 gene. We further analyzed the regulation of the 9E3/cCAF gene that contains two Elk-1 binding sites [Li et al., 1999, 2000]. While the Egr-1 promoter contains five SREs and multiple TCF binding sites, the 9E3/cCAF promoter does not contain a SRE. HEK293 cells were infected with a lentivirus encoding a luciferase reporter gene, controlled by the 9E3/cCAF promoter (Fig. 3F) and with a lentivirus encoding $R\alpha_q$. In addition, cells were either mock infected or infected with a lentivirus encoding REST/Elk-1ΔC. Treatment of the cells with CNO stimulated transcription of the reporter gene (Fig. 3G, left panels), indicating that stimulation of $G\alpha_q$-coupled receptors induce transcription of genes regulated either by SREs or by TCFs lacking SRF binding sites.

STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS TRIGGERS AN UPREGULATION OF AP-1 ACTIVITY

Stimulation of either $G\alpha_q$-coupled muscarinic acetylcholine receptor or AT_1 angiotensin II receptors triggers an upregulation of activator protein-1 (AP-1) activity [Rössler et al., 2008; Thiel and Rössler, 2011]. AP-1 is a dimeric transcription factor complex composed of members of the Jun, Fos, and ATF families of basic region leucine zipper transcription factors. We assessed whether stimulation of a $G\alpha_q$-coupled designer receptor increased AP-1 activity as well. To measure AP-1 regulated transcription, a collagenase promoter/luciferase reporter gene was implanted into the chromatin of HEK293 cells. The collagenase promoter contains the sequence motif 5′-TGAGTCA-3′, known as 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-responsive element (TRE) [Angel et al., 1987a, b], a high affinity binding site for the c-Jun:c-Fos

Fig. 1. Stimulation of $G\alpha_q$-coupled designer receptor $R\alpha_q$ with CNO enhances Egr-1 promoter activity and stimulates the biosynthesis of biologically active Egr-1. A: Induction of Egr-1 biosynthesis in CNO-stimulated HEK293 cells expressing $R\alpha_q$. Cells were cultured for 24 h in medium containing 0.05% serum. Stimulation with CNO (1 μM) was performed with medium containing 0.05% serum. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. The antibody directed against HDAC1 was used as a loading control. B and C: The newly synthesized Egr-1 protein is biologically active. HEK293 cells were infected with a recombinant lentivirus encoding an Egr-1 responsive reporter gene. A schematic depiction of the integrated provirus is shown (B). The implanted transcription unit encoded the luciferase reporter gene, controlled by a minimal promoter consisting of four binding sites for Egr-1, a TATA box and an initiator element. The U3 region of the 5′ LTR of the transfer vector is deleted. The location of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and the HIV flap element are shown. Additionally, cells were infected with a lentivirus expressing $R\alpha_q$. The infected cells were stimulated with CNO for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration (C). D: Carbachol, but not CNO, induces an upregulation of Egr-1 activity in human SH-SY5Y neuroblastoma cells. The cells were infected with a recombinant lentivirus encoding the Egr-1 responsive reporter gene $EBS1^4$luc. The infected cells were cultured in medium containing 0.05% serum for 24 h. Stimulation with CNO (1 μM) or carbachol (100 μM) was performed for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. E–G: Transcriptional regulation of an Egr-1 promoter/luciferase reporter gene. E: Schematic representation of integrated proviruses encoding an Egr-1 promoter/luciferase reporter gene. The serum response elements (SREs), and the cyclic AMP response element (CRE) within the Egr-1 promoter are depicted. F: HEK293 cells were infected with recombinant lentiviruses expressing the Egr-1 promoter/luciferase reporter gene. In addition, the cells were infected with a lentivirus encoding $R\alpha_q$. The infected cells were stimulated with CNO for 24 h. G: SH-SY5Y neuroblastoma cells were infected with recombinant lentiviruses expressing the Egr-1 promoter/luciferase reporter gene. Cells were stimulated with CNO (1 μM) or carbachol (100 μM) for 24 h as indicated. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. H and I: Regulation of a serum-response element (SRE) element controlled reporter gene by $R\alpha_q$ stimulation. H: Schematic representation of integrated proviruses encoding a SRE-containing luciferase reporter gene. The implanted transcription unit encoded the luciferase reporter gene, controlled by a minimal promoter consisting of the two proximal SREs # 1 and 2 of the Egr-1 promoter. I: HEK293 cells were infected with a recombinant lentivirus encoding the SRE-containing responsive reporter gene and with a lentivirus expressing $R\alpha_q$. The infected cells were stimulated with CNO (1 μM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

heterodimer. The AP-1 transcription factor was originally described as a heterodimer of c-Jun and c-Fos [Chiu et al., 1988; Curran and Franza, 1988]. A schematic depiction of the integrated provirus is seen in Figure 4A, including the sequence of the wild-type and the mutated TRE. Figure 4B shows that stimulation of a $G\alpha_q$-coupled designer receptor with CNO enhanced transcription of an AP-1-responsive reporter gene. In contrast, reporter gene transcription was not stimulated in cells expressing a collagenase promoter/luciferase reporter gene that contained a mutated TRE within the collagenase promoter. The specificity of ligand–receptor interaction was proven in SH-SY5Y neuroblastoma cells. Stimulation of the cells with carbachol induced AP-1 activity in the cells via stimulation of muscarinic acetylcholine receptors. In contrast, CNO treatment did not lead to an upregulation of AP-1 activity in SH-SY5Y cells (Fig. 4C).

STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS ENHANCES c-Fos PROMOTER ACTIVITY AND INDUCES THE BIOSYNTHESIS OF c-Fos

The TRE found in the collagenase promoter was identified as binding site for heterodimers of the basic region leucine zipper proteins c-Jun and c-Fos [Chiu et al., 1988; Curran and Franza, 1988]. We therefore analyzed expression and biological activity of these proteins in HEK293 cells that expressed a $G\alpha_q$-coupled designer receptor. Cells were infected with a lentivirus encoding a c-Fos-promoter/luciferase reporter gene (Fig. 5A). In addition, we infected the cells with a lentivirus encoding $R\alpha_q$. Stimulation of the cells with CNO enhanced c-Fos promoter activity (Fig. 5B). Next, we analyzed nuclear extracts of HEK293 cells expressing $R\alpha_q$. Figure 5C shows that stimulation with CNO strikingly enhanced c-Fos expression with a peak expression occurring 3 h following stimulation. The occurrence of different immunoreactive bands suggests that the biosynthesis of c-Fos as a result of $G\alpha_q$-coupled designer receptor stimulation was accompanied by phosphorylation of c-Fos.

SUPPRESSION OF TCF ACTIVITY BLOCKS THE TRANSCRIPTION OF A CHROMATIN-EMBEDDED c-Fos PROMOTER/LUCIFERASE REPORTER GENE FOLLOWING STIMULATION OF A $G\alpha_q$-COUPLED DESIGNER RECEPTOR

The c-Fos promoter contains a SRE and regulation of c-Fos expression by TCFs has been shown [Shaw et al., 1989; Thiel and Rössler, 2011]. To analyze the impact of the SRE, we infected HEK293 cells with a lentivirus encoding the c-Fos promoter/luciferase reporter gene and with a lentivirus encoding the dominant-negative mutant of Elk-1, REST/Elk-1ΔC. In addition, cells were infected with a lentivirus encoding $R\alpha_q$. Figure 5D shows that expression of REST/Elk-1ΔC reduced the upregulation of c-Fos

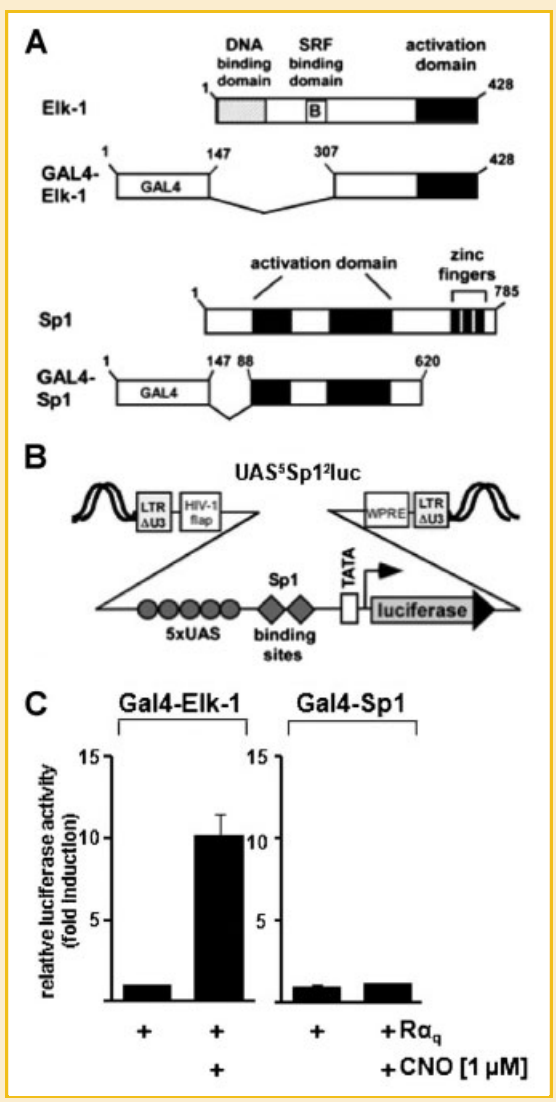

Fig. 2. CNO stimulation upregulates the transcriptional activation potential of Elk-1 in HEK293 cells expressing the $G\alpha_q$-coupled designer receptor $R\alpha_q$. A: Schematic representation of the modular structure of Elk-1, GAL4-Elk-1, Sp1, and GAL4-Sp1. The DNA binding domain of Elk-1 is localized on the N-terminus while the transcriptional activation domain is localized on the C-terminus. The regulatory domain is located within this transcriptional activation domain encompassing the key phosphoacceptor sites S383 and S389. Elk-1 binds with its B-domain to SRF, allowing the formation of the ternary Elk-1-SRF complex. The B-domain also couples the C-terminal phosphorylation of Elk-1 with enhanced DNA binding via the Ets domain. The GAL4-Elk-1 fusion protein lacks the DNA and SRF binding domains, but retains the phosphorylation-regulated C-terminal activation domain of Elk-1. The truncated Elk-1 is expressed as a fusion protein together with the N-terminal DNA-binding domain of GAL4. The Sp1 mutant GAL4-Sp1 includes the N-terminal activation domain of Sp1 (amino acids 88–620), fused to the GAL4 DNA binding domain. The zinc finger domain of Sp1 has been deleted. B: Schematic representation of the integrated provirus encoding the luciferase reporter under the control of the minimal promoter, consisting of two Sp1 binding sites, a TATA box and an initiator element. Upstream of the minimal promoter, five GAL4 binding sites (upstream activating sequence, UAS) were inserted. C: HEK293 cells were double-infected with a lentivirus encoding a GAL4-responsive luciferase reporter gene and a lentivirus encoding either GAL4-Elk-1 or GAL4-Sp1. In addition, the cells were infected with a lentivirus expressing $R\alpha_q$. The infected cells were stimulated with CNO (1 μM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

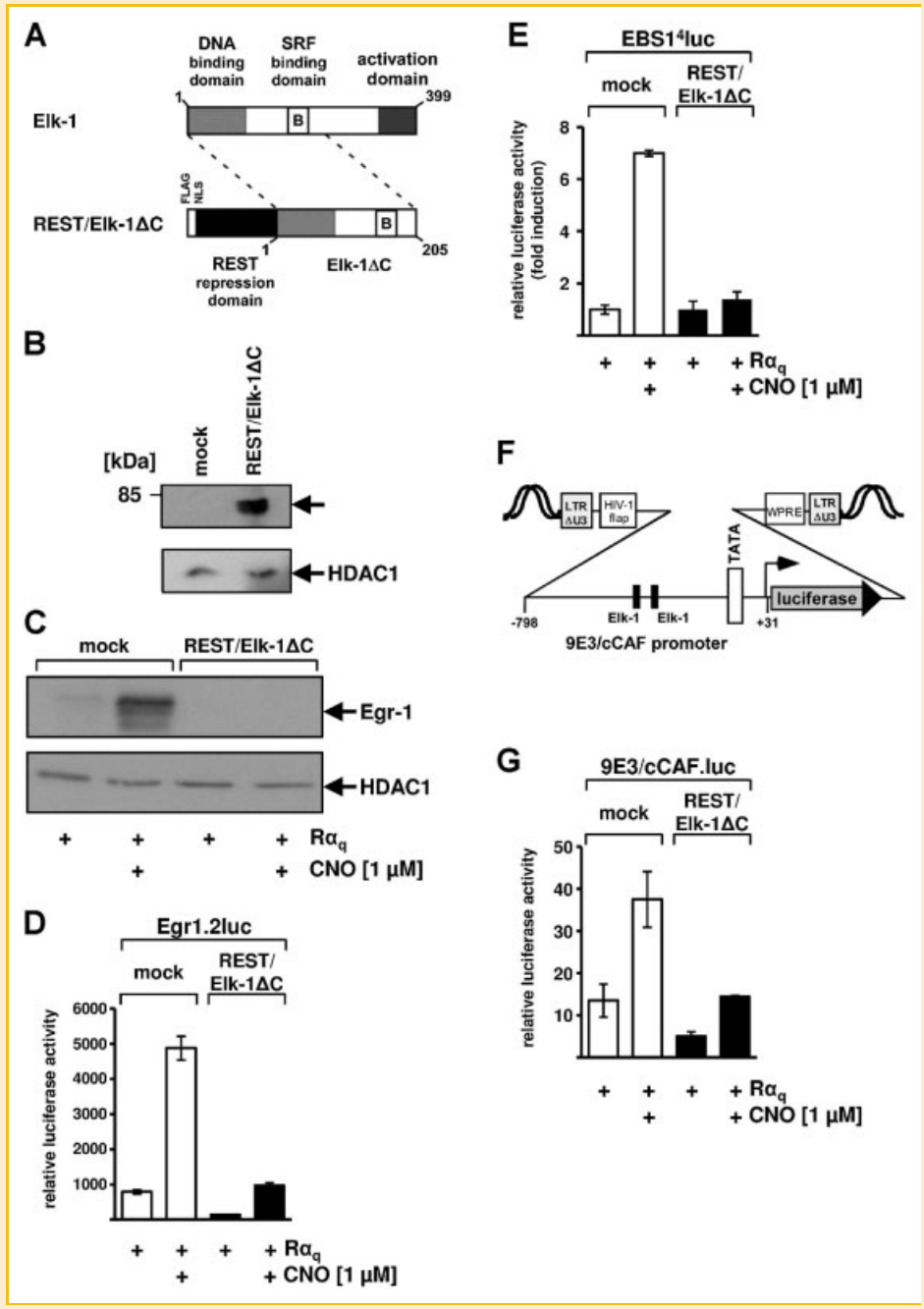

Fig. 3. TCFs play an essential role for inducing Egr-1 expression and upregulating Egr-1 promoter activity in CNO-stimulated HEK293 cells expressing a $G\alpha_q$-coupled designer receptor. A: Schematic representation of the modular structure of Elk-1 and the dominant-negative mutant REST/Elk-1ΔC. The mutant lacks the phosphorylation-regulated activation domain, but retains the DNA and SRF binding domains. The truncated Elk-1 is expressed as a fusion protein together with a transcriptional repression domain derived from the transcriptional repressor REST. B: Western blot analysis of mock-infected HEK293 cells or cells infected with a recombinant lentivirus encoding REST/Elk-1ΔC. The Western blot was probed with an antibody against the FLAG-tag. Molecular-mass markers in kDa are shown on the left. The antibody directed against HDAC1 was used as a loading control. C: Expression of REST/Elk-1ΔC blocks CNO-induced upregulation of Egr-1 in HEK293 cells expressing $R\alpha_q$. Cells were either mock-infected or infected with a recombinant lentivirus encoding REST/Elk-1ΔC. In addition, cells were infected with a lentivirus encoding $R\alpha_q$. The cells were stimulated with CNO (1 μM) as indicated. Nuclear extracts were prepared and subjected to Western blot analysis. The blot was incubated with an antibody directed against Egr-1. The antibody directed against HDAC1 was used as a loading control. D: Expression of a dominant-negative mutant of Elk-1 reduces the activity of the Egr-1 promoter. HEK293 cells were infected with lentiviruses encoding an Egr-1 promoter/luciferase reporter gene and the $G\alpha_q$-coupled designer receptor $R\alpha_q$. Cells were either mock infected or infected with a lentivirus encoding REST/Elk-1ΔC. The cells were serum-starved for 24 h in medium containing 0.05% serum. Stimulation with CNO (1 μM) was performed for 24 h. E: Expression of REST/Elk-1ΔC reduced Egr-1 activity in HEK293 cells expressing a stimulated $G\alpha_q$-coupled designer receptor. HEK293 cells were infected with recombinant lentiviruses encoding the Egr-1-responsive EBS1⁴luc transcription unit and the designer receptor $R\alpha_q$. In addition, cells were either mock infected or infected with a lentivirus encoding REST/Elk-1ΔC. The cells were serum-starved for 24 h in medium containing 0.05% serum. Stimulation with CNO (1 μM) was performed for 24 h. Cells were harvested, cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. F and G: Expression of a dominant-negative mutant of Elk-1 blocks transcription of an Elk-1-regulated reporter gene lacking SRF binding sites. F: Schematic representation of integrated proviruses encoding a 9E3/cCAF promoter/luciferase reporter gene. Luciferase expression is controlled by sequences from −789 to +31 derived from the chicken 9E3/cCAF gene. The Elk-1 binding sites within the 9E3/cCAF regulatory region are depicted. G: HEK293 cells were infected with recombinant lentiviruses encoding both a 9E3/cCAF promoter/luciferase reporter gene and the designer receptor $R\alpha_q$. In addition, cells were either mock infected or infected with a lentivirus encoding REST/Elk-1ΔC. The cells were serum-starved for 24 h in medium containing 0.05% serum. Stimulation with CNO (1 μM) was performed for 24 h.

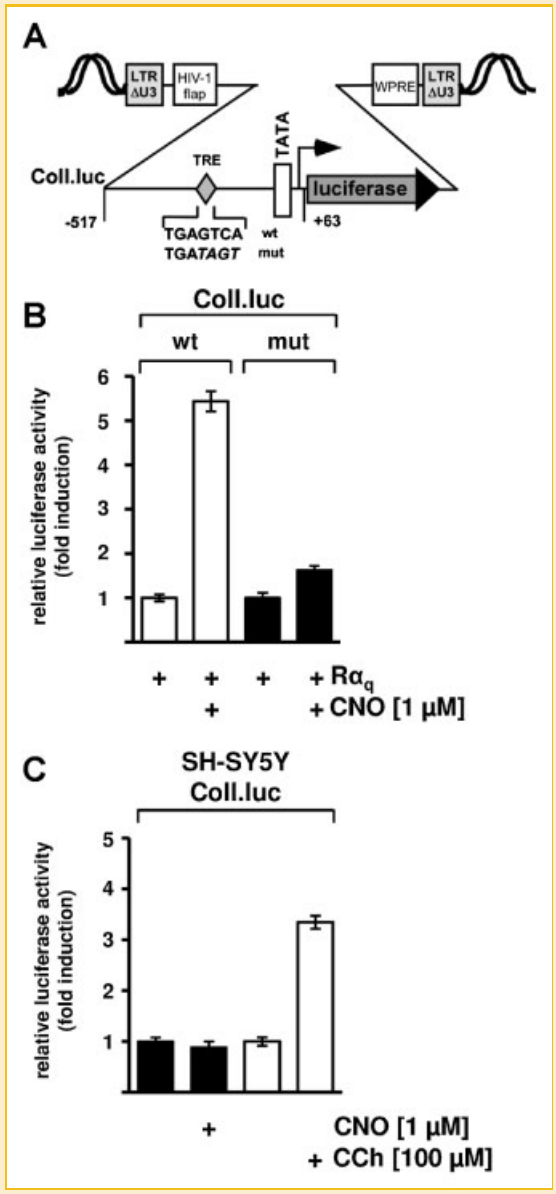

Fig. 4. Upregulation of AP-1 activity as a result of stimulation of a $G\alpha_q$-coupled designer receptor with CNO. A: Schematic representation of the integrated provirus encoding a collagenase promoter/luciferase reporter gene. The promoter was inserted upstream of the luciferase reporter gene. The location and sequence of the wild type and mutated 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) within the collagenase promoter is depicted. B: HEK293 cells were infected with recombinant lentiviruses encoding a collagenase promoter/luciferase reporter gene with an intact (wild-type, wt) or a mutated (mut) TRE. In addition, cells were infected with a lentivirus encoding the $R\alpha_q$ designer receptor. The cells were serum-starved for 24 h and then stimulated with CNO (1 μM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. C: SH-SY5Y neuroblastoma cells were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The infected cells were serum-starved for 24 h and then stimulated with either CNO (1 μM) or carbachol (CCh, 100 μM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

promoter/luciferase reporter gene transcription in CNO stimulated HEK293 cells that expressed $R\alpha_q$. The relative luciferase values indicate that expression of REST/Elk-1ΔC reduced reporter gene transcription under both basal and stimulated conditions. We conclude that TCFs control c-Fos expression following stimulation of $G\alpha_q$-coupled designer receptors.

STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS WITH CNO INDUCES THE BIOSYNTHESIS AND PHOSPHORYLATION OF c-Jun

Having shown that c-Fos is synthesized and activated following stimulation of $G\alpha_q$-coupled receptors with CNO, we assessed the expression of c-Jun under these conditions. Figure 5E shows that stimulation of $G\alpha_q$-coupled designer receptors induced an upregulation of c-Jun expression. c-Jun is a substrate for c-Jun N-terminal protein kinases and phosphorylation of c-Jun is required for activation of the transcriptional activation potential of c-Jun. Figure 5E shows that stimulation of $R\alpha_q$ with CNO induced the phosphorylation of c-Jun, indicating that c-Jun was activated as a result of stimulation.

c-Jun AND TCFs REGULATE AP-1 ACTIVITY FOLLOWING STIMULATION OF $G\alpha_q$-COUPLED DESIGNER RECEPTORS

We expressed a dominant-negative mutant of c-Jun, c-JunΔN, in HEK293 cells to assess the role of c-Jun in the regulation of AP-1 activity in HEK293 cells expressing $R\alpha_q$. The modular structure of c-Jun and c-JunΔN is shown in Figure 5F. This mutant fails to activate transcription because it lacks the transcriptional activation domain. Rather, the mutant inhibits DNA-binding of its wild-type bZIP counterpart by blocking the cognate sites for DNA-binding. The biological activity of these mutants has been demonstrated [Steinmüller et al., 2001; Steinmüller and Thiel, 2003; Thiel and Rössler, 2011]. Expression of c-JunΔN was verified in HEK293 cells infected with a c-JunΔN encoding lentivirus (Fig. 5G). Expression of c-JunΔN significantly reduced the upregulation of AP-1 activity in $R\alpha_q$ expressing HEK293 cells that had been stimulated with CNO (Fig. 5H), indicating that c-Jun or a c-Jun dimerization partner is involved in the upregulation of AP-1-mediated gene transcription following stimulation of a $G\alpha_q$-coupled designer receptor with the designer drug CNO.

Given the fact that TCFs are required to induce c-Fos expression following activation of $R\alpha_q$ with CNO, the impact of TCF activation on the regulation of AP-1 activity in $R\alpha_q$ expressing HEK293 cells was assessed using the dominant-negative mutant of Elk-1, REST/Elk-1ΔC. Figure 5K shows that the CNO-induced upregulation of AP-1 activity in HEK293 cells expressing $R\alpha_q$ was blocked in presence of REST/Elk-1ΔC. Together, these data underline that AP-1 activity is controlled by TCFs and c-Jun in cells expressing a stimulated $G\alpha_q$-coupled designer receptor.

RAF CONNECTS $G\alpha_q$-COUPLED DESIGNER RECEPTOR STIMULATION WITH ENHANCED TRANSCRIPTION OF EGR-1 AND AP-1 REGULATED GENES

Stimulation of $G\alpha_q$-coupled receptors induces an elevation of the intracellular Ca^{2+} concentration, due to the activation of phospholipase C and the generation of IP_3. As a result Ca^{2+}-dependent isoforms of protein kinase C are activated, that, in turn, activate the

ERK signaling pathway involving direct or indirect activation of Raf [Kolch et al., 1993; Schönwasser et al., 1998; Corbit et al., 2003]. The signaling cascade is schematically depicted in Supplement Figure S1. The importance of Raf in regulation of Egr-1 and AP-1 mediated gene transcription was shown in experiments involving HEK293 cells that expressed a ΔB-Raf/estrogen receptor fusion protein, a conditionally active form of BRaf [Thiel et al., 2009] (Fig. 6A). Stimulation of the cells with 4OHT induced an upregulation of transcription of chromatin-embedded Egr-1 and AP-1-responsive reporter genes (Fig. 6B). Stimulation of the ΔB-Raf/estrogen receptor fusion protein also activated the transcriptional activation potential of Elk-1, while the transcriptional activation potential of Sp1 remained unchanged (Fig. 6C). We assessed the role of Raf in stimulus-transcription coupling by expressing DA-Raf1, a splice form of A-Raf that functions as an antagonist of the Ras/Raf-ERK pathway [Yokoyama et al., 2007]. Figure 6D shows the modular structure of A-Raf and DA-Raf1 proteins. The myc-tagged DA-Raf1 protein was identified in extracts from HEK293 cells infected with a DA-Raf1 encoding lentivirus (Fig. 6E). Expression of DA-Raf1 significantly reduced the upregulation of Egr-1 expression following stimulation of $G\alpha_q$-coupled designer receptors with CNO (Fig. 6F). Likewise, the upregulation of Egr-1 and AP-1 activities as a result of $R\alpha_q$ stimulation was impaired in HEK293 cells expressing DA-Raf1 (Fig. 6G and H). Thus, Raf is essential to connect $G\alpha_q$-coupled receptor stimulation with enhanced transcription of Egr-1 and AP-1 controlled genes.

CYTOPLASMIC AND NUCLEAR ERK IS REQUIRED TO CONNECT $G\alpha_q$-COUPLED RECEPTOR STIMULATION WITH ENHANCED TRANSCRIPTION

Raf activates ERK via phosphorylation and activation of MAP kinase kinase (Fig. S1). HEK293 cells expressing $R\alpha_q$ were incubated with PD98059 to assess the role of ERK within the signaling cascade that connects CNO-induced stimulation of a $G\alpha_q$-coupled designer with enhanced transcription. PD98059 is a compound that inhibits phosphorylation of the MAP kinase kinase by Raf. As a control, we analyzed transcription of Egr-1 and AP-1 responsive reporter genes in HEK293 cells expressing a conditionally active version of BRaf. Figure 7A shows that preincubation of the cells with PD98059 efficiently reduced the transcriptional activity of Egr-1 and AP-1 following activation of the ΔBRaf:ER fusion protein with 4OHT. Likewise, preincubation of HEK293 cells expressing activated $G\alpha_q$-coupled designer receptors with PD98059 significantly reduced Egr-1 and AP-1 activity (Fig. 7B), indicating that suppression of the cytosolic ERK signaling pathway attenuated $G\alpha_q$-coupled designer receptor stimulation of gene transcription.

The phosphorylated and activated ERK translocates into the nucleus and influences the gene transcriptional program of the cells by phosphorylating transcriptional regulatory proteins. In the nucleus, MKP-1 is part of a negative feedback loop leading to the inactivation of ERK via dephosphorylation, as depicted schematically in Figure S1 [Rössler et al., 2008; Rössler and Thiel, 2009; Mayer et al., 2011]. Figure 7C shows that expression of MKP-1 attenuated the CNO-induced upregulation of Egr-1 expression in HEK293 cells expressing $G\alpha_q$-coupled designer receptors. In contrast, expression of protein phosphatase 2C (PP2C) had no effect on Egr-1 expression. In addition, MKP-1 expression prevented the upregulation of Egr-1 and AP-1 transcriptional activity in HEK293 cells that expressed activated $G\alpha_q$-coupled designer receptors (Figs. 7D and E), indicating that MKP-1 functioned as a negative feedback effector in stimulus-transcription coupling.

$G\alpha_q$-COUPLED DESIGNER RECEPTOR STIMULATION INDUCES CRE/CREB-REGULATED TRANSCRIPTION

Activation of the ERK signaling pathway has been shown to induce transcription of CRE-regulated genes via activation of CREB. ERK does not phosphorylate CREB directly, but phosphorylates and activates the protein kinase MSK that in turn phosphorylates CREB [Deak et al., 1998]. However, experiments analyzing gene transcription regulated by stimulation of either M3 muscarinic acetylcholine receptors or AT_1 angiotensin II receptors did not show an upregulation of CREB regulated transcription [Rössler et al., 2008; Thiel and Rössler, 2011]. Thus, we assessed the regulation of a CRE-regulated reporter gene following stimulation of a $G\alpha_q$-coupled designer receptor with the designer drug CNO. As a model reporter gene, we used a luciferase gene under control of four copies of the CRE derived from the c-Fos gene upstream of a minimal promoter. In this transcription unit, activation of transcription is only regulated by the CREs. A schematic representation of the integrated provirus is shown in Figure 8A. Transcription of the reporter gene was stimulated by CNO in HEK293 cells expressing $R\alpha_q$ (Fig. 8B). The upregulation of CRE-mediated gene transcription was impaired in cells, expressing a dominant-negative mutant of CREB, GST-CREB (Fig. 8C and D), indicating that $G\alpha_q$-coupled designer receptor stimulation activated CREB-regulated gene transcription. These data were corroborated by experiments analyzing the transcriptional activation potential of CREB in CNO-treated HEK293 cells that expressed, in addition to $R\alpha_q$, a GAL4-CREB fusion protein (Fig. 8E), together with a GAL4-responsive reporter gene. The experiment showed that the transcriptional activation potential of CREB was enhanced following stimulation of $R\alpha_q$ with CNO (Fig. 8F).

DISCUSSION

Transgenic experiments involving expression of a $G\alpha_q$-coupled designer receptor exclusively activated by a designer drug (termed $R\alpha_q$) in pancreatic β-cells or neurons revealed that these designer receptors represent a powerful chemical-genetic tool to investigate GPCR signaling and function [Alexander et al., 2009; Guettier et al., 2009]. The objective of this study was to investigate the signaling transduction following stimulation of $R\alpha_q$ by CNO, a compound that has been reported to be pharmacologically inert. Recently, we showed that stimulation of $G\alpha_q$-coupled AT_1 angiotensin II receptors, muscarinic acetylcholine receptors, or gonadotropin-releasing hormone receptors has a profound effect on transcriptional regulation [Mayer et al., 2008; Rössler et al., 2008; Thiel and Rössler, 2011]. Thus, we focused our study on the analysis of transcriptional changes as a result of $G\alpha_q$-coupled designer receptor activation.

This study showed that stimulation of $R\alpha_q$ by CNO activates expression of the transcription factor Egr-1, a protein that couples intracellular signaling with altered gene transcription [Thiel and Cibelli, 2002; Rössler et al., 2006]. Together with previous results [Mayer et al., 2008; Rössler et al., 2008; Thiel and Rössler, 2011], we conclude that activation of Egr-1 biosynthesis is a common theme of $G\alpha_q$-coupled receptor stimulation. Furthermore, we have demonstrated that the newly synthesized Egr-1 is biologically active, using a chromosomally integrated Egr responsive reporter gene.

Promoter studies showed that stimulation of $G\alpha_q$-coupled designer receptors triggered an activation of the Egr-1 promoter. The Egr-1 promoter contains five SREs and multiple TCF binding

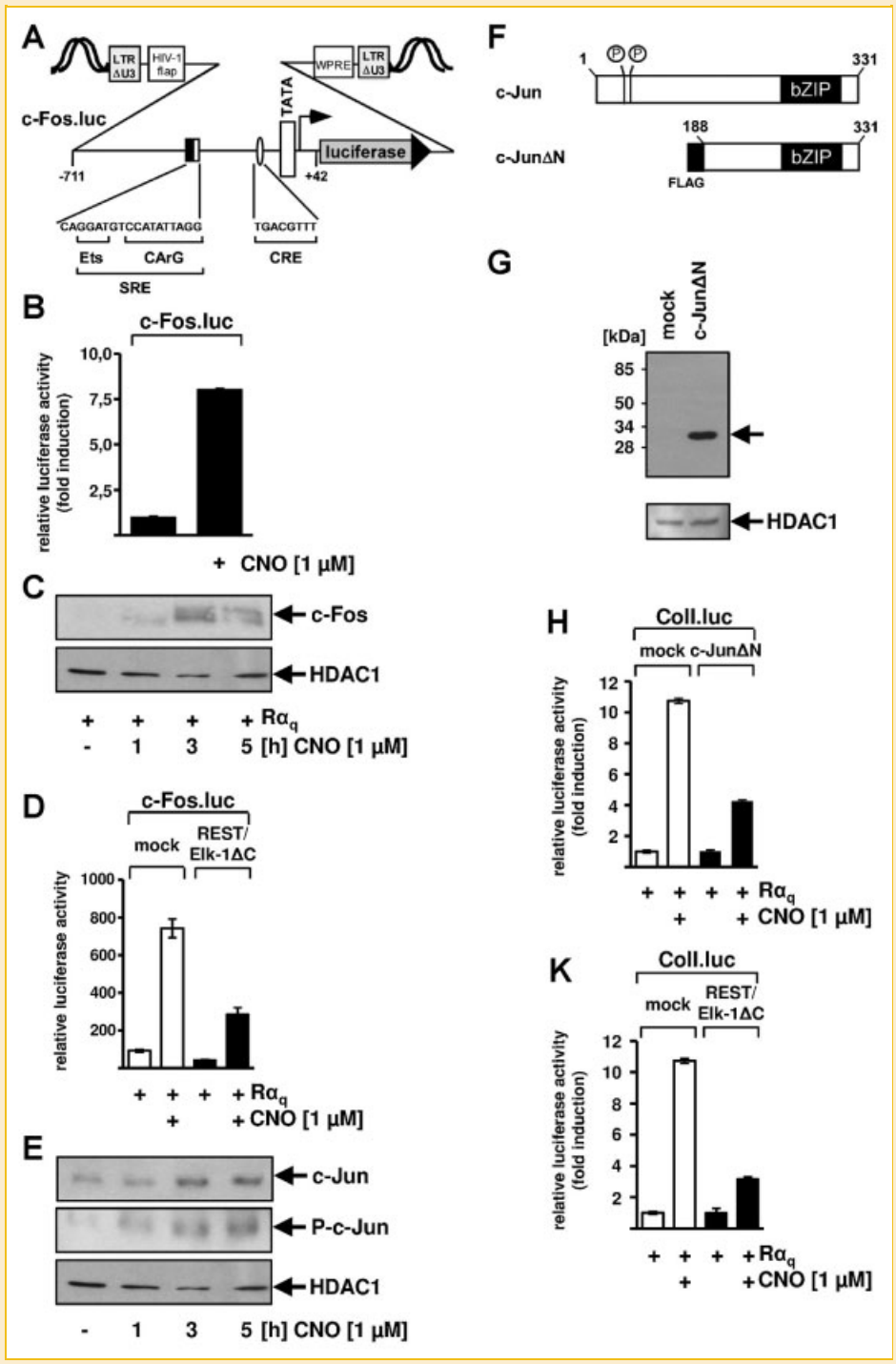

Fig. 5.

sites. These TCFs play a key role in stimulus-transcription coupling. They are substrates for multiple protein kinases and function as a molecular convergence point of cellular signaling cascades to convert the hormone-induced signaling cascade into a change in gene transcription [Shaw and Saxton, 2003]. The first indication that Elk-1 may be involved in the upregulation of Egr-1 expression as a result of stimulation of cells expressing $R\alpha_q$ was provided by the observation that the transcriptional activation potential of Elk-1 increased as a result of CNO stimulation. To prove the necessity of TCF activation in $R\alpha_q$ signaling, loss-of-function experiments were performed using a dominant negative version of Elk-1. Due to its binding to DNA and SRF, the Elk-1 mutant most likely also inhibited the activity of two other TCFs, SAP-1 and SAP-2. These experiments revealed that expression of REST/Elk-1ΔC impaired the activation of the Egr-1 promoter and the subsequent biosynthesis of Egr-1 in HEK293 cells expressing stimulated $G\alpha_q$-coupled designer receptors. Together with previous observations [Rössler et al., 2008; Rössler and Thiel, 2009; Thiel and Rössler, 2011], we conclude that TCF activation is a key step in connecting $G\alpha_q$-coupled receptors stimulation with enhanced Egr-1 biosynthesis.

Analysis of gene transcription following $G\alpha_q$-coupled receptor stimulation showed an upregulation of AP-1 activity [Rössler et al., 2008; Rössler and Thiel, 2009]. To measure AP-1 transcriptional activity, a collagenase promoter/luciferase reporter gene was implanted into the chromatin of HEK293 cells expressing $R\alpha_q$. This promoter contains an AP-1 binding site in the proximal promoter region and has frequently been used to monitor AP-1 activity [Angel et al., 1987a, b; Steinmüller et al., 2001; Steinmüller and Thiel, 2003; Mayer et al., 2008; Müller et al., 2010; Thiel and Rössler, 2011]. The results showed that stimulation of HEK293 cells expressing $R\alpha_q$ with CNO is accompanied by a rise in AP-1 activity. We also showed that expression of both c-Jun and c-Fos, the classical constituents of AP-1, was upregulated under these circumstances as well. Transcription of c-Fos was controlled by TCFs targeting the SRE within the c-Fos gene promoter. In addition, we showed that impairment of TCF activity reduced the AP-1 activity in $R\alpha_q$ expressing cells that had been stimulated with CNO. Thus, TCFs play a key role in regulating stimulus-transcription coupling by connecting $R\alpha_q$ stimulation with transcription of Egr-1 and AP-1 regulated genes. This study additionally showed that the newly synthesized c-Jun is phosphorylated and thus activated. Experiments involving expression of a dominant negative mutant of c-Jun revealed that c-Jun is an important regulator of AP-1 activity in HEK293 cells expressing $R\alpha_q$ that had been stimulated with CNO. Together, these data show that AP-1 activity is controlled by both c-Jun and TCFs following stimulation of $G\alpha_q$ coupled designer receptors.

Stimulation of the ERK signaling pathway may increase the transcriptional activity of CREB via activation of the CREB kinase MSK [Deak et al., 1998]. Accordingly, we showed in this study that CRE-regulated transcription is upregulated following stimulation of a $G\alpha_q$-coupled designer receptor with CNO. In line with this, we measured an elevated transcriptional activation potential of CREB as a result of $R\alpha_q$ stimulation. Similarly, enhanced CREB phosphorylation and CRE-regulated transcription have been observed in neuroblastoma cells following activation of $G\alpha_q$-coupled M3 muscarinic acetylcholine receptors [Rosethorne et al., 2008]. However, a recent study showed that stimulation of $G\alpha_q$-coupled AT_1 angiotensin II receptors in H295R adrenocortical cells with angiotensin II failed to activate CRE controlled reporter gene transcription and only marginally stimulated the transcriptional activation potential of CREB [Thiel and Rössler, 2011]. This outcome may be explained by a low expression of CREB in these cells [Nogueira and Rainey, 2010], indicating that the expression levels of signaling molecules (e.g. CREB and related proteins, inhibitory CRE-binding proteins) may decide whether the CRE/CREB signaling pathway is activated following $G\alpha_q$-coupled receptor activation.

Signaling mediated by $G\alpha_q$-coupled receptors involves a rise in intracellular Ca^{2+}, the activation of protein kinase C, and the subsequent activation of ERK. Using genetic and pharmacological tools, this study has shown that Raf activation is required in CNO-stimulated HEK293 cells expressing $R\alpha_q$ for an agonist-induced

Fig. 5. Stimulation of $G\alpha_q$-coupled designer receptors with CNO upregulates c-Fos and c-Jun expression. A and B: Transcriptional upregulation of an integrated c-Fos promoter/luciferase reporter gene in CNO-stimulated HEK293 cells expressing $R\alpha_q$. A: Schematic representation of integrated proviruses encoding a c-Fos promoter/luciferase reporter genes. The cyclic AMP response element (CRE) and the SRE are depicted. B: HEK293 cells were infected with a recombinant lentivirus encoding the c-Fos promoter/luciferase reporter gene. In addition, cells were infected with a lentivirus encoding $R\alpha_q$. The cells were serum-starved for 24 h and then stimulated with CNO (1 μM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. C: Induction of c-Fos biosynthesis in CNO-stimulated HEK293 cells expressing $R\alpha_q$. Cells expressing $R\alpha_q$ were cultured for 24 h in medium containing 0.05% serum and then stimulated with CNO (1 μM) for 1, 3 or 5 h as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against c-Fos. The antibody directed against HDAC1 was used as a loading control. D: Expression of a dominant-negative mutant of Elk-1 blocks the transcriptional activation of c-Fos promoter/luciferase gene transcription. HEK293 cells were double-infected with lentiviruses encoding the c-Fos promoter/luciferase reporter gene and the Elk-1 mutant REST/Elk-1ΔC. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). In addition, cells were infected with a lentivirus encoding $R\alpha_q$. The cells were serum-starved for 24 h and then stimulated with CNO (1 μM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. E: Expression and phosphorylation of c-Jun in CNO-stimulated human HEK293 cells expressing $R\alpha_q$. The infected cells were cultured for 24 h in medium containing 0.05% serum. Stimulation was performed with medium containing CNO (1 μM). Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with antibodies directed against either c-Jun, phosphorylated c-Jun, or HDAC1 as indicated. F–I: Essential role of c-Jun and TCF for controlling AP-1 activity in CNO-stimulated HEK293 cells expressing $R\alpha_q$ designer receptors. F: Modular structure of c-Jun and the dominant-negative form c-JunΔN. The dominant-negative mutant encompasses amino acid residues 188–331 of c-Jun, retaining the basic region leucine zipper domain (bZIP) responsible for dimerization and DNA-binding, but lacking the NH_2-terminal transcriptional activation domain. G: Western blot analysis of HEK293 cells infected with a lentivirus encoding c-JunΔN. As a control, mock-infected cells were analyzed. Western blots were probed with the antibody directed against the FLAG epitope. Molecular-mass markers in kDa are shown on the left. The antibody directed against HDAC1 was used as a loading control. H and K: HEK293 cells were double-infected with lentiviruses encoding a collagenase promoter/luciferase reporter gene and either c-JunΔN (H) or the Elk-1 mutant REST/Elk-1ΔC (K). As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). In addition, cells were infected with a lentivirus encoding $R\alpha_q$. The cells were serum-starved for 24 h. Stimulation was performed with medium containing CNO (1 μM) for 24 h.

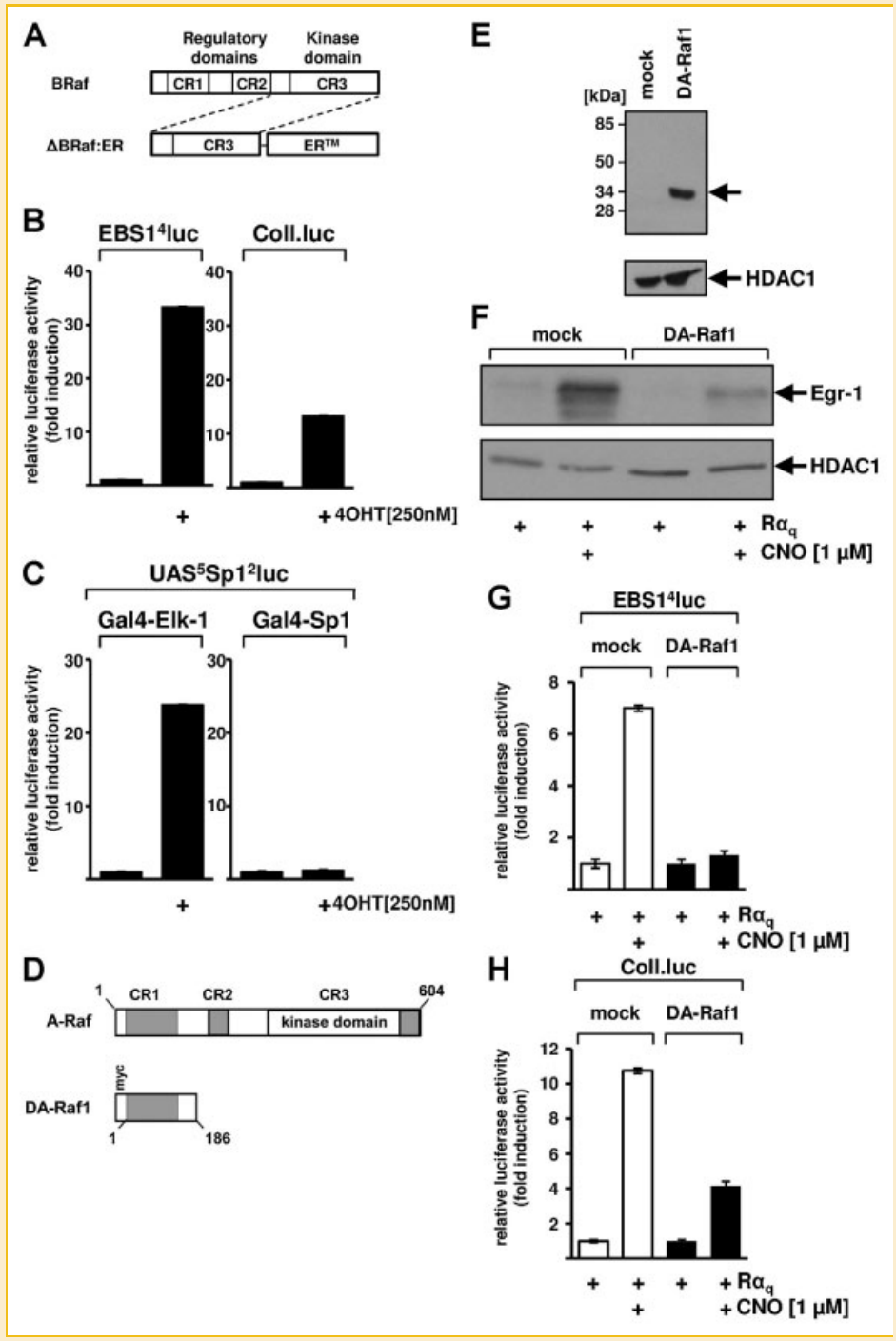

Fig. 6. Essential role of Raf for transcriptional activation in cells expressing stimulated $G\alpha_q$-coupled designer receptors. A: Schematic representation of the modular structure of BRaf and the conditionally active form ΔBRaf:ER. The functional domains of BRaf (CR3, CR2, and CR1) are depicted. CR1 is a cysteine-rich region and functions as binding site for activated Ras (Ras-GTP) at the cell membrane. CR2 is rich in serine and threonine residues and negatively regulates the biological activity of the catalytic domain. CR3 encompasses the protein kinase domain. Fusion of the catalytic CR3 domain to the hormone binding domain of the estrogen receptor generates the ΔRaf-1:ER fusion protein. B: Stimulation of ΔBRaf:ER in HEK293 cells activates Egr-1 and AP-1 controlled transcription. HEK293/ΔBRaf:ER cells were infected with lentiviruses encoding either the Egr-1 responsive $EBS1^4luc$ reporter gene (left panel) or the AP-1 responsive collagenase promoter/luciferase reporter gene (right panel). The infected cells were stimulated with 4OHT (250 nM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities which were normalized to the protein concentrations. C: Stimulation of ΔBRaf:ER in HEK293 cells upregulates the transcriptional activation potential of Elk-1. HEK293/ΔBRaf:ER cells were infected with lentiviruses encoding either a GAL4-Elk-1 fusion protein (left panel) or a GAL4-Sp1 fusion protein. In addition, cells were infected with a lentivirus encoding the GAL4-responsive reporter UAS^5Sp1^2luc. The infected cells were stimulated with 4OHT (250 nM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities which were normalized to the protein concentrations. D–H: Expression of DA-Raf1, a splice form of A-Raf, attenuates CNO-induced upregulation of Egr-1 and AP-1 activity in $R\alpha_q$-expressing HEK293 cells. D: Modular structure of A-Raf and the DA-Raf1. E: Expression of DA-Raf1 in lentiviral-infected HEK293 cells is detected using an antibody against the N-terminal myc-tag. Molecular-mass markers in kDa are shown on the left. F: HEK293 cells were infected with a lentivirus encoding DA-Raf1. As a control HEK293 cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). In addition, cells were infected with a lentivirus encoding $R\alpha_q$. Cells were cultured for 24 h in medium containing 0.05% serum. Stimulation was performed with medium containing CNO (1 μM). Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with antibodies directed against either Egr-1, or HDAC1 as indicated. G and H: HEK293 cells were double-infected with lentiviruses encoding $R\alpha_q$ and DA-Raf1 as indicated. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). In addition, cells were infected with a lentivirus encoding either the Egr-1 responsive $EBS1^4luc$ reporter gene (G) or the AP-1 responsive collagenase promoter/luciferase reporter gene (H). The cells were serum-starved for 24 h. Stimulation was performed with medium containing CNO (1 μM) for 24 h.

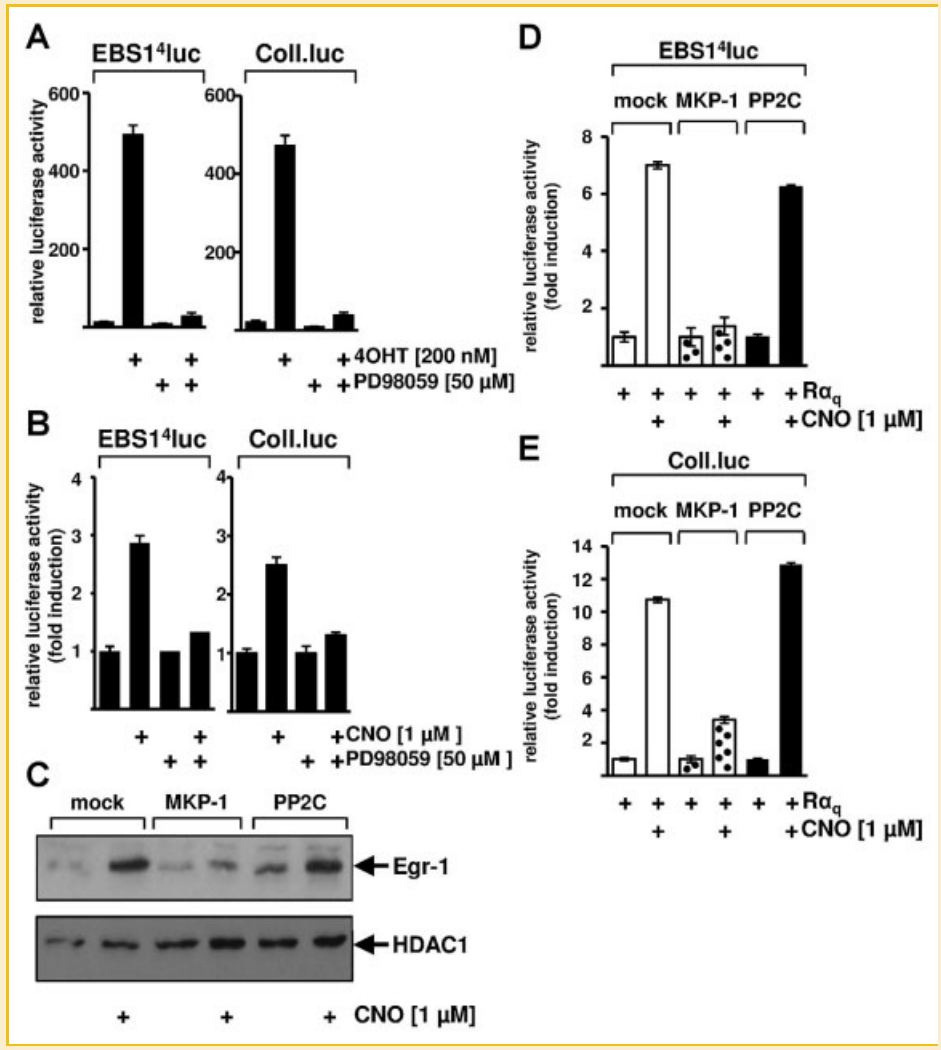

Fig. 7. Essential role of ERK in stimulus-transcription coupling mediated by a $G\alpha_q$-coupled designer receptor. A: Preincubation of HEK293/ΔBRaf:ER cells with PD98059 attenuates ΔBRaf:ER-induced upregulation of Egr-1 and AP-1 activity. HEK293/ΔBRaf:ER cells were infected with lentiviruses encoding either the Egr-1 responsive EBS1^{4}luc reporter gene (left panel) or the AP-1 responsive collagenase promoter/luciferase reporter gene (right panel). The cells were preincubated for 3 h with PD98059 (50 μM) as indicated. Stimulation with 4OHT (200 nM) was performed for 24 h. Cell extracts were prepared and analyzed for luciferase activities, which were normalized to the protein concentrations. B: HEK293 cells were infected with lentiviruses encoding either the Egr-1 responsive EBS1^{4}luc reporter gene (left panel) or the AP-1 responsive collagenase promoter/luciferase reporter gene (right panel). In addition, cells were infected with a lentivirus encoding $R\alpha_q$. The cells were preincubated for 3 h with PD98059 (50 μM). Stimulation with CNO (1 μM) was performed for 24 h. Cell extracts were prepared and analyzed for luciferase activities, which were normalized to the protein concentrations. C: Expression of MKP-1 attenuates $G\alpha_q$-coupled designer receptor induced stimulus-transcription coupling. HEK293 cells encoding $R\alpha_q$ were infected with lentiviruses encoding either MKP-1 or protein phosphatase 2C (PP2C). As a control HEK293 cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). Cells were cultured for 24 h in medium containing 0.05% serum. Stimulation was performed with medium containing CNO (1 μM). Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with antibodies directed against either Egr-1 or HDAC1 as indicated. D and E: Expression of MKP-1 attenuates upregulation of Egr-1 and AP-1 transcriptional activity in HEK293 cells expressing an activated $G\alpha_q$-coupled designer receptor. HEK293 cells were infected with a lentivirus encoding $R\alpha_q$ and with lentiviruses encoding either the Egr-1 responsive EBS1^{4}luc reporter gene (D) or the AP-1 responsive collagenase promoter/luciferase reporter gene (E). In addition, cells were infected with lentiviruses either MKP-1 or PP2C as indicated. As a control, cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 h. Stimulation was performed with medium containing CNO (1 μM) for 24 h.

upregulation of Egr-1 and AP-1 activity. The experiments clearly showed that inhibition of MEK phosphorylation by Raf via the compound PD98059 blocked the elevation of Egr-1 and AP-1 activity as a result of $R\alpha_q$ stimulation with CNO. Likewise, expression of an A-Raf splice form impaired the signaling cascade that connects $G\alpha_q$ coupled designer receptor stimulation with enhanced transcription of Egr-1 and AP-1 target genes.

The phosphorylation of ERK as a result of $G\alpha_q$ coupled designer receptor stimulation has been described recently [Alvarez-Curto et al., 2011] and this is corroborated by the necessity of Raf in the signaling chain. In addition, this study showed that expression of MKP-1, a dual-specific phosphatase that dephosphorylates and inactivates ERK in the nucleus, blocked the upregulation of Egr-1 and AP-1 activity following stimulation of $G\alpha_q$ coupled designer

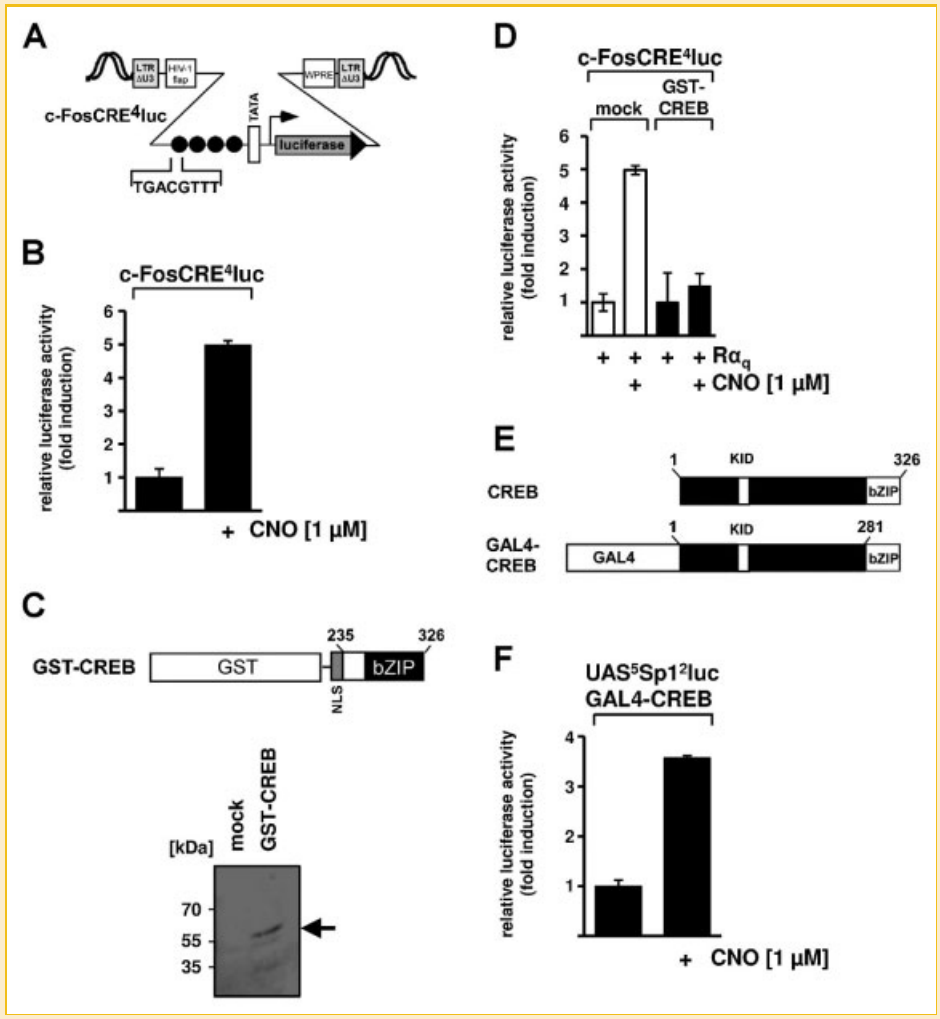

Fig. 8. Activation of CRE/CREB-regulated transcription in HEK293 cells expressing an activated $G\alpha_q$-coupled designer receptor. A: Schematic representation of the integrated provirus encoding a CRE-controlled luciferase reporter gene. The regulatory region contains a minimal promoter consisting of the human immunodeficiency virus TATA box, the adenovirus major late promoter initiator element, and four copies of CRE derived from the c-Fos promoter. B: HEK293 cells were infected with recombinant lentiviruses encoding the CRE-controlled reporter gene and the $R\alpha_q$ designer receptor. The cells were serum-starved for 24 h and then stimulated with CNO (1 µM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. C and D: The upregulation of CRE-regulated transcription following activation of $R\alpha_q$ is attenuated in HEK293 cells expressing a dominant-negative mutant of CREB. C: Schematic representation and expression of the modular structure of a GST-CREB fusion protein, a dominant-negative mutant of CREB. HEK293 cells were infected with a GST-CREB encoding lentivirus. Nuclear extracts of the infected cells were prepared and analyzed for GST-CREB expression using anti-GST-antibodies. D: HEK293 cells were infected with lentiviruses encoding a CRE-controlled reporter gene and the $G\alpha_q$-coupled designer receptor $R\alpha_q$. Cells were either mock infected or infected with a lentivirus encoding GST-CREB. The cells were serum-starved for 24 h in medium containing 0.05% serum. Stimulation with CNO (1 µM) was performed for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. E and F: Stimulation of a $G\alpha_q$-coupled designer receptor leads to an enhanced transcriptional activation potential of CREB. E: Schematic representation of the modular structure of CREB and GAL4-CREB. F: HEK293 cells were infected with three lentiviruses encoding a GAL4-responsive luciferase reporter gene, a GAL4-CREB fusion protein, and the designer receptor $R\alpha_q$. The cells were incubated with medium containing 0.05% serum for 24 h and then stimulated with CNO (1 µM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

receptors. Thus, MKP-1 functions as a nuclear shut-off device that interrupts the signaling cascade connecting receptor stimulation with transcription of Egr-1 and AP-1 controlled genes.

Transgenic mice had been generated expressing $G\alpha_q$ coupled designer receptors in pancreatic β-cells or neurons, respectively [Alexander et al., 2009; Guettier et al., 2009]. These studies showed that the use of designer receptors is powerful tools for analysis of the physiological responses to $G\alpha_q$ coupled receptor stimulation in animal models. The in vitro analysis presented here shed light to the fact that activation of gene transcription is an integral part of $G\alpha_q$ coupled receptor activation. This fact should be kept in mind in the interpretation of the phenotypes of transgenic mice expressing activated $G\alpha_q$ coupled designer receptors.

ACKNOWLEDGMENTS

We thank Jürgen Wess for the $R\alpha_q$ expression vector, Oliver Rössler for drafting Figure S1, Karl Bach for excellent technical help, and Libby Guethlein for critical reading of the manuscript.

REFERENCES

Alexander GM, Rogan SC, Abbas AI, Armbruster BN, Pei Y, Allen JA, Nonneman RJ, Hartmann J, Moy SS, Nicolelis MA, McNamara JO, Roth BL. 2009. Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. Neuron 63:27–39.

Al-Sarraj A, Day RM, Thiel G. 2005. Specificity of transcriptional regulation by the zinc finger transcription factors Sp1, Sp3 and Egr-1. J Cell Biochem 94:153–167.

Alvarez-Curto E, Prihandoko R, Tautermann CS, Zwier JM, Pediani JD, Lohse MJ, Hoffmann C, Tobin AB, Milligan G. 2011. Developing chemical genetic approaches to explore G-protein coupled receptor function: validation of the use of a receptor activated solely by synthetic ligand (RASSL). Mol Pharmacol 80:1033–1046.

Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, Jonat C, Herrlich P, Karin M. 1987a. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49: 729–739.

Angel P, Baumann I, Stein B, Delius H, Rahmsdorf HJ, Herrlich P. 1987b. 12-O tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer located in the 5′ flanking region. Mol Cell Biol 7:2256–2266.

Cesari F, Brecht S, Vintersten K, Vuong LG, Hofmann M, Klingel K, Schnorr J-J, Arsenian S, Schild H, Herdegen T, Wiebel FF, Nordheim A. 2004. Mice deficient for the Ets transcription factor Elk-1 show normal immune responses and mildly impaired neuronal gene activation. Mol Cell Biol 24:294–305.

Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M. 1988. The c-fos protein interacts with c-jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 54:541–552.

Cibelli G, Policastro V, Rössler OG, 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.

Conklin BR, Hsiao E, Claeysen S, Dumuis A, Srinivasan S, Forsayeth JR, Guettier J-M, Chang WC, Pei Y, McCarthy KD, Nissenson RA, Wess J, Bockaert J, Roth BL. 2008. Engineering GPCR signaling pathways with RASSLs. Nat Meth 5:673–678.

Corbit KC, Trakul N, Eves EM, Diaz B, Marshall M, Rosner MR. 2003. Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. J Biol Chem 278: 13061–13068.

Curran T, Franza BR, Jr. 1988. Fos and jun: the AP-1 connection. Cell 55: 395–397.

Deak M, Clifton AD, Lucocq JM, Alessi DR. 1998. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J 17:4426–4441.

Ekici M, Keim A, Rössler OG, Hohl M, Thiel G. 2012. Chromatin structure and expression of the AMPA receptor subunit GluR2 in human glioma cells: major role of REST and Sp1. J Cell Biochem 113:528–543.

Guettier J-M, Gautam D, Scarselli M, Ruiz de Azua I, Li JH, Rosemond E, Ma X, Gonzalez FJ, Armbruster BN, Lu H, Roth BL, Wess J. 2009. A chemical-genetic approach to study G protein regulation of β cell function in vivo. Proc Nat Acad Sci USA 106:19197–19202.

Hermans E. 2003. Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. Pharmacol Ther 99: 25–44.

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.

Keim A, Rössler OG, Rothhaar TL, Thiel G. 2012b. Arsenite-induced apoptosis of human neuroblastoma cells requires p53 but occurs independently of c-Jun. Neuroscience 206:25–38.

Keim A, Müller I, Thiel G. 2012a. Efficient genetic manipulation of 1321N1 astrocytoma cells using lentiviral gene transfer. J Neurosci Meth 206:138–142.

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marmé D, Rapp UR. 1993. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 364:249–252.

Lagerström MC, Schiöth HB. 2008. Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov 7:339–357.

Li Q-J, Vaingankar S, Green HM, Martins-Green M. 1999. Activation of the 9E3/cCAF chemokine by phorbol esters occur via multiple signal transduction pathways that converge to MEK1/ERK2 and activate Elk1 transcription factor. J Biol Chem 274:15454–15465.

Li Q-J, Vaingankar S, Sladek FM, Martins-Green M. 2000. Novel nuclear target for thrombin: activation of the Elk1 transcription factor leads to chemokine gene expression. Blood 96:3696–3706.

Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 295:868–872.

Mayer SI, Deixheimer V, Nishida E, Kitajima S, Thiel G. 2008. Expression of the transcriptional repressor ATF3 in gonadotrophs is regulated by Egr-1, CREB, and ATF2 after gonadotropin-releasing hormone receptor stimulation. Endocrinology 149:6311–6325.

Mayer SI, Rössler OG, Endo T, Charnay P, Thiel G. 2009. Epidermal growth factor-induced proliferation of astrocytes requires Egr transcription factors. J Cell Sci 122:3340–3350.

Mayer SI, Müller I, Mannebach S, Endo T, Thiel G. 2011. Signal transduction of pregnenolone sulfate in insulinoma cells. J Biol Chem 286:10084–10096.

Müller I, Endo T, Thiel G. 2010. Regulation of AP-1 activity in glucose-stimulated insulinoma cells. J Cell Biochem 110:1481–1494.

Nogueira EF, Rainey WE. 2010. Regulation of aldosterone synthase by activator transcription factor/cAMP response element-binding protein family members. Endocrinology 151:1060–1070.

Pei Y, Rogan SC, Yan F, Roth BL. 2008. Engineering GPCRs as tools to modulate signal transduction. Physiology 23:313–321.

Rosethorne EM, Nahorski SR, Challiss RAJ. 2008. Regulation of cyclic AMP response-element binding-protein (CREB) by $G_{q/11}$-protein-coupled receptors in human SH-SY5Y neuroblastoma cells. Biochem Pharmacol 75:942–955.

Rössler OG, Thiel G. 2009. Thrombin induces Egr-1 expression in fibroblasts involving elevation of the intracellular Ca^{2+} concentration, phosphorylation of ERK and activation of ternary complex factor. BMC Mol Biol 10:40.

Rössler OG, Stefano L, Bauer I, Thiel G. 2006. Stimulus-transcription coupling in the nervous system – The zinc finger protein Egr-1. In: Thiel G, editor. Transcription factors in the nervous system—development, brain function and disease. Weinheim, German: Wiley-VCH. pp 379–395.

Rössler OG, Henß I, Thiel G. 2008. Transcriptional response to muscarinic acetylcholine receptor stimulation: regulation of Egr-1 biosynthesis by ERK, Elk-1, MKP-1, and calcineurin in carbachol-stimulated human neuroblastoma cells. Arch Biochem Biophys 470:93–102.

Russo MW, Sevetson BR, Milbrandt J. 1995. Identification of NAB1, a repressor of NGFI-A and Krox20-mediated transcription. Proc Natl Acad Sci USA 92:6873–6877.

Schönwasser DC, Marais RM, Marshall CJ, Parker P. 1998. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol Cell Biol 18:790–798.

Shaw PE, Saxton J. 2003. Ternary complex factors: prime targets for mitogen-activated protein kinases. Int J Biochem Cell Biol 35:1210–1226.

Shaw PE, Schroter H, Nordheim A. 1989. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. Cell 56:563–572.

Spohn D, Rössler OG, Philipp SE, Raubuch M, Kitajima S, Griesemer D, Hoth M, Thiel G. 2010. Thapsigargin induces expression of activating transcription factor 3 in human keratinocytes involving Ca^{2+} ions and c-Jun N-terminal protein kinase. Mol Pharmacol 78:865–876.

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.

Steinmüller L, Cibelli G, Moll JR, Vinson C, Thiel G. 2001. Regulation and composition of activator protein 1 (AP-1) transcription factors controlling collagenase and c-Jun promoter activities. Biochem J 360:599–607.

Svaren J, Sevetson BR, Apel ED, Zimonjic DB, Popescu NC, Milbrandt J. 1996. NAB2, a corepressor of NGFI-A (egr-1) and Krox20, is induced by proliferative and differentiative stimuli. Mol Cell Biol 16:3545–3553.

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, Rössler OG. 2011. Immediate-early transcriptional response to angiotensin II in human adrenocortical cells. Endocrinology 152:4211–4223.

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.

Thiel G, Al Sarraj J, Vinson C, Stefano L, Bach K. 2005. Role of basic region leucine zipper transcription factors cyclic AMP response element binding protein (CREB), CREB2, activating transcription factor 2 and CAAT/enhancer binding protein alpha in cyclic AMP response element-mediated transcription. J Neurochem 92:321–336.

Thiel G, Ekici M, Rössler OG. 2009. Regulation of cellular proliferation, differentiation and cell death by activated Raf. Cell Commun Signal 7:8.

Wess J, Han S-J, Kim S-K, Jacobson KA, Li JH. 2008. Conformational changes involved in G-protein-coupled-receptor activation. Trends Pharmacol Sci 29:616–625.

Yokoyama T, Takano K, Yoshida A, Katada F, Sun P, Takenawa T, Andoh T, Endo T. 2007. DA-Raf1, a competent intrinsic dominant-negative antagonist of the Ras-ERK pathway, is required for myogenic differentiation. J Cell Biol 177:781–793.