Transforming Growth Factor-β–Mediated Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Expression and Apoptosis in Hepatoma Cells Requires Functional Cooperation between Smad Proteins and Activator Protein-1

Kerstin Herzer,[1] Anne Grosse-Wilde,[2] Peter H. Krammer,[3] Peter R. Galle,[1] and Stephan Kanzler[4]

[1]I. Department of Internal Medicine, Johannes Gutenberg University, Mainz, Germany; [2]Fred Hutchinson Cancer Research Center, Seattle, Washington; [3]Division of Immunogenetics, German Cancer Research Center, Heidelberg, Germany; and [4]II. Department of Internal Medicine, Leopoldina Clinic, Schweinfurt, Germany

Abstract

Transforming growth factor-β (TGF-β) has been shown to induce apoptotic cell death in normal and transformed hepatocytes. We recently identified tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) as an important mediator of TGF-β–induced apoptosis in hepatoma cells. In this study, we have further explored the mechanism by which TGF-β up-regulates TRAIL expression. The 5′-flanking region of the *TRAIL* gene was isolated and characterized. Deletion mutants of the 5′-untranslated region of the *TRAIL* gene revealed a region comprising nucleotides −1950 to −1100 responsible for TRAIL induction following treatment with TGF-β. Within this region, we have identified an activator protein-1 (AP-1) site indispensable for TGF-β–mediated induction of TRAIL. Activation of this AP-1 site is mediated by a JunD·FosB heterodimer. Expression of DNSmad4, DNJunD, or DNFosB significantly impairs TGF-β–mediated activation of the TRAIL promoter. Furthermore, with tRNA interference targeting Smad4, junD, FosB, we could abolish TRAIL expression and, subsequently, TGF-β–induced TRAIL-mediated apoptosis in hepatoma cells. Our results reveal a new AP-1 site within the TRAIL promoter functionally involved in TGF-β–induced TRAIL expression and apoptosis in hepatomas and thus provide evidence for the underlying mechanism by which TGF-β might regulate cell death in liver cancer. (Mol Cancer Res 2008;6(7):1169–77)

Received 2/8/08; revised 4/12/08; accepted 4/28/08.

Grant support: Deutsche Krebshilfe and Deutsche Forschungsgemeinschaft grants (S. Kanzler).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Stephan Kanzler, II. Department of Medicine, Leopoldina Clinic, Gustav-Adolf-Str. 8, 97422 Schweinfurt Germany. Phone: 49-9721-7202484; Fax: 49-9721-7202484. E-mail: skanzler@leopoldina.de

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1541-7786.MCR-08-0073

Introduction

Transforming growth factor-β (TGF-β) and its family members are multifunctional cytokines that exert profound effects on cell division, differentiation, migration, adhesion, organization, and cell death. Thus, TGF-β is one of the most potent regulators of apoptosis (1, 2).

TGF-β signals via transmembrane receptors, e.g., the type I TGF-β receptor, to intracellular mediators of the Smad family, the so-called R-Smads, that are phosphorylated on serine residues and form heteromeric complexes with a common mediator Smad4. Consequently, this complex translocates to the nucleus, where Smads regulate gene expression either directly or in association with a number of coactivators and corepressors. An inhibitory molecule, Smad7, blocks this cascade to prevent TGF-β–mediated changes in gene expression (3). TGF-β can also signal through other pathways (e.g., the mitogen-activated protein kinase pathways; ref. 4), although the mechanisms for activation of these pathways seem diverse. Cross-talk between the Smad signaling cascade and other pathways also adds complexity to the system.

It has been shown that Smad proteins and the AP-1 complex synergize to activate the TGF-β1–responsive promoters (5). Recent studies indicate that Smad3 directly binds c-Jun and c-Fos of the AP-1 complex and that both Smad3 and Smad4 bind all three Jun proteins, c-Jun, JunB, and JunD (6). The AP-1 complex seems to be involved in cell proliferation and survival, and a role of this multicomponent complex is also suggested in apoptosis of various cells (6, 7). It has been shown that the AP-1 complex is crucially involved in TGF-β1–dependent apoptosis (8, 9).

Both death receptors and TGF-β receptors play an important role in the development of liver disease (9, 10). We previously examined a potential role of various death receptor/ligand systems in TGF-β–induced apoptosis and have identified the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) as an important mediator of TGF-β–induced apoptosis in hepatoma cells (11). TRAIL belongs to a family of death ligand systems and has attracted attention for its ability to preferentially kill a wide variety of tumor cell lines (12, 13), whereas most normal cells are resistant to TRAIL both *in vitro* and *in vivo* (14, 15). This study reveals the underlying mechanism of

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.

TRAIL induction by TGF-β, which is mediated by a newly identified AP-1 site in the TRAIL promoter, recognized by Jun-Fos heterodimers. Our results show that both Smad proteins and the AP-1 complex cooperatively play a critical role in TGF-β1–dependent induction of TRAIL expression.

Results

The TRAIL Promoter Is a Direct Target of TGF-β Signaling

We previously showed that TRAIL mRNA is rapidly induced by TGF-β in hepatoma cell lines (8). Treatment of cells with cycloheximide before TGF-β treatment did not impair induction of TRAIL mRNA. This indicates that *de novo* protein synthesis is not required. In the same study, we also showed that TRAIL mRNA is a primary target of TGF-β signaling.

To investigate in more detail the underlying mechanism of TGF-β–dependent regulation of TRAIL expression, the 5′ flanking region of the *TRAIL* gene was isolated and characterized (Fig. 1A). A DNA fragment corresponding to the −1950 to −1 region relative to the translational start site of the TRAIL promoter was inserted upstream of a luciferase reporter gene to determine the transcriptional induction of the TRAIL promoter by TGF-β. When Huh7 cells, transfected with this reporter construct, were stimulated with TGF-β, TRAIL expression was stimulated severalfold (Fig. 1B).

To determine whether Smad proteins are directly involved in TGF-β–induced TRAIL expression, we investigated the influence of the TGF-β signaling components on TRAIL mRNA levels by LightCycler PCR. Smad2 does not show any effect on the induction of TRAIL expression. mRNA levels of TRAIL, however, show an increase after overexpression of Smad3 and Smad4 (Fig. 1C). TRAIL mRNA expression becomes significantly increased after expression of Smad2/3 together with Smad4, especially after stimulation with TGF-β, indicating that these signaling components are crucially involved in TGF-β–induced TRAIL expression.

Together with our previous data, showing that that the effect of TGF-β on TRAIL mRNA accumulation is not due to mRNA degradation, these results imply that TGF-β signaling induces transcription of the *TRAIL* gene.

A Newly Identified AP-1 Site Mediates TGF-β–Induced TRAIL Expression

To further analyze the underlying mechanism of TGF-β–mediated TRAIL induction, we tested different TRAIL

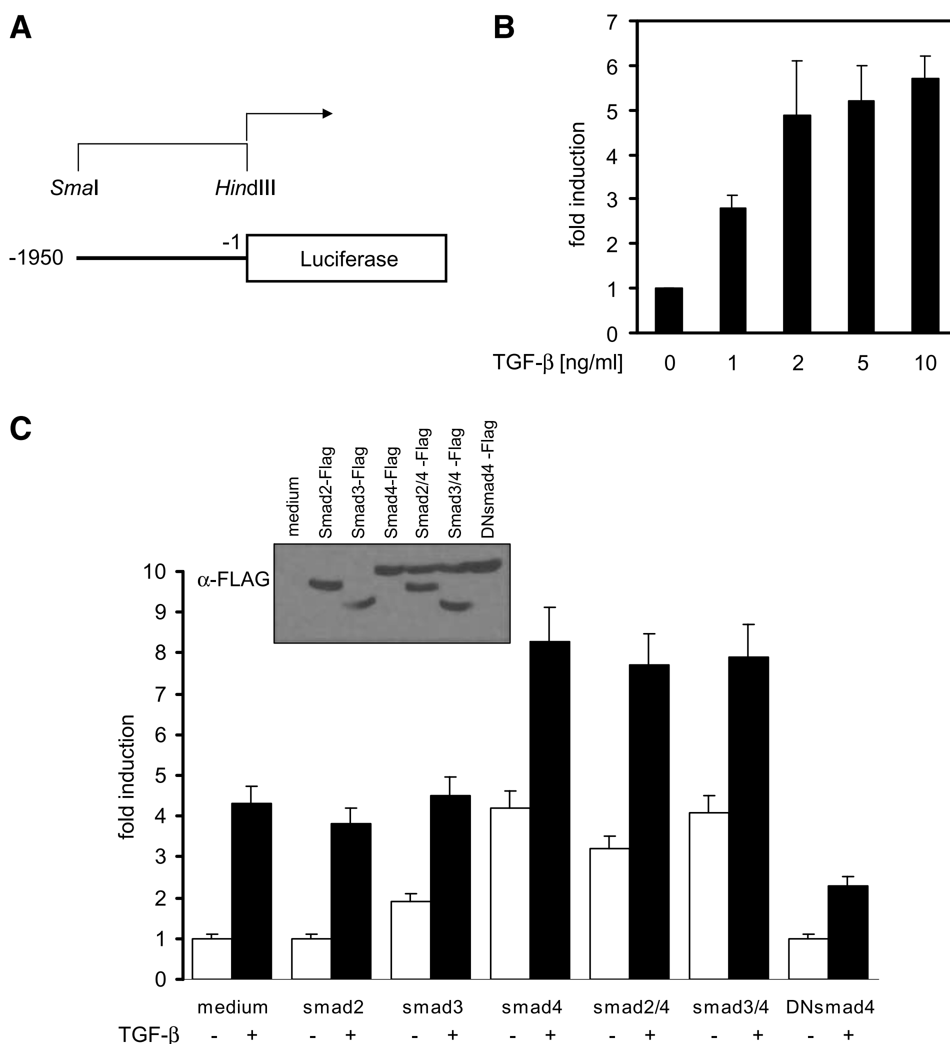

FIGURE 1. The TRAIL promoter is directly addressed by TGF-β signaling. **A.** Illustration of a luciferase reporter driven by the TRAIL promoter. Huh7 cells were transfected with the TRAIL promoter reporter construct and incubated with various concentrations of TGF-β. **B.** *X*-fold induction of luciferase activity relative to medium control. Huh7 cells were transfected with Flag-tagged expression plasmids for Smad2, Smad3, Smad4, and DNSmad4 alone or in combination as indicated. Twenty-four hours after transfection, cells were treated with 2 ng/mL TGF-β or left untreated. Twenty-four hours after treatment, RNA was isolated from one half of the samples, reverse transcribed, and subjected to quantitative PCR to analyze mRNA expression levels of TRAIL, using β-actin for normalization. **C.** The other half of the treated cells were subjected to Western blot for control of expression levels of the respective plasmid. The experiments shown are each representative of four experiments.

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.

promoter deletion mutants in a luciferase reporter gene assay of transfected Huh7 cells upon treatment of cells with TGF-β.

Inducibility of TRAIL promoter activity decreased significantly after one third of the promoter fragment was deleted. This indicates that this portion of the promoter contains transcription factor binding sites important for TRAIL induction (Fig. 2A). Interestingly, inspection of this region revealed three consensus binding sites (5′-TGAG/CTCA-3′) for the transcription factor AP-1. The exact position of the sequences are circled in Fig. 2B. Because the AP-1 complex has been shown to be important for TGF-β–induced apoptosis (16-18), we next tested whether these sites in the TRAIL promoter are functional AP-1 sites in TGF-β–induced TRAIL regulation. Therefore, we prepared −1950 to −1 promoter constructs with mutated AP-1 binding sites. Each wild-type consensus sequence (5′-CTGAGTCA-3′) was changed at three different positions, as shown in Fig. 2B. Upon TGF-β stimulation of Huh7 cells after transfection with the mutated promoter constructs, the inducibility of the m_3-mutant (−1327/−1321) was almost completely abolished when compared with the wild-type promoter construct (Fig. 2C), indicating that this particular AP-1 site is crucial for TRAIL induction by TGF-β.

The AP-1 complex is formed by different components affecting the reaction to different extracellular stimuli (18). We therefore cotransfected different Jun and Fos expression constructs with the different TRAIL promoter luciferase reporter constructs. Cotransfection of exogenous JunD and FosB gave a pronounced stimulation of the −1950/−1 construct but not of any one of the shorter promoter constructs (not shown) or the construct containing the m_3AP-1 site (Fig. 2D), further supporting a crucial role for this AP-1 site located in the upstream one third of the promoter. Furthermore, only JunD and FosB but none of the other AP-1 family members showed an activating effect on the promoter.

To further clarify whether transactivation by JunD and FosB is required for the induction of the TRAIL promoter, we cotransfected dominant negative mutants of JunD, FosB, and other AP-1 family members, together with the different luciferase reporter constructs. The dominant negative molecules effectively interfere with the activation of the AP-1 complex because they retain the dimerization domain but are lacking the transactivation domain and can no longer activate AP-1 target genes. The effect of inhibition is shown in Fig. 2D. Dominant negative JunD, FosB, and DNSmad4 drastically inhibited the activation of the TRAIL promoter upon treatment with TGF-β. Moreover, TGF-β treatment of hepatoma cells led to induction of protein and mRNA levels of JunD and FosB but not of c-Jun and c-Fos (Fig. 3A and B). These results underline the necessity of JunD·FosB activation for the observed up-regulation of the *TRAIL* gene.

Thus, we aimed to further determine the specificity of the dimer binding to the AP-1 site of the TRAIL promoter and did gel shift analyses with oligonucleotides comprising the AP-1 site (CTGAGTCA). These oligonucleotides form a complex with nuclear extracts from TGF-β–treated but not from untreated Huh7 cells (Fig. 3C). The complex formation could be competed with consensus AP-1 oligonucleotides but not with unlabeled oligonucleotides comprising the mutated m_3AP-1 sequence. Antibodies to JunD and to FosB inhibited complex formation in nuclear extracts from Huh7 cells. Neither antibodies to other transcription factors (data not shown) nor an isotype-matched immunoglobulin specificity control influenced the formation of the complexes (Fig. 3C). Thus, the AP-1 site located at position −1327 to −1321 in the TRAIL promoter is a specific AP-1 site and functional in TGF-β signaling with clear selectivity for Jun-Fos heterodimers. Moreover, right next to the indicated AP-1 sequence maps a Smad binding element at position −1336 to −1331. Therefore, we further tested DNA interaction of the whole complex with the TRAIL promoter by chromatin immunoprecipitation. PCR amplification of samples immunoprecipitated with anti-JunD, anti-FosB, and anti-Smad4 antibodies revealed the presence of the respective protein at position −1410 to −1301 of the 5′ flanking region of the *TRAIL* gene (Fig. 3D), which contains not only the specific AP-1 sequence but also shows occupation of the Smad binding element just upstream the responsive AP-1 sequence by Smad4. These data confirm that AP-1 binding to the target site in the TRAIL promoter is specific and induced by TGF-β. Moreover, these data define FosB, JunD, and Smad4 as centrally involved in TGF-β–induced activation of the TRAIL promoter.

Silencing of AP-1 Components and Smad4 Severely Impairs TGF-β–Induced TRAIL Expression and Apoptosis

Next, we confirmed the requirement for Smad4, JunD, and FosB in TGF-β–dependent TRAIL regulation in hepatoma cells using RNA interference. Twenty-one nucleotide short interfering RNA (siRNA) duplexes for Smad4, JunD, or FosB were transfected into Huh7 cells. After 48 hours, the expression of the respective protein was strongly suppressed, whereas cells transfected with a control siRNA showed unchanged expression (Fig. 4A). Interestingly, silencing of Smad4, JunD, or FosB expression did not only significantly decrease (10-20%) TRAIL expression upon TGF-β exposure (Fig. 4B). Transfected cells also got resistant to TGF-β–mediated apoptosis, as cell death was diminished to ~20% to 40% upon knockdown of Smad4 or the AP-1 components (Fig. 4C) compared with control (nonsilencing) siRNA-treated cells. Surprisingly, the effect of FosB siRNA on abrogation of TGF-β–mediated TRAIL induction is greater than that of Smad4 (Fig. 4B) whereas the effect of FosB siRNA on abrogation of TGF-β–mediated apoptosis is less than that of Smad4. However, TGF-β–mediated effects are complex and the components that are involved are diverse. When FosB expression is inhibited, the effect on TRAIL expression is slightly more intriguing than on apoptosis inhibition. We suspect that this is due to a redundancy in mechanisms and pathways that lead to TGF-β–induced cell death, whereas TGF-β–mediated TRAIL regulation is rather obliged to FosB expression.

TRAIL expression as well as apoptosis that was impaired by tRNA interference could be rescued by coexpression of functional murine homologous of the respective gene, demonstrating specificity of the siRNA constructs used and, thus, of the observed effect.

To assess the functional consequence of suppressed TRAIL expression, we used cytotoxicity assays. If hepatoma cells were pretreated with the siRNA as indicated before TGF-β stimulation, CEM cell lysis was severely diminished compared with coincubation with TGF-β–stimulated hepatoma cells pretreated with control siRNA. Thus, ~45% of CEM cells, as TRAIL-sensitive target cells, underwent apoptosis upon

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.

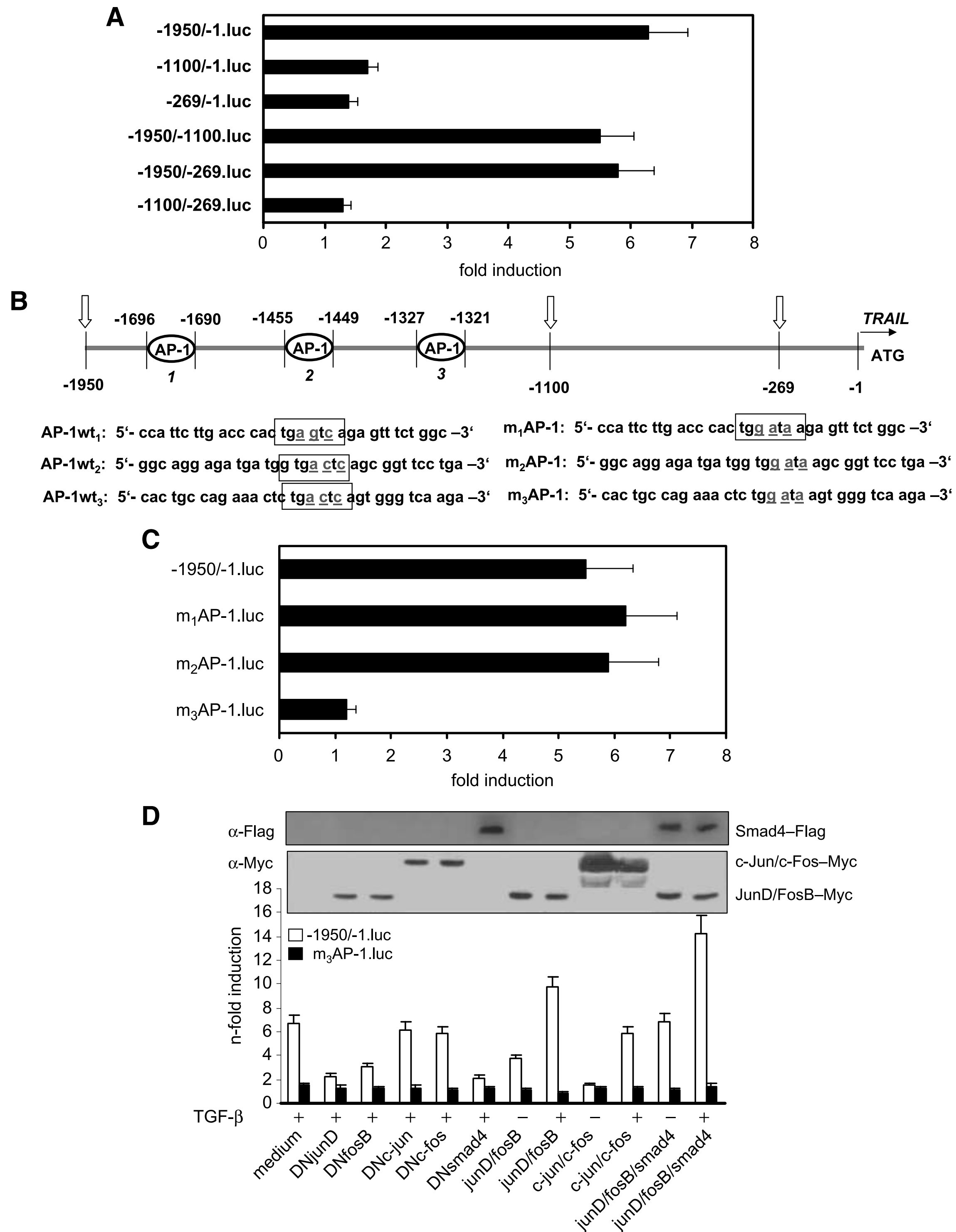

FIGURE 2. Mutations in the AP-1 site of the TRAIL promoter destroy the inducibility by TGF-β. **A.** Huh7 cells were transfected with the deletion constructs described in **B** and luciferase activity was measured following 36 h of treatment with TGF-β (2 ng/mL). **B.** Overview of the 5′-untranslated region of the *TRAIL* gene. Mutations in the AP-1 site of the −1950/−1 construct ($m_{1\text{-}3}$AP-1) were introduced using the QuickChange mutagenesis Kit (Stratagene), indicated as circled sequences. Gray arrows, −1950/−1, −1100/−1, and −269/−1 deletion constructs. **C.** Huh7 cells were transfected with the $m_{1\text{-}3}$AP-1 constructs described in **B** and luciferase activity was measured following 36 h of treatment with TGF-β (2 ng/mL). Huh7 cells were cotransfected with the TRAIL reporter plasmid; expression vectors for Myc-tagged Jun, Fos, Flag-tagged Smad4, or the respective dominant negative mutant; and a Renilla luciferase plasmid for normalization, as indicated. Firefly luciferase activities were measured after treatment of transfected cells with TGF-β (2 ng/mL) or left untreated. Half of each probe was subjected to Western blot for expression control of the respective plasmid. **D.** Firefly luciferase activities were normalized for transfection efficiency and cell survival against Renilla luciferase activity from the cotransfected plasmid. Columns, mean values of duplicate assays from four independent experiments; bars, SD.

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.

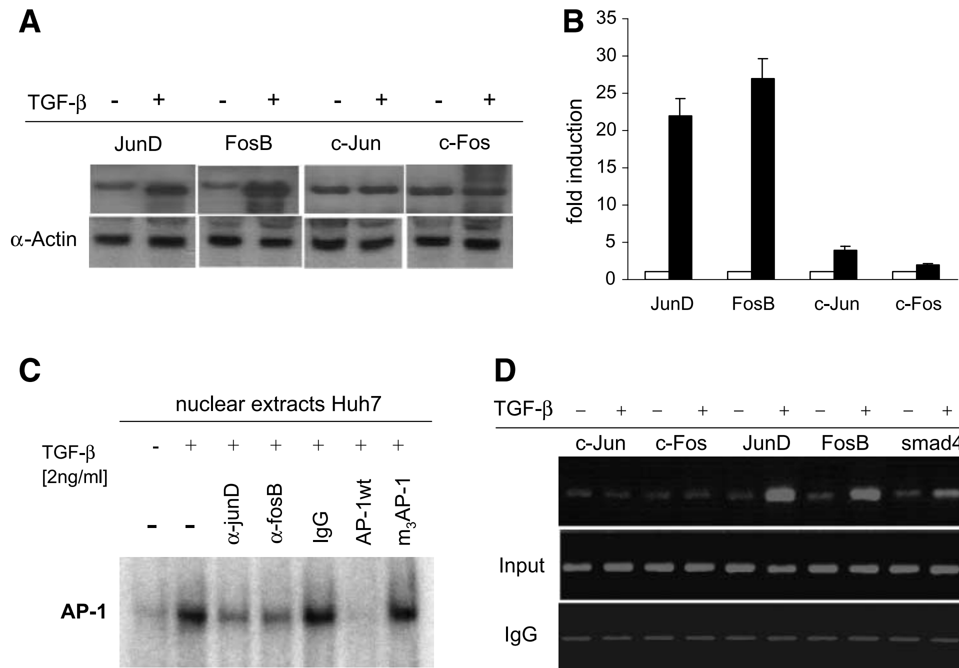

FIGURE 3. Expression and DNA interaction of AP-1 components and Smad4 after treatment with TGF-β. Huh7 cells were treated with TGF-β (2 ng/mL) or not for 36 h and subjected to Western blot analysis (**A**) or RT-PCR (**B**). Huh7 cells were treated with TGF-β (2 ng/mL) for 24 h and the nuclear extracts were subjected to an electrophoretic mobility shift assay of the −1950/−1020 region of the human TRAIL promoter sequence. In presence of antibodies against JunD or FosB, complex formation is inhibited. wtAP-1 or m_3AP-1 are the TRAIL promoter regions used for competition experiments. The experiment shown is representative for three performed (ref. 21; **C**). Huh7 cells were incubated with TGF-β (2 ng/mL) for 48 h before performing a chromatin immunoprecipitation assay. Chromatin extracts were immunoprecipitated by antibodies against JunB, FosD, or Smad4. The association of JunB, FosD, and Smad4 with the TRAIL promoter was detected by immunoprecipitation followed by PCR amplification of the −1410 to −1301 region of the TRAIL promoter region. Five microliters of each of the analyzed samples were used for PCR amplification as the input control (*Input*) before immunoprecipitation. Input sample DNA was PCR amplified before immunoprecipitation and the negative control was immunoprecipitated with control IgG (**D**).

coincubation with TGF-β–stimulated TRAIL-expressing Huh7 cells, but not with unstimulated hepatoma cells (Fig. 4D). Our results clearly show a significant decrease of apoptosis of CEM cells upon coincubation with those hepatoma cells that were pretreated with siRNA for Smad4, JunD, or FosB before TGF-β stimulation, compared with Huh7 cells that were pretreated with control siRNA. Compromised TRAIL expression by silencing these signal transduction components correlates clearly to our previously published results, using TRAIL-R2-Fc to efficiently and specifically inhibit TGF-β–induced apoptosis in hepatoma cells (11). Therefore, TGF-β–induced TRAIL expression and function is dependent on functional expression of Smad4 as well as of the AP-1 components JunD and FosB.

Taken together, our data indicate that TGF-β–induced apoptosis works via an autocrine loop that involves the induction of TRAIL. TRAIL induction is achieved by TGF-β–induced activation of the Jun-Fos heterodimeric AP-1 transcription factor, resulting in up-regulation of the activity of the promoter that drives the expression of TRAIL via a specific binding site for AP-1 located at −1327 to −1321 relative to the translational start site.

Discussion

In a previous study, we showed that TRAIL plays an important role in TGF-β–induced apoptosis in liver tumor cells (11). Here, we elucidate the underlying molecular mechanism and show that this process involves activation of the transcription factor AP-1, which stimulates the TRAIL promoter. Activation of AP-1 is mediated by Jun and Fos through Smads, the central components of TGF-β signaling. Thus, the principal findings of this study explain the molecular mechanisms by which TGF-β regulates TRAIL expression and reveal that (*a*) the transcription factor AP-1 binds to the 5′-flanking region of the TRAIL promoter; (*b*) mutation of an AP-1 binding sequence abrogates TGF-β–mediated enhancement of TRAIL promoter activity; (*c*) TGF-β enhances AP-1 nd Smad4 DNA binding activity to a specific genomic sequence in the TRAIL promoter; and (*d*) silencing of Smad4, JunD, or FosB expression effectively suppresses TGF-β–mediated TRAIL protein up-regulation and apoptosis. Thus, the conclusion that TGF-β–mediated induction of TRAIL involves functional cooperation between Smad proteins and AP-1 is based on three sets of data. First, DNSmad4, a Smad mutant that is unable to bind DNA, did not synergize with JunD in transcription assays. Second, silencing of Smad4 expression by siRNA abolishes TGF-β–induced up-regulation of TRAIL on the cell surface and inhibits apoptosis induction. Third, TGF-β induces occupation of a Smad binding element (CAGACA) right next to the indicated AP-1 sequence by Smad4 on the TRAIL promoter.

Components of the AP-1 transcription complex were recognized early as transcriptional targets of TGF-β signaling before the mechanisms of TGF-β signal transduction were known. Our results confirm previous results, suggesting that

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.

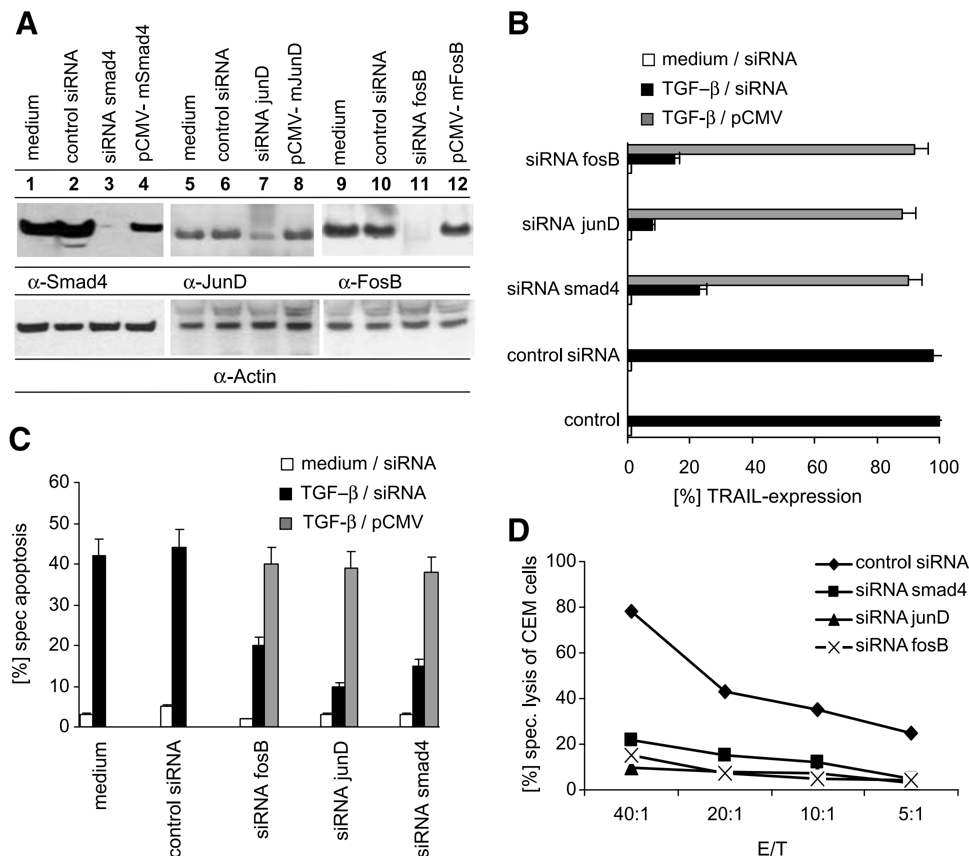

FIGURE 4. The expression and function of TRAIL are impaired by RNA interference of TGF-β signaling components. Smad4, JunD, and FosB were silenced in Huh7 cells by transfection with siRNA against the respective protein, a corresponding overexpression plasmid (pCMV-mSmad4/ pCMV-mJunD/ pCMV-mFosB), or both. Cells were incubated in the absence (*lanes 1, 5*, and *9*) or the presence of control (nonsilencing) siRNA (*lanes 2, 6*, and *10*), Smad4 siRNA (*lane 3*), JunD siRNA (*lane 7*), FosB siRNA (*lane 11*), or the corresponding murine DNA for rescue (*lanes 4, 8*, and *12*). Forty-eight hours after transfection, cells were treated with TGF-β; 36 h afterward, protein expression was assessed by Western blot (**A**). Cells were treated as in **A** and TRAIL expression was analyzed by fluorescence-activated cell sorting staining of TRAIL with TRAIL-R2-Fc or huIgG1 (**B**) or cell death was quantified by analyzing the percentage of cells with subdiploid DNA content, as described by Nicoletti et al., 1991 (**C**). Huh7 cells were treated as in **A** with the siRNA as indicated, treated with or without 2 ng/mL TGF-β for 36 h, washed three times with PBS, and coincubated with ^{51}Cr-labeled CEM cells as targets in a standard chromium release assay. Points, mean values of duplicate samples from four-step titrations at effector/target (*E/T*) ratios as indicated of one of three representative experiments (**D**).

TGF-β–mediated apoptosis is, in part, mediated by AP-1–associated gene expression and extend those by revealing *TRAIL* as a target gene mediating cell death in this context. Smad4, JunD, and FosB are shown to be indispensable for activation of the TRAIL promoter by TGF-β and, thus, TRAIL expression and function. Nevertheless, although chromatin immunoprecipitation analysis indicated TGF-β–dependent DNA interaction of Smad4 and the AP-1 components in direct neighborhood, we could not detect any on-DNA interactions between Smad and AP-1 proteins by electrophoretic mobility shift assay. However, these results are in agreement with previous studies excluding that Smad/AP-1 heterocomplexes may form on either CAGA boxes or AP-1 sites (8, 19). Their functional cooperation with respect to AP-1–driven transcription likely results from additional, yet unknown, mechanisms, such as a cooperative recruitment of different members of the basal transcription machinery. Moreover, it has been shown and discussed by Shi et al. (20) that simultaneous DNA binding of Smad and AP-1 proteins may not be compatible with the three-dimensional structure acquired by these factors when bound to DNA. In agreement with our findings, Wong et al. (19) showed that no Smad3/c-Jun heterocomplexes were formed despite the synergistic transcriptional effect of these proteins on promoter activity.

The responses to Smad and AP-1 signals in the context of TGF-β–mediated gene expression are diverse, and notable differences were observed between the abilities of JunD, JunB, and c-Jun to modulate Smad4-driven promoter transactivation (17). Moreover, consistent with our observations, JunD and FosB have been shown to cooperate with Smad4 at an AP-1 binding site (16).

By reporter gene analysis, we identified a 5′-flanking sequence in the TRAIL promoter that is important for the regulation of TRAIL promoter activity by TGF-β, containing three putative AP-1 binding sites. Reporter gene analysis revealed that mutation of one of these sites completely abrogated TRAIL promoter activity in response to TGF-β. Taken together, TGF-β seems to activate *TRAIL* gene

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.

transcription by facilitating AP-1–TRAIL promoter binding at a specific AP-1 site in the −1950/−1100 flanking region of this death receptor gene. These data raise the question in how far resistance of certain tumors to TGF-β–induced apoptosis may be tightly connected to defects in TRAIL regulation, e.g., by sequence changes in certain regulatory elements. Those mutations may develop during carcinogenesis, providing a growth advantage for the tumor. Mutation analyses of the respective sequence in a large set of tumor samples are on their way. However, given the potential importance of TRAIL in cancer therapy, these data may be important in developing strategies to enhance expression of this death ligand in other cell types. Tumor-selective activation of molecules involved in TRAIL expression may be an attractive approach for enhancing TRAIL-mediated cancer therapy.

Materials and Methods

Cell Lines

The hepatoma cell lines used were obtained from the American Type Culture Collection. Cells were maintained as described before (14).

Plasmids

The Smad expression plasmids were kindly provided by C-H. Heldin (Ludwig Cancer Research Institute, Uppsala, Sweden) and were described elsewhere (22).

The expression constructs for Jun and Fos were kindly provided by P. Angel (German Cancer Research Center, Heidelberg, Germany) and have been described previously (23, 24).

The sequence of the human TRAIL promoter was retrieved by a BLAST search with the first 100 bp of the 5′ end of the open reading frame of human TRAIL. Normal human genomic DNA of a healthy individual was used as template. Primers were purchased from MWG Biotech GmbH. Primer sequences were as follows: *TRAIL* sense, 5′-GACGAAGAGAGTATGAACAGCC-3′; *TRAIL* antisense, 5′-GGTCCATGTCTATCAAGTGCTC-3′, yielding a PCR product of 603 bp. The sequence amplified by the primers spans all three introns of TRAIL, thereby facilitating the differentiation between cDNA and genomic DNA. The human TRAIL promoter fragments were then cloned between the *Sma*I site and the *Hin*dIII site of the pGL3 basic luciferase expression vector (Promega). The different 5′ deletion mutants of the TRAIL promoter were produced by PCR amplification of regions of the human TRAIL promoter from nucleotide −1 relative to the translational start site to various deletion end points. The oligonucleotides used for cloning were as follows: 3′TLP(−1) (CATCAAGCTTGATCCTGTCAGAGTCTGACT), 5′TLP(−1100) (CATCGAGCTCGAGATTGTGCCATTGCACCA), 5′TLP(−1950) (CATCGAGCTCGAGATGTTGACTTCCTGCAC), and 5′TLP(−269) (CATCGAGCTCAAGGGGTGCATGGATCCTGA). Normal human genomic DNA of a healthy individual was used as template. The various human TRAIL promoter fragments were then placed between the *Sma*I site and the *Hin*dIII site of the pGL3 basic luciferase expression vector (Promega). Successful cloning was confirmed by sequencing.

Mutations in the AP-1 site of the −1950/−1 construct (m_{1-3}AP-1) were introduced using the QuikChange mutagenesis kit (Stratagene). The primers for the mutagenesis reaction (MWG Biotech GmbH) were m_1AP-1 sense (5′-CCATTCTTGACCCACTGGATAAGAGTTTCTGGC-3′) and m_1AP-1 antisense (5′-GCCAGAAACTCTTATCCAGTGGGTCAAGAATGG-3′), m_2AP-1 sense (5′-GGCAGGAGATGATGGTGGATAAGCGGTTCCTGA-3′) and m_2AP-1 antisense (5′-TCAGGAACCGCTTATCCACCATCATCTCCTGCC-3′), and m_3AP-1 sense (5′-CACTGCCAGAAACTCTGGATAAGTGGGTCAAGA-3′) and m_3AP-1 antisense (5′-TCTTGACCCACTTATCCAGAGTTTCTGGCAGTG-3′). Underlined nucleotides represent the mutated sites compared with the wild-type TRAIL promoter sequence. The mutations were confirmed by automated sequencing.

Western Blot

Western Blot was done essentially as described (11). Antibodies to detect Smad4, FosB, JunD, c-Fos, c-Jun, Myc, and Flag were obtained from Santa Cruz Biotechnology or Abcam.

RNA Interference

For RNA interference, siRNA duplexes for Smad4, JunD, and FosB were obtained from Qiagen. Chemically synthesized control (nonsilencing) siRNA was also purchased from Qiagen. Transfection of siRNA was carried out with TransMessenger reagent (Qiagen) in six-well plates. siRNA was condensed with Enhancer R and formulated with 4 μL of TransMessenger reagent, according to the manufacturer's instructions. The transfection complex was diluted in 900 μL of DMEM and was added directly to the cells. It was replaced with DMEM containing 10% fetal bovine serum after 3 h. Cells were analyzed 48 h after transfections.

The knockdown was controlled by Western blot. To exclude off-target effects of the siRNAs, cells were transfected with siRNA against the protein alone, an overexpression plasmid of the homologous murine protein (p-CMV-mSmad4, p-CMV-mFosB, p-CMV-mJunD) alone, or both, and cultured for 48 h before further treatment and analysis. Plasmids were purchased from BioCat.

Transient Transfection and Luciferase Assay

We did transient transfection of hepatoma cells using FuGene (Roche Diagnostics), following the manufacturer's instructions.

For luciferase assays, cells were plated on six-well plates. The firefly luciferase reporter construct was cotransfected with the control vector pcDNA3, the respective expression plasmid, and/or stimulated with TGF-β after transfection, as indicated. One hundred nanograms of the Renilla luciferase vector (Promega) were cotransfected to determine transfection efficiencies. Cells were harvested and the assay was done according to the manufacturer's instructions. Luciferase activity was measured using the DuoLumat LB9507 (Berthold). Duplicate measurements were done for all experiments.

Protein determination was done using the BCA assay (Bio-Rad GmbH).

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.

Quantitative Real-time PCR

Quantitative PCR was done using a LightCycler System according to the manufacturer's instructions (Roche Diagnostics) using the following primers: TRAIL (5′-GGTCCATGTCTATCAAGTGCTC-3′ and 5′-GACGAAGAGAGTAGTAACAGC-3′), JunD (5′-GAGAAGGCTCAGCAAGAAG-3′ and 5′-TGTCTGGTATGATCCTTCT-3′), FosB (5′-TTCTCCGAACGTGTCACGTAT-3′ and 5′-ACGTGACACGTTCGGAGAATA-3′), c-Jun (5′-CGCTGCTGAGCATATATCATA-3′ and 5′-ATTGCTGGCCTACAGGCCTCC-3′), c-Fos (5′-TCCTCAGCAGCCATTACTGC-3′ and 5′-GCTGCAGAACATTACCCGTA-3′), and β-actin (5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′).

Flow Cytometry

Cells were rinsed twice with PBS 36 h after stimulation, detached from culture dishes with PBS containing 20 mmol/L EDTA, washed, and resuspended in PBS supplemented with 5% FCS. Approximately 5×10^5 cells were used per sample. Incubations with primary and secondary antibodies were done for 30 min at 4°C, followed by two washing steps with PBS/5% FCS after each incubation. Analysis was done with a Becton Dickinson fluorescence-activated cell sorting flow cytometer and CellQuest software.

Cells were incubated in 50 μL of PBS/5% FCS with TRAIL-R2-Fc for TRAIL staining or, as the respective control, in the presence of huIgG (Southern Biotechnology Associates) at a concentration of 10 μg/mL. Biotinylated secondary goat anti-mouse or goat anti-human antibodies (Southern Biotechnology Associates) and streptavidin-phycoerythrin (PharMingen) were used in a final volume of 50 μL at a dilution of 1:200.

Determination of Cell Death

As a direct measurement of cell death, DNA fragmentation was quantified by determination of the percentage of cells with subdiploid DNA content, essentially as described (11).

Cell-Mediated Cytotoxicity Assay

Cell-mediated lysis was quantitated using standard chromium-51 release assay (25). In brief, hepatoma cells were stimulated or not with 2 ng/mL TGF-β (Sigma) for 30 to 40 h, subsequently washed thrice with PBS, detached with 2 mmol/L EDTA, and counted. CEM cells were labeled with 100 mCi/mL ^{51}Cr for 1 h in culture medium. Effector cells and target cells were coincubated for 16 h. Spontaneous release was determined by incubating target cells alone; total release was determined by directly counting labeled cells. Percentage cytotoxicity was calculated as follows: % specific lysis = (experimental cpm − spontaneous cpm/total cpm − spontaneous cpm) × 100. Duplicate measurements of four-step titrations of effector cells were used for all experiments.

Electrophoretic Mobility Shift Assay and Competition Analyses

Nuclear extracts from hepatoma cells were prepared as previously described (26). Briefly, 4×10^7 cells were lysed in 10 mmol/L Tris-HCl (pH 7.4)/2 mmol/L $MgCl_2$/140 mmol/L NaCl/0.5 mmol/L DTT/0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF)/0.1% Triton X-100. Sucrose density gradient centrifugation was done and the nuclear fraction was resolved in 20 mmol/L HEPES (pH 7.9)/25% glycerol/0.42 mol/L NaCl/1.5 mmol/L $MgCl_2$/0.2 mmol/L EDTA/0.5 mmol/L DTT/0.5 mmol/L PMSF. After 30 min, nuclear membranes were pelleted and the supernatant was stored in liquid nitrogen after determination of the protein content using the Bio-Rad protein assay. Double-stranded oligonucleotides comprising the AP-1 site in the TRAIL promoter were end-labeled with T4 polynucleotide kinase (MBI Fermentas) using 5,000 Ci/mmol of [-32^P]ATP (Amersham GmbH). Sequences of the single-stranded oligonucleotides were sense 5′-CCATTCTTGACCCACTGAGTCAGAGTTTCTGGC-3′ and antisense 5′-GCCAGAAACTCTGACTCAGTGGGTCAAGAATGG-3′. Free nucleotides were removed with Microspin G-50 columns (Pharmacia GmbH). Binding reactions were carried out at 4°C for 30 min using 5 μg of nuclear protein in a buffer containing 100 ng of bovine serum albumin (Roche)/μL, 50 ng of poly(deoxyinosinic-deoxycytidylic acid) (Roche)/μL, 2 mmol/L DTT (Life Technologies), 500 μmol/L Pefabloc (Roche), 1 μg of aprotinin (Roche)/μL, 25 mmol/L HEPES (Sigma), 5 mmol/L $MgCl_2$ (Sigma), 35 mmol/L KCl (Sigma), and 3×10^4 cpm of the labeled oligonucleotide. For competition analyses, 1 μg of antibody was added to the binding reaction. Samples were analyzed on a 6% non-denaturing polyacrylamide gel in 0.5% Tris borate-EDTA.

Chromatin Immunoprecipitation

Formaldehyde cross-linking and chromatin immunoprecipitation were done according to the manufacturer's protocol (Upstate). Antibodies against JunD, FosB, and Smad4 or a control IgG (Santa Cruz Biotechnology) were used to precipitate chromatin from 5×10^6 Huh7 cells. After reverse cross-linking and purification, DNA was recovered and amplified by PCR using the following primers (MWG Biotech GmbH): forward 5′-TCCTAAGTCCACTGCCAGAAACTC-3′ and reverse 5′-TTCCATTCTTGACCCACTGAGTCA-3′. PCR was carried out as follows: 1 μL of DNA, 0.5 μL of each primer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphates, 1.25 units *Taq* DNA polymerase (Bio-Rad), and 1× Taq buffer (Bio-Rad). After 40 amplification cycles, the PCR products were analyzed by ethidium bromide staining of a 2% agarose gel.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank S. Weyer and J. Gersbach for excellent technical assistance, M. Li-Weber and H. Walczak for helpful discussion, T.G. Hofmann for precious suggestions, and P. Angel and C-H. Heldin for providing the reagents.

References

1. Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer. Nat Rev Cancer 2003;3:807–21.

2. Kanzler S, Galle PR. Apoptosis and the liver. Semin Cancer Biol 2000;10:173–84.

3. Shi Y, Massague J. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 2003;113:685–700.

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.

4. Moustakas A, Heldin CH. Non-Smad TGF-β signals. J Cell Sci 2005;118: 3573 – 84.

5. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-β-induced transcription. Nature 1998;394:909 – 13.

6. Liberati NT, Datto MB, Frederick JP, et al. Smads bind directly to the Jun family of AP-1 transcription factors. Proc Natl Acad Sci U S A 1999;96:4844 – 9.

7. Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997;9:240 – 6.

8. Verrecchia F, Vindevoghel L, Lechleider RJ, Uitto J, Roberts AB, Mauviel A. Smad3/AP-1 interactions control transcriptional responses to TGF-β in a promoter-specific manner. Oncogene 2001;20:3332 – 40.

9. Bissell DM, Roulot D, George J. Transforming growth factor β and the liver. Hepatology 2001;34:859 – 67.

10. Walczak H, Krammer PH. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res 2000;256:58 – 66.

11. Herzer K, Ganten TM, Schulze-Bergkamen H, et al. Transforming growth factor β can mediate apoptosis via the expression of TRAIL in human hepatoma cells. Hepatology 2005;42:183 – 92.

12. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 1995;3:673 – 82.

13. Walczak H, Degli-Esposti MA, Johnson RS, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 1997;16:5386 – 97.

14. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat Med 1999;5:157 – 63.

15. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155 – 62.

16. Yamamura Y, Hua X, Bergelson S, Lodish HF. Critical role of Smads and AP-1 complex in transforming growth factor-β-dependent apoptosis. J Biol Chem 2000;275:36295 – 302.

17. Wisdom R. AP-1: one switch for many signals. Exp Cell Res 1999;253: 180 – 5.

18. Hocevar BA, Howe PH. Regulation of AP-1 activity by TGF-β. Methods Mol Biol 2000;142:97 – 108.

19. Wong C, Rougier-Chapman EM, Frederick JP, et al. Smad3-4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor β. Mol Cell Biol 1999;19:1821 – 30.

20. Shi Y, Wang YF, Jayaraman L, Yang H, Massague J, Pavletich NP. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-β signaling. Cell 1998;94:585 – 94.

21. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cycometry. J Immunol Methods 1991;139:271 – 9.

22. Nakao A, Imamura T, Souchelnytskyi S, et al. TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad4. EMBO J 1997;16: 5353 – 62.

23. Angel P, Hattori K, Smeal T, Karin M. The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell 1988;55:875 – 85.

24. Angel P, Allegretto EA, Okino ST, et al. Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. Nature 1988;332:166 – 71.

25. Schendel DJ, Wank R, Dupont B. Standardization of the human *in vitro* cell-mediated lympholysis technique. Tissue Antigens 1979;13:112 – 20.

26. Haglund RE, Rothblum LI. Isolation, fractionation and reconstitution of a nuclear extract capable of transcribing ribosomal DNA. Mol Cell Biochem 1987; 73:11 – 20.

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.

AACR American Association for Cancer Research

Molecular Cancer Research

Transforming Growth Factor-β–Mediated Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Expression and Apoptosis in Hepatoma Cells Requires Functional Cooperation between Smad Proteins and Activator Protein-1

Kerstin Herzer, Anne Grosse-Wilde, Peter H. Krammer, et al.

Mol Cancer Res 2008;6:1169-1177.

Updated version	Access the most recent version of this article at: http://mcr.aacrjournals.org/content/6/7/1169

Cited articles	This article cites 26 articles, 6 of which you can access for free at: http://mcr.aacrjournals.org/content/6/7/1169.full#ref-list-1
Citing articles	This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://mcr.aacrjournals.org/content/6/7/1169.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/6/7/1169. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

Downloaded from mcr.aacrjournals.org on October 22, 2020. © 2008 American Association for Cancer Research.