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Interleukin-1β and Tetradecanoylphorbol Acetate-Induced Biosynthesis of Tumor Necrosis Factor α in Human Hepatoma Cells Involves the Transcription Factors ATF2 and c-Jun and Stress-Activated Protein Kinases

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Abstract The proinflammatory cytokine tumor necrosis factor (TNF) α is mainly produced in cells from the monocyte/macrophage lineage. TNFα is also a key signaling molecule in the liver functioning as an important physiological and pathogenic mediator. In hepatocytes or human hepatoma cells TNFα is expressed at extremely low levels but TNFα biosynthesis can be induced by interleukin (IL)-1β or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Here, we show that IL-1β and TPA stimulated TNFα gene transcription in hepatoma cells mediated by a composite TPA-responsive element/cAMP response element. Both IL-1β and TPA triggered phosphorylation and activation of the basic region leucine zipper transcription factors c-Jun and ATF2 and expression of dominant-negative mutants of c-Jun and ATF2-reduced TNFα promoter activity and secretion of TNFα. Expression of the nuclear dual-specific MAP kinase phosphatase-1 (MKP-1) blocked TNFα promoter activity and TNFα secretion following IL-1β or TPA stimulation, indicating that MKP-1 functions as a nuclear shut-of-device of IL-1β and TPA-induced TNFα expression.
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Key words: ATF2; c-Jun; MEKK1; MKP-1; p38 protein kinase; TNFα promoter

The gene encoding the cytokine tumor necrosis factor α (TNFα) is among the earliest genes expressed in lymphocytes following activation through the antigen receptor. TNFα shows a pleiotropic spectrum of bioactivities involving the regulation of immune responses, proliferation, and programmed cell death. Most importantly, TNFα functions as a major proinflammatory mediator and dysregulated TNFα function has been connected with a variety of diseases, including Crohn's disease and rheumatoid arthritis [Jones and Moreland, 1999; Bell and Kamm, 2000]. The analysis of TNFα-deficient mice revealed that TNFα is a key player of the innate immune system, as shown by an impaired defense against certain intracellular pathogens in TNFα-deficient animals [Marino et al., 1997].

TNFα binds to two surface receptors, TNF receptor 1 and 2. The TNF receptor 1, that is highly expressed in liver, contains a protein–protein interaction domain termed death domain in its cytoplasmic portion, connecting the TNF receptor 1 with caspase activation and apoptosis. However, TNFα alone is not able to induce liver cell death. Rather, hepatocytes require sensitization to TNFα-toxicity by

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compounds that block NF-κB activation or RNA or protein synthesis [Tilg and Diehl, 2000; Liedtke et al., 2002]. The proapoptotic function of TNFα is obviously masked by concomitant activation of the transcription factor NF-κB that protects liver cells from TNFα-induced apoptosis. Gene targeting experiments showed that NF-κB has a protective function in the liver [Beg et al., 1995]. Massive liver apoptosis occurred in RelA knockout mice, leading to embryonic death. Interestingly, crossbreeding of $RelA^{-/-}$ mice with TNFα receptor 1 knockout mice rescued the animals [Doi et al., 1999], indicating that TNFα-induced activation of NF-κB during liver development protects against apoptosis. TNFα also induces transcription of genes encoding proinflammatory cytokines such as interleukin-8, resulting in the extravasation of neutrophils in the liver that damage the organ. TNFα may also play a prominent role in viral hepatitis, alcoholic liver disease, and fulminant hepatic failure (reviewed in [Bradham et al., 1998]). Thus, TNFα clearly functions as a mediator of hepatotoxicity. However, the fact that TNFα activates the hepatoprotective transcription factor NF-κB, and the observation that hepatocyte proliferation during liver regeneration requires TNFα signaling via activation of the TNF receptor-1 [Yamada et al., 1997], indicate that TNFα functions as a "two-edged sword" in the liver [Bradham et al., 1998].

Unstimulated hepatocytes synthesize extremely low amounts of TNFα mRNA. However, signaling molecules such as IL-1β or the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induce TNFα gene transcription and subsequently TNFα biosynthesis [Frede et al., 1996]. Thus, an analysis of the regulation of TNFα biosynthesis, including the study of the signaling cascade that connects extracellular stimulation with enhanced TNFα gene transcription, and the identification of the responsible gene regulatory proteins, is essential for the understanding of the physiology and pathophysiology of TNFα in the liver. Members of the mitogen-activated protein (MAP) kinases have been identified to be responsible for TNFα gene expression in lymphocytes and non-lymphocyte tissues. In T lymphocytes, extracellular signal-regulated protein kinase (ERK), c-Jun-*N*-terminal kinase (JNK), and p38 MAP kinase have been shown to cooperate to regulate TNFα gene expression [Hoffmeyer et al., 1999]. In embryonic stem cell-derived cardiac myocytes, it has been suggested that JNK represses TNFα gene expression, whereas p38 MAP kinase positively regulates TNFα biosynthesis [Minamino et al., 1999]. A variety of transcription factors including NF-κB, AP1, AP2, nuclear factor of activated T cells, Sp1, Egr-1, c-Jun, CAAT/enhancer binding protein β and ATF2 have been proposed to control TNFα gene transcription in various cell types [Rhoades et al., 1992; Tsai et al., 1996, 2000; Yao et al., 1997; Zagariya et al., 1998; Falvo et al., 2000]. We have studied the intracellular signaling pathways and the transcription factors responsible for stimulus-induced TNFα gene expression in hepatoma cells. The results show that stress-activated protein kinases and the basic region leucine zipper transcription factors c-Jun and ATF2 are integral parts of the signaling cascades that connect IL-1β and phorbol ester stimulation of hepatoma cells with enhanced TNFα gene transcription.

MATERIALS AND METHODS

Reporter Constructs

The TNFα promoter/luciferase construct pTNFα(−1311)luc [Rhoades et al., 1992] was a kind gift of James S. Economou, UCLA, Los Angeles, CA, USA. The reporter plasmid pTNFα $(TRE/CRE)^2$luc that contains two copies of the composite TRE/cAMP response element (CRE) of the human TNFα gene, was generated by subcloning of two copies of the synthetic annealed oligonucleotides 5′-TCG AGC CTC CAG ATG AGC TCA TGG GTT TCT G-3′ and 5′-TCG ACA GAA ACC CAT GAG CTC ATC TGG AGG C-3′ into the Xho I and Sal I-sites of plasmid pHIVTATA-CAT [Thiel et al., 1996]. This sequence was subsequently multimerized as described, excised together with the minimal promoter and cloned into pGL3-Basic (Promega). The transcription unit present in this reporter plasmid contains 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.

Expression Vectors

The expression vectors encoding dominant-negative mutants of ATF2, ATF4, CREB, c-Jun,

and C/EBP [Olive et al., 1997; Ahn et al., 1998; Steinmüller et al., 2001; Vinson et al., 2002; Thiel et al., 2004] have been described elsewhere. An expression plasmid encoding MEKK1Δ, a truncated form of MEK kinase 1 that lacks amino acids 1–351 [Minden et al., 1994] was a kind gift of Michael Karin, University of California, San Diego, CA, USA. The coding region was cloned into plasmid p3XFLAG-CMV-7.1 (Sigma) generating the plasmid pCMV-FLAG-MEKK1Δ. Expression vectors encoding FLAG-tagged p38 MAP kinase and a constitutively active form of MAP kinase kinase 6 (MKK6b(E)) have been described [Han et al., 1996; Jiang et al., 1996] and were kind gifts from Jiahuai Han, Department of Immunology, The Scripps Research Institute, La Jolla, CA, USA. The expression vector encoding IκBS32A/S36A was a kind gift of Thomas Wirth, University of Ulm, Germany. Expression plasmids encoding constitutively active MAP kinase kinase 1 [Mansour et al., 1994] and MKP-1 [Sun et al., 1993] were kind gifts of Natalie G. Ahn and Sam Mansour, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO, USA and Nicholas K. Tonks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA, respectively.

Cell Culture, Transfections and Reporter Gene Assays

Human HepG2 hepatoma cells were cultured and transfected as described [Steinmüller et al., 2001]. The amounts of expression vectors transfected are indicated in the figure legends. The luciferase reporter plasmids (1 μg/plate) and the internal reference plasmid pRSVβ (2 μg/plate) were transfected into cells grown on 60 mm plates. Twenty-four hours post-transfection, the serum concentration was lowered to 0.05% (for the stimulation with TPA) or 0.0% (for the stimulation with IL-1β) and the cells were incubated for further 24 h. Stimulation with TPA (50 ng/ml, dissolved in DMSO) was performed for 8 h. Stimulation with IL-1β (1 ng/ml, Strathmann Biotech, Hannover, dissolved in sterile water as a 5 μg/ml stock solution) was performed for 8 h. Lysates were prepared using cell culture lysis buffer (Promega) and β-galactosidase and luciferase activities were measured as described [Thiel et al., 2000]. Each experiment was repeated at least two times with consistent results.

Lentiviral Gene Transfer

The lentiviral transfer vectors pFUW-MKP-1 and pFUW-A-ATF2, encoding MKP-1 and the dominant-negative ATF2 mutant A-ATF2, respectively, were generated via subcloning of the coding region into plasmid pFUW [Lois et al., 2002]. The viral particles were produced by transient transfection of 293T/17 cells as described [Stefano et al., 2006].

Western Blots

Nuclear extracts were prepared as described [Kaufmann and Thiel, 2002]. Twenty micrograms of nuclear proteins were separated by SDS–PAGE and the blot was incubated with antibodies directed against either c-Jun (Upstate # 06–225, dilution 1:1,000), or ATF2 (Santa Cruz # sc-6233, dilution 1:3,000), or the phosphorylated forms of c-Jun (Upstate # 06-659, dilution 1: 2,500) and ATF2 (Santa Cruz # sc-8398, dilution 1:1,000). Blots were developed with a horseradish peroxidase conjugated anti-mouse (for anti-phospho-ATF2) or anti-rabbit secondary antibody (for the c-Jun, phospho-c-Jun and ATF2 antibodies) and ECL (Amersham, Freiburg, Germany).

Reverse Transcriptase-Polymerase Chain Reactin (RT-PCR)

RNA was isolated as described [Chomczynski and Sacchi, 1987]. One microgram total RNA from unstimulated, IL-1β, and TPA-stimulated HepG2 cells was reverse transcribed. The following gene-specific primers were used: TNFα: 5′-AGC CTC TTC TCC TTC CTG AT-3′ and 5′-GAG GAC CTG GGA GTA GAT GA-3′ (product size 317 nucleotides); β-actin: 5′-TTC AAC TCC ATC ATG AAG TGT GAC GTG-3′ and 5′-CTA AGT CAT AGT CCG CCT AGA AGC ATT-3′ (product size 309 bp). The PCR conditions were: denaturation (95°C, 30 s), annealing (β-actin: 57°C; TNFα: 59°C, 30 s), primer extension (72°C, 1 min) for 40 (TNFα) or 30 (β-actin) cycles. Amplified products were resolved by electrophoresis on 2% agarose gels and stained with ethidiumbromide.

ELISA

Twenty-four hours after stimulation with IL-1β (1 ng/ml) or TPA (50 ng/ml) cell culture supernatants were collected and immediately frozen at −70°C. Human TNFα was quantified using the BD OptEIA™ human ELISA Set (BD

Biosciences, San Diego, CA) according to the instructions.

RESULTS

Interleukin-1β and Phorbol Ester Stimulate TNFα Gene Transcription in Human HepG2 Hepatoma Cells

HepG2 hepatoma cells were cultured in serum-free or serum-reduced medium for 24 h and then stimulated with IL-1β or TPA for 3 h. The cells were harvested and the RNA was isolated and analyzed by RT-PCR. Figure 1A shows that the treatment of hepatoma cells with IL-1β or TPA led to an upregulation of TNFα mRNA. Next, we used the ELISA technique to quantify the secreted TNFα protein following stimulation (Fig. 1B). Treatment of the cells with TPA triggered a significant secretion of TNFα into the cell culture medium. The biological activity of IL-1β towards TNFα secretion in HepG2 cells was much weaker and TNFα concentration secreted was close to the detection limit of the assay.

Interleukin-1β and Phorbol Ester Enhance TNFα Gene Promoter Activity in Human HepG2 Hepatoma Cells

To identify genetic elements and transcription factors responsible for the IL-1β or TPA-responsiveness of the TNFα gene, we analyzed a human TNFα promoter/luciferase reporter gene in transient transfection experiments. In humans, the TNFα promoter consists of an intervening sequence of ≈1,300 nucleotides located between the TNFβ and TNFα open reading frames. We used a reporter plasmid encompassing −1311 nucleotides of the regulatory region of the TNFα gene (Fig. 2A). HepG2 cells were transfected with the TNFα promoter/luciferase reporter plasmid pTNFα(−1311)luc together with the internal reference plasmid pRSVβ. Cells were serum-starved for 24 h and then stimulated with either IL-1β (1 ng/ml) or TPA (50 ng/ml) for 8 h. Cell extracts were prepared and the relative luciferase activities (luciferase light units divided by β-galactosidase units) were determined. Figure 2B shows that the addition of IL-1β or phorbol ester to the cells induced reporter gene transcription controlled by the regulatory region of the human TNFα gene. Luciferase activity was enhanced by a factor of ≈2 (IL-1β) and 6 (TPA). Thus,

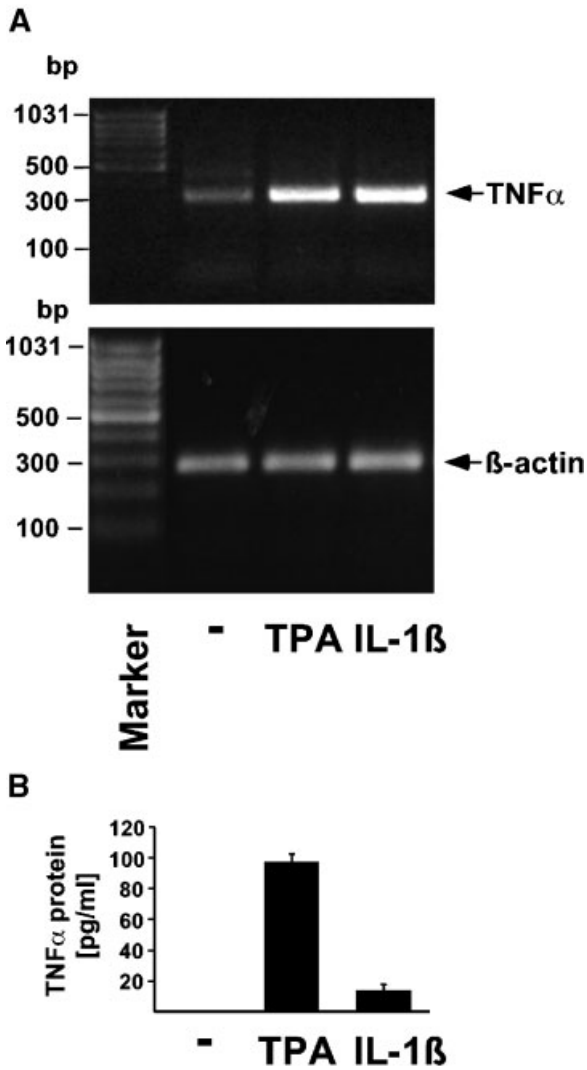

Fig. 1. Upregulation of TNFα mRNA and protein concentrations in HepG2 hepatoma cells as a result of IL-1β and TPA stimulation. **A**: HepG2 cells were stimulated with IL-1β (1 ng/ml) or TPA (50 ng/ml) for 3 h. Total RNA was isolated, the mRNA reverse transcribed and the cDNA analyzed by PCR using primers to detect TNFα and β-actin. **B**: HepG2 cells were cultured in serum-reduced or serum-free medium for 24 h and then treated with TPA (50 ng/ml) or IL-1β (1 ng/ml) for 24 h. Secreted TNFα in the cell culture medium was detected by ELISA.

IL-1β and TPA trigger signaling cascades that converge on the TNFα gene.

The proximal region of the human TNFα promoter contains a genetic element encompassing the sequence 5′-TGAGCTCA-3′ which is very similar to the classical phorbol 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE, sequence 5′-TGAGTCA-3′) or the CRE (sequence 5′-TGACGTCA-3′). In cells derived from the immune system this motif has been reported to function as an integrative

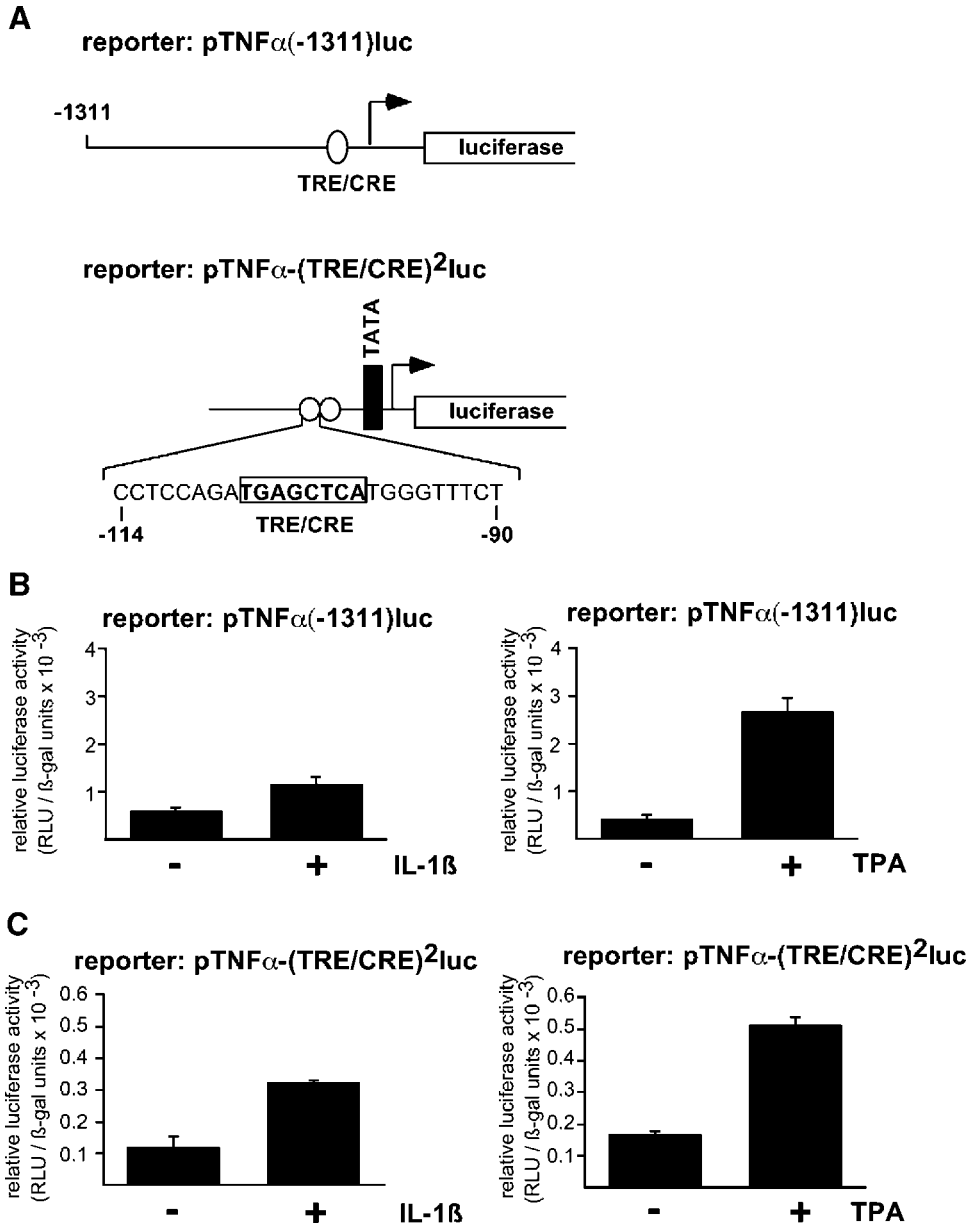

Fig. 2. IL-1β and phorbol ester enhance TNFα promoter activity in human hepatoma cells. **A**: The reporter plasmid pTNFα(−1311)luc contains the luciferase reporter gene and the regulatory region derived from the human TNFα gene. The reporter plasmid pTNFα(TRE/CRE)^{2}luc contains two copies of the composite TRE/CRE motif of the TNFα gene upstream of a minimal promoter consisting of the human immunodeficiency virus TATA box and the adenovirus major late promoter initiator element. **B**, **C**: The reporter plasmids pTNFα(−1311)luc (B) or pTNFα(TRE/CRE)^{2}luc (C) were transfected into HepG2 cells together with the internal standard plasmid pRSVβ Cells were incubated in serum-free or serum-reduced medium for 24 h and then stimulated with IL-1β (1 ng/ml) or TPA (50 ng/ml) for 8 h. Cells were harvested, cell extracts were prepared and analyzed for β-galactosidase and luciferase activities. Luciferase activity was normalized to β-galactosidase activity, which served as control for transfection efficiency. Data are means ± SD for n = 4 experiments.

genetic element for diverse signal transduction pathways including stimulation by antigens, calcium, or lipopolysaccharides [Tsai et al., 1996, 2000]. To analyze the biological role of this composite TRE/CRE motif in IL-1β or TPA-stimulated hepatoma cells, we created a reporter gene containing two copies of this element upstream of a minimal promoter. The transcription unit of this reporter gene termed pTNFα (TRE/CRE)^{2}luc is depicted in Figure 2A. Figure 2C shows that the stimulation of HepG2 cells with IL-1β or phorbol ester upregulated

reporter gene transcription controlled by the composite TRE/CRE element of the TNFα promoter.

IL-1β and Phorbol Ester Stimulation Triggers Phosphorylation and Activation of ATF2 and c-Jun in Human Hepatoma Cells

In the cell, ATF2 and c-Jun have to be phosphorylated in order to function as transcriptional activators. ATF2 contains on its *N*-terminus a phosphorylation-dependent transcriptional activation domain [Livingstone et al., 1995] that is controlled via phosphorylation of threonine residues 69 and 71 by the stress-activated protein kinases c-Jun-*N*-terminal protein kinase (JNK) and p38 [Gupta et al., 1995; van Dam et al., 1995]. Likewise, phosphorylation of serine residues 63 and 73 of c-Jun by c-Jun *N*-terminal kinase (JNK) is necessary to turn c-Jun into a biologically active transcriptional activator. We used phospho-specific antibodies to detect phosphorylation of ATF2 and c-Jun following stimulation of HepG2 cells with Il-1β or TPA. Figure 3 shows the detection of phosphorylated ATF2 and c-Jun in IL-1β and TPA-treated hepatoma cells. The overall levels of ATF2 did not change significantly as a result of IL-1β treatment. TPA, in contrast enhanced the levels of c-Jun in HepG2 cells. We conclude that IL-1β and TPA convert ATF2 and c-Jun into biologically active transcription factors.

Dominant-Negative Mutants of ATF2 and c-Jun Impair IL-1β and TPA-Stimulated TNFα Promoter Activity in Human Hepatoma Cells

The composite TRE/CRE motif of the TNFα gene functions in A20 B cells as a binding site for a heterodimer consisting of the basic region leucine zipper (bZIP) transcription factors ATF2 and c-Jun [Tsai et al., 1996; Falvo et al., 2000]. Moreover, constitutively active mutants of c-Jun and ATF2 transactivate the TNFα promoter in hepatoma cells [Al Sarraj et al., 2005]. To verify the involvement of ATF2 and/or c-Jun in the IL-1β and TPA-triggered transcriptional activation of the TNFα promoter, we used dominant-negative mutants termed A-ZIPs. These mutants inhibit DNA-binding of the

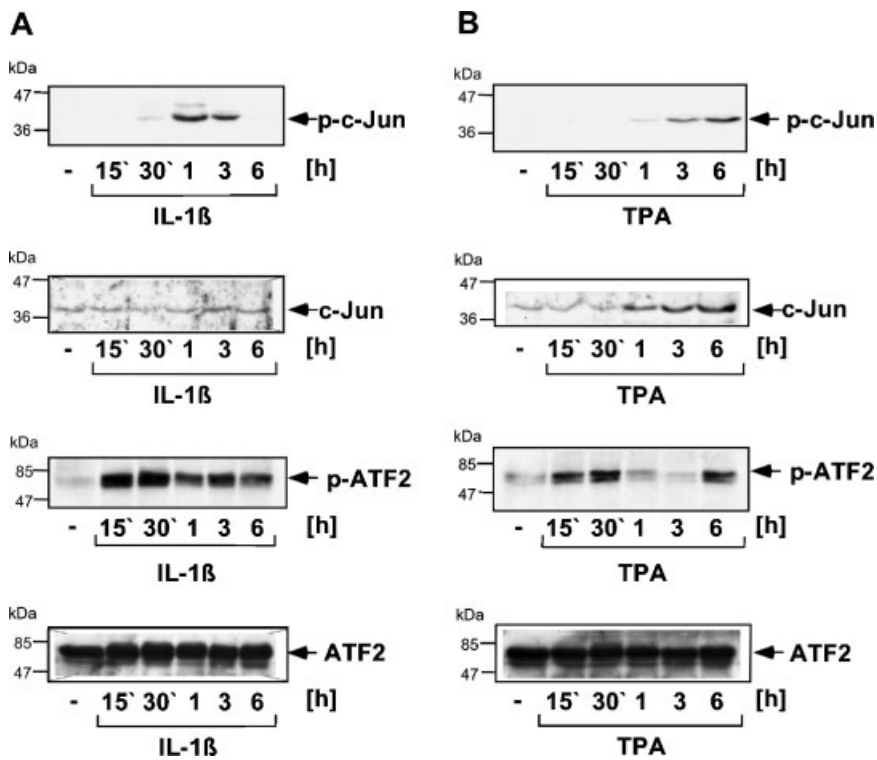

Fig. 3. IL-1β and TPA-dependent phosphorylation of c-Jun and ATF2 in hepatoma cells. HepG2 cells were treated with IL-1β (1 ng/ml) (**A**) or TPA (50 ng/ml) (**B**). The cells were harvested as indicated and nuclear extracts were prepared. Nuclear proteins were analyzed by Western blotting using antibodies for the detection of c-Jun and ATF2 and phospho-specific antibodies to detect the phosphorylated forms of c-Jun and ATF2.

wild-type bZIP proteins in a leucine zipper-dependent fashion [Vinson et al., 2002; Thiel et al., 2004]. Figure 4 shows that A-ATF2 was the most effective reagent in inhibiting TPA- and IL-1β-stimulated transcription of the reporter gene under these conditions. A-ATF2 functions as a dominant-negative mutant for both ATF2 and c-Jun, due to the ability of ATF2 to either form homodimers or heterodimers with c-Jun. In addition, the IL-1β and TPA-mediated upregulation of TNFα promoter/luciferase reporter gene transcription was impaired by A-Fos, a dominant-negative to c-Jun. Dominant-negative mutants for CREB, ATF4, and C/EBP did not significantly reduce reporter gene transcription. A-C/EBP even enhanced transcription of the TNFα promoter/luciferase reporter gene, presumably by sequestering inhibitory cofactors away from the transcription units.

To study the role of ATF2 in stimulus-induced TNFα secretion we generated recombinant lentiviruses expressing A-ATF2. HepG2 cells infected with a A-ATF2-encoding lentivirus showed a significant reduction of TPA- or IL-1β-induced secretion of TNFα in comparison to mock-infected cells (Fig. 4B). Collectively, these data show a link between the stimulation of the cells with IL-1β or TPA, the phosphorylation of ATF2 and c-Jun, the upregulation of TNFα gene transcription via the phosphorylated forms of ATF2 and c-Jun, and the subsequent secretion of TNFα into the cell culture medium.

Expression of the Dual-Specific Phosphatase MKP-1 Impairs IL-1β and TPA-Induced TNFα Gene Transcription

The nuclear phosphatase MKP-1 dephosphorylates the protein kinases ERK, p38 and

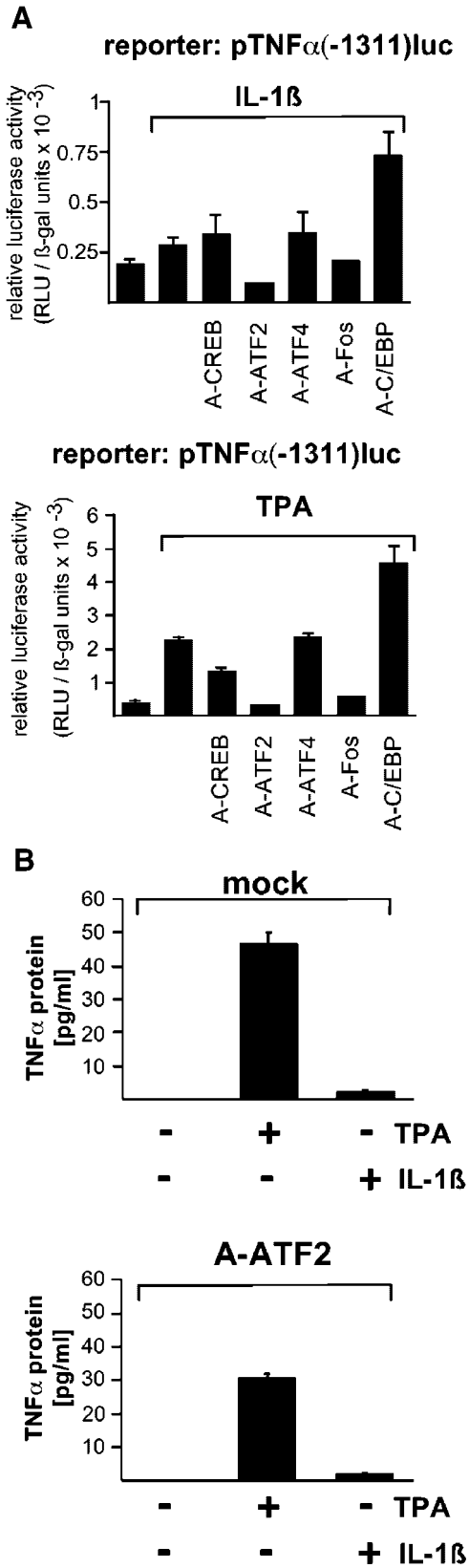

Fig. 4. Role of dominant-negative ATF2 and c-Jun mutants upon TNFα promoter activity and TNFα secretion following stimulation with IL-1β or TPA. **A**: HepG2 cells were transfected with the pTNFα(−1311)luc reporter plasmid, the pRSVβ reference plasmid and 50 ng/plate of expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2, or A-C/EBP. Cells were stimulated with IL-1β (1 ng/ml) (**upper panel**) or TPA (50 ng/ml) (**lower panel**) as indicated. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined. **B**: Lentiviruses encoding A-ATF2 were pseudotyped with the vesicular stomatitis virus glycoprotein and used to infect HepG2 cells. As a control mock-infected cells were analyzed. The cells were serum-starved for 24 h and then treated with TPA (50 ng/ml) or IL-1β (1 ng/ml) for 24 h. Secreted TNFα in the cell culture medium was detected by ELISA.

JNK [Sun et al., 1993; Hirsch and Storck, 1997]. We performed transient transfection experiments using a MKP-1 expression vector in order to substantiate the involvement of these MAP kinases in the IL-1β and TPA-triggered signaling cascade. Fig. 5A reveals that expression of MKP-1 blocked the IL-1β and TPA-induced upregulation of TNFα promoter activity. Moreover, MKP-1 overexpression also impaired the transcriptional upregulation of the reporter gene controlled by the composite TRE/CRE motif (Fig. 5B), indicating that activation of MAP kinases is necessary to connect IL-1β and TPA stimulation with TNFα gene transcription mediated by the TRE/CRE genetic motif.

Next, we tested the effect of MKP-1 expression on TNFα secretion using a recombinant lentivirus. HepG2 cells infected with a

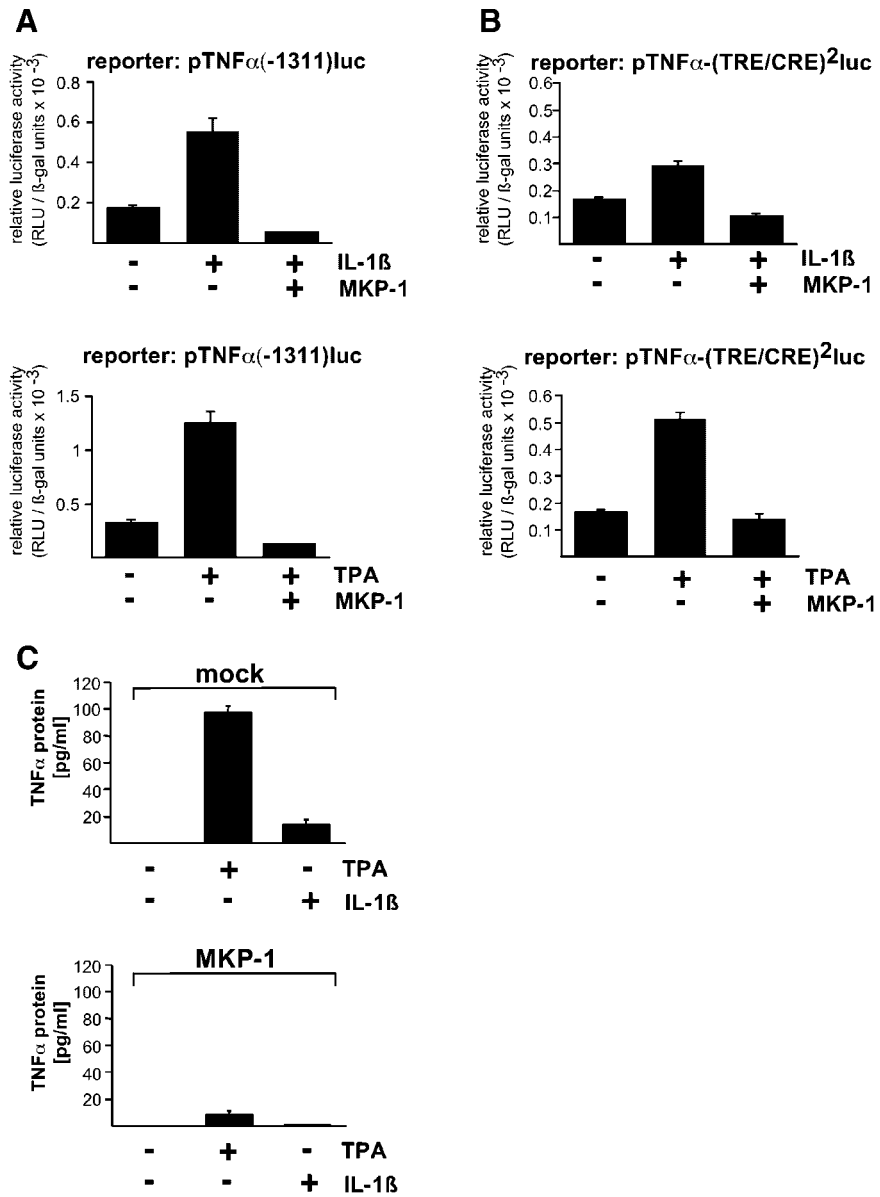

Fig. 5. IL-1β and TPA-induced upregulation of the TNFα promoter activity and TNFα secretion is blocked by overexpression of MAP kinase phosphatase-1. The reporter plasmids pTNFα(−1311)luc (**A**) or pTNFα(TRE/CRE)^{2}luc (**B**) were transfected into HepG2 cells together with the internal standard plasmid pRSVβ and either with the ''empty'' expression vector pCMV5 or an expression vector encoding MKP-1 (1 μg plasmid/plate). Cells were stimulated with IL-1β (1 ng/ml) or TPA (50 ng/ml) as indicated. Cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined. **C**: HepG2 cells were infected with recombinant lentiviruses encoding MKP-1. As a control mock-infected cells were analyzed. The cells were serum-starved for 24 h and then treated with TPA (50 ng/ml) or IL-1β (1 ng/ml) for 24 h. Secreted TNFα in the cell culture medium was detected by ELISA.

MKP-1-encoding lentivirus showed a significant reduction of TPA- and IL-1β-induced secretion of TNFα (Fig. 5C), indicating that phosphorylated MAP kinases are integral parts of the signaling cascade connecting IL-1β and TPA stimulation with TNFα biosynthesis and secretion.

Activation of Extracellular Signal-Regulated Protein Kinase (ERK) Increases TNFα Promoter Activity Independent of the TRE/CRE Motif

ERK was selectively activated in HepG2 cells via expression of a constitutively active MAP kinase kinase (MAPKKΔN3-S218E-S222D), the kinase that phosphorylates and activates ERK. Activation of MAP kinase kinase by Raf occurs via phosphorylation of the serine residues at positions 218 and 222. These serines have been changed to glutamic and aspartic acid residues, respectively. Moreover, residues 32–51 that are thought to stabilize the inactive state of the kinase, had been deleted [Mansour et al., 1994] (Fig. 6A). Transfection experiments revealed that the activation of ERK strongly stimulated TNFα gene promoter activity in human hepatoma cells (Fig. 6B). The upregulation of TNFα promoter activity was attenuated by expressing MKP-1 (Fig. 6B, right bar). Reporter gene transcription controlled by the composite TRE/CRE motif was not altered following activation of the ERK signaling pathway (Fig. 6C) indicating that TNFα gene transcription is controlled by the ERK signaling pathway using genetic elements distinct from the TRE/CRE motif.

The Constitutively Active MAP3 Kinase Mutant MEKK1Δ Stimulates TNFα Promoter/Luciferase Gene Transcription Involving ATF2 and c-Jun

The activity of c-Jun *N*-terminal kinase (JNK) is controlled by the MAP3 kinase mitogen-activated/extracellular signal responsive kinase kinase (MEK) kinase-1 (MEKK1). JNK, in turn, is able to phosphorylate c-Jun and ATF2. To stimulate JNK activity, we expressed a truncated form of MEK kinase 1 (MEKK1Δ) in HepG2 cells. Experimentally, MEKK1Δ strongly enhanced the transcriptional activation potential of a fusion protein consisting of the GAL4 DNA-binding domain and the c-Jun transactivation domain (GAL4-c-Jun) as well as the transcriptional activation potential of a GAL4-ATF2 fusion protein (data not shown). We tested two different concentrations of MEKK1Δ-encoding

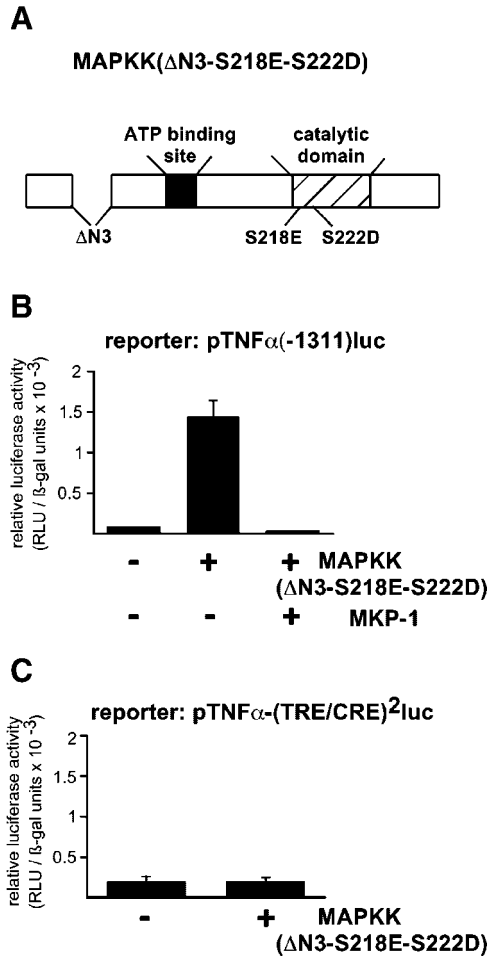

Fig. 6. Expression of a constitutively active MAP kinase kinase activates TNFα promoter/luciferase reporter gene transcription independent of the composite TRE/CRE motif. **A**: Schematic representation of the modular structure of the constitutively active MAP kinase kinase MAPKKΔN3-S218E-S222D. The ATP-binding site and the catalytic domain are indicated. The mutant kinase carries two point mutations (S218E, S222D) and a deletion of amino acids 32–51. **B**, **C**: The reporter plasmids pTNFα(−1311)luc (B) or pTNFα(TRE/CRE)^{2}luc (C) were transfected into HepG2 cells together with the internal standard plasmid pRSVβ and either the ''empty'' expression vector pCMV5 or an expression vector encoding MAPKKΔN3-S218E-S222D (0.25 μg plasmid/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined.

expression vector. Figure 7A reveals that transfection of 2 ng of expression plasmid was sufficient to enhance the TNFα promoter activity in human hepatoma cells. The stimulating effect of MEKK1Δ expression upon TNFα promoter activity was attenuated by expressing MKP-1 (data not shown). Expression of MEKK1Δ also stimulated transcription of the

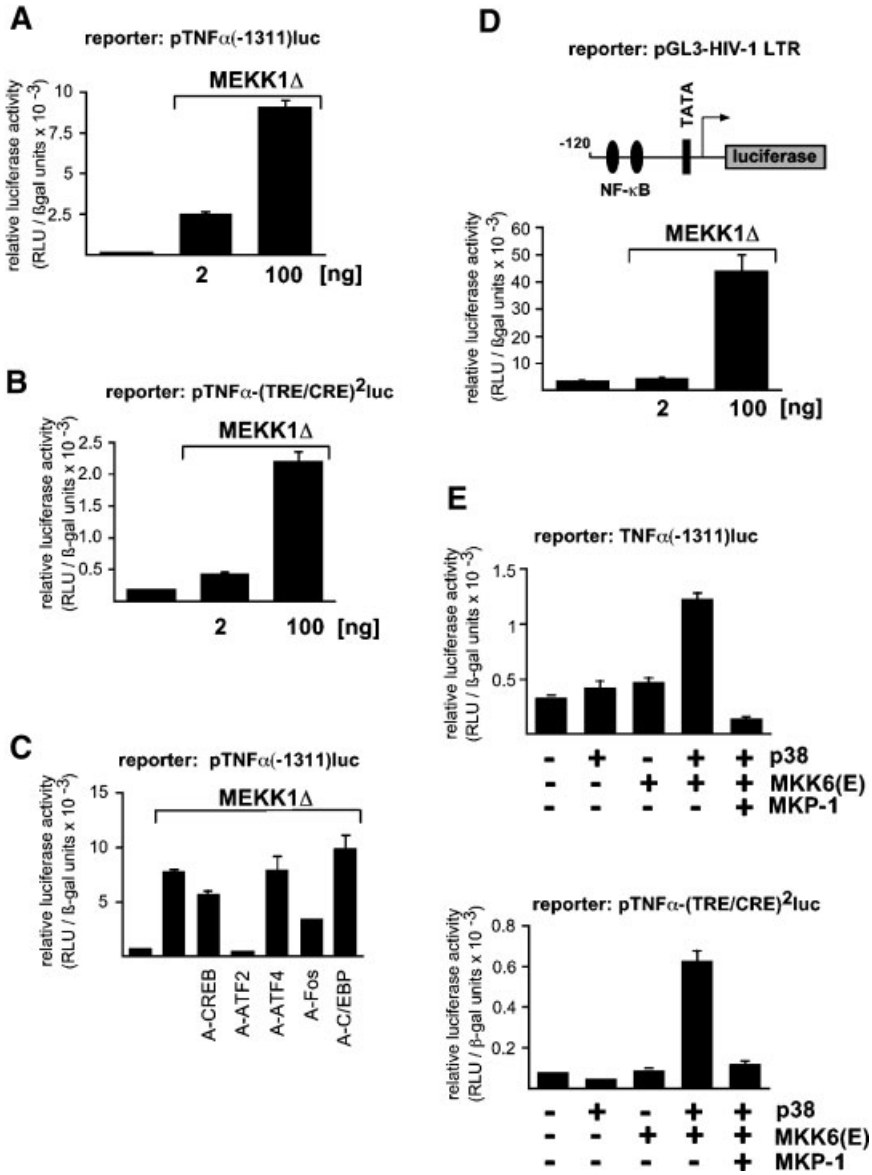

Fig. 7. Role of MEKK1, MAP kinase kinase 6, and p38 MAP kinase on TNFα promoter activity. **A**: The reporter plasmid pTNFα(−1311)luc was transfected into HepG2 cells together with the internal standard plasmid pRSVβ and an expression vector encoding MEKK1Δ (2 or 100 ng plasmid/plate). **B**: The reporter plasmid pTNFα(TRE/CRE)^{2}luc was transfected into HepG2 cells together with the internal standard plasmid pRSVβ and an expression vector encoding MEKK1Δ (2 or 100 ng plasmid/plate). **C**: Dominant-negative mutants of ATF2 and c-Jun impair MEKK1Δ-induced transcriptional activation of the TNFα promoter/luciferase reporter gene. HepG2 cells were transfected with the pTNFα(−1311)luc reporter plasmid, the pRSVβ reference plasmid, an expression vector encoding MEKK1Δ (2 ng plasmid/plate) and 50 ng/plate of expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2, or A-C/EBP. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined. **D**: Higher concentrations of MEKK1Δ activate NF-κB controlled reporter gene transcription. The NF-κB responsive reporter plasmid pGL3-HIV-1-LTR contains the proximal region of the HIV-1 LTR as regulatory region. The two binding sites for NF-κB are indicated. The pGL3-HIV-1-LTR reporter plasmid was transfected into HepG2 cells together with the internal standard plasmid pRSVβ and an expression vector encoding MEKK1Δ (2 or 100 ng plasmid/plate). **E**: The reporter plasmids pTNFα(−1311)luc (**upper panel**) or pTNFα(TRE/CRE)^{2}luc (**lower panel**) were transfected into HepG2 cells together with MKK6(E) (25 ng/plate) and a p38 MAP kinase expression vector (50 ng/plate) as indicated. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined.

luciferase reporter gene controlled by the TRE/CRE element (Fig. 7B). This effect of MEKK1Δ on the activity of the TNFα promoter was completely blocked by A-ATF2 and partially by A-Fos (Fig. 7C). The results confirmed the previous observation that either c-Jun or ATF2 or a heterodimer of ATF2 and c-Jun mediated the MEKK1Δ-induced upregulation of TNFα promoter/luciferase reporter gene transcription via the TRE/CRE site. Expression of MEKK1Δ has been reported to activate the transcription factor NF-κB [Yao et al., 1997]. However, higher concentrations of MEKK1Δ-encoding expression vector were needed, as demonstrated with an NF-κB-responsive reporter plasmid (Fig. 7D). This series of experiments revealed that we were able to separate the JNK and the NF-κB signaling pathways by using very low amounts of MEKK1Δ expression vector.

Stimulation of TNFα Promoter/Luciferase Gene Transcription by p38 MAP Kinase and a Constitutively Active Mutant of MAP Kinase Kinase 6

The results obtained with the dominant-negative A-ZIP mutants shed light on the important role of ATF2 in connecting IL-1β and TPA stimulation with enhanced TNFα gene transcription. ATF2 is activated by phosphorylation of the threonine residues 69 and 71, catalyzed by the protein kinases p38 MAP kinase or c-Jun *N*-terminal protein kinase. We tested whether an activation of the p38 MAP kinase signaling pathway influenced TNFα promoter/luciferase reporter gene transcription. To activate p38 protein kinase we expressed a constitutively active mutant of MAP kinase kinase 6, MKK6(E). Figure 7E shows that expression of MKK6(E) was not sufficient to increase reporter gene transcription. Expression of MKK6(E) in the presence of p38 MAP kinase elevated TNFα promoter/luciferase gene transcription (Fig. 7E, upper panel) and also enhanced transcription of a reporter gene controlled by the TRE/CRE motif (Fig. 7E, lower panel), indicating that the activation of p38 protein kinase stimulated TNFα gene transcription involving ATF2 and the TRE/CRE site.

Role of NF-κB in IL-1β and TPA-Induced TNFα Gene Transcription

IL-1β and TPA are strong activators of NF-κB and the regulation of TNFα gene transcription by NF-κB has been documented. Figure 8A shows that IL-1β and TPA treatment enhanced the transcriptional activity of an NF-κB-responsive reporter gene and this upregulation was attenuated by IκBS32AS36A, a non-degradable form of IκB. In contrast, IL-1β and TPA-induced reporter gene transcription under control of the TRE/CRE motif of the TNFα gene was not impaired by IκBS32AS36A, indicating that NF-κB targets other genetic elements within the TNFα regulatory region (Fig. 8B).

DISCUSSION

TNFα is a key signaling molecule in the liver, not only regulating hepatocyte proliferation during liver regeneration, but also functioning as a key stimulus for various liver diseases. TNFα mRNA and protein are not at all or barely detectable in hepatocytes, but TNFα biosynthesis can be induced by some extracellular signaling molecules. Among these molecules, IL-1β and TPA have been described to induce TNFα biosynthesis in human HepG2 hepatoma cells [Frede et al., 1996], an established cell culture model for liver parenchymal cells. IL-1β activates, among others, the protein kinase JNK and induces the translocation of the transcription factor NF-κB into the nucleus. Phorbol esters are polycyclic esters that mimic diacylgycerol and thereby irreversibly activate protein kinase C. As a result, intracellular signaling cascades are induced, including the activation of mitogen and stress-regulated protein kinases and the translocation of NF-κB. The objective of this study was to analyze the stimulus-induced activation of TNFα biosynthesis in hepatoma cells.

A key element within the TNFα gene regulatory region is the composite TRE/CRE motif that functions in B cells and macrophages as an integrative genetic element for diverse signal transduction pathways including stimulation by antigens, calcium, or lipopolysaccharides [Tsai et al., 1996, 2000]. We tested the biological importance of this genetic element by analyzing the activity of a reporter gene solely controlled by the composite TRE/CRE motif and showed that stimulus-transcription coupling initiated by IL-1β or TPA involves this motif in human hepatoma cells. Constitutively active mutants of ATF2 and c-Jun transactivated reporter genes under the control of the human TNFα promoter and dominant-negative mutants of

ATF2 and c-Jun blocked both the IL-1β and TPA-induced signaling cascade directed towards the TNFα gene. Moreover, we showed that lentiviral expression of A-ATF2, a dominant-negative ATF2 mutant, attenuated secretion of TNFα into the culture medium following stimulation with TPA or IL-1β. Hence, the composite TRE/CRE functions as an integrative genetic element in hepatoma cells for both the IL-1β and TPA-triggered signaling pathways.

Both the signaling cascades induced by IL-1β and TPA in HepG2 cells triggered phosphorylation and activation of the bZIP transcription factors ATF2 and c-Jun, connecting stimulation of the cells with alterations in the gene expression pattern. The fact that overexpression of the dual-specificity phosphatase MKP-1 blocked the stimulus-induced upregulation of TNFα promoter activity and the secretion of TNFα into the culture medium implicates that MAP kinases are involved in connection of the cytoplasmic signaling cascade with nuclear targets. Expression of a constitutively active MAP kinase kinase induced an upregulation of TNFα gene transcription, but the composite TRE/CRE motif was not involved in connecting the ERK signaling pathway with TNFα gene transcription.

The biological activities of ATF2 and c-Jun are regulated by stress-activated protein kinases, and ATF2 and c-Jun play an important role in the cellular stress response. In fact, overexpression of a constitutively active mutant of the MAP2 kinase MKK6, together with the expression of the p38 MAP kinase, upregulated transcription of a TNFα promoter/reporter gene. Moreover, a reporter gene was also activated that contained only the composite TRE/CRE motif of the TNFα gene as regulatory sequence. The MAP3 kinase MEKK1 has been shown to be required for IL-1-induced JNK activation [Xia et al., 2000]. Expression of a truncated form of MEKK1 (MEKK1Δ) in hepatoma cells induced an upregulation of TNFα promoter/reporter gene transcription, and experiments involving dominant-negative mutants revealed that either c-Jun or ATF2 or heterodimers of both bZIP proteins are responsible for the transcriptional effect of MEKK1Δ

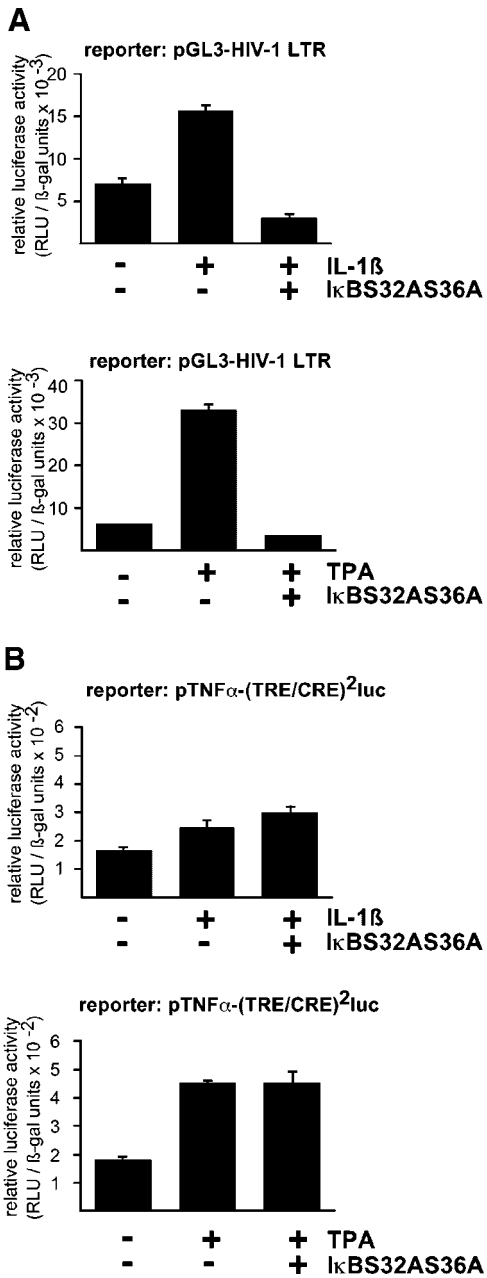

Fig. 8. Il-1β and TPA-induced activation of NF-κB upregulates TNFα promoter activity independent of the composite TRE/CRE motif of the TNFα gene. **A**: IL-1β and TPA activate NF-κB controlled transcription in HepG2 cells. The NF-κB responsive reporter plasmid pGL3-HIV-1-LTR was transfected into HepG2 cells together with the internal standard plasmid pRSVβ. Cells were stimulated with IL-1β (1 ng/ml) or TPA (50 ng/ml) as indicated. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined. NF-κB activation and upregulation of TNFα promoter activity was impaired following expression of the undegradable IκB mutant IκBαS32A/S36A (1 μg plasmid/plate). **B**: The reporter plasmid pTNFα(TRE/CRE)^{2}luc was transfected into HepG2 cells in the presence or absence of an expression vector encoding IκBαS32A/S36A. Cells were stimulated with IL-1β (1 ng/ml) or TPA (50 ng/ml) as indicated. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined.

expression. We conclude that the activation of stress-activated protein kinases converge to the TNFα gene in human hepatoma cells, involving the transcription factors ATF2 and c-Jun.

MEKK1 is upstream of several signaling pathways including the regulation of JNK and ERK activity and the activation of NF-κB [Hirano et al., 1996; Lee et al., 1997; Karandikar et al., 2000]. This pleiotropic activity has raised questions about the specificity [Karin and Delhase, 1998]. The analysis of MEKK1-deficient embryonic stem cells revealed that MEKK1 signaling channels to the activation of JNK, with marginal contribution to ERK and NF-κB activation [Xia et al., 2000]. Here, we showed by titration experiments that expression of low concentrations of the truncated form of MEKK1 (MEKK1Δ) (transfection of 2 ng expression plasmid/60 mm plate) was sufficient to activate TNFα gene transcription via the activation of ATF2 and c-Jun without the activation of NF-κB-controlled reporter genes.

In conclusion, our data shed light to the importance of the transcription factors c-Jun and ATF2 and the stress-activated protein kinases JNK and p38 MAP kinase in the stimulus-regulated transcription of the TNFα gene in hepatoma cells. The composite TRE/CRE motif functions as an integrative genetic element for both the IL-1β and TPA-induced signaling cascades. In addition, activators of the ERK signaling cascade as well as activators of NF-κB strongly upregulate TNFα gene transcription using other genetic elements within the TNFα regulatory region.

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