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Inhibition of the Transforming Growth Factor β (TGFβ) Pathway by Interleukin-1β Is Mediated through TGFβ-activated Kinase 1 Phosphorylation of SMAD3

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Transforming growth factor β is the prototype of a large family of secreted factors that regulate multiple biological processes. In the immune system, TGFβ acts as an anti-inflammatory and immunosuppressive molecule, whereas the cytokine interleukin (IL)-1β is a crucial mediator of inflammatory responses and induces proinflammatory genes and acute phase proteins. Here, we present evidence for the existence of a direct inhibitory interaction between the IL-1β and TGFβ signaling cascades that is not dependent on IL-1β–induced SMAD7 expression. IL-1β and its downstream mediator TAK1 inhibit SMAD3-mediated TGFβ target gene activation, whereas SMAD3 nuclear translocation and DNA binding in response to TGFβ are not affected. IL-1β transiently induces association between TAK1 and the MAD homology 2 domain of SMAD3, resulting in SMAD3 phosphorylation. Furthermore, IL-1β alleviates the inhibitory effect of TGFβ on in vitro hematopoietic myeloid colony formation. In conclusion, our data provide evidence for the existence of a direct inhibitory effect of the IL-1β-TAK1 pathway on SMAD3-mediated TGFβ signaling, resulting in reduced TGFβ target gene activation and restored proliferation of hematopoietic progenitors.

INTRODUCTION

The response of a cell to environmental changes is dependent on its ability to integrate the input from multiple signaling pathways to generate the appropriate biological response. Various signaling molecules exert opposite effects on cells, such as the proinflammatory cytokine interleukin (IL)-1β and the anti-inflammatory secreted factor transforming growth factor β (TGFβ). TGFβ signaling is mediated through transmembrane receptors with Ser/Thr kinase activity (Massague, 1998). On binding of extracellular TGFβ to its type II receptor (TβRII), a type I TGFβ receptor is recruited and activated by TβRII. The activated receptor complex activates intracellular mediators of TGFβ signaling, receptor-regulated SMAD proteins (R-SMADs). These activated R-SMADs form a complex with coSMADs (SMAD4) and translocate to the nucleus where the activated SMAD complex, often in cooperation with (DNA-binding) cofactors, modulates transcriptional activity of target genes (Wrana, 2000). Cross-talk between TGFβ/SMAD signaling and other cascades has been demonstrated to target nuclear translocation of SMADs as well as SMAD interaction with cofactors (reviewed in Moustakas *et al*., 2001). Mitogen-activated protein kinase (MAPK) cascades have been shown to target R-SMADs, resulting in either enhanced TGFβ responses (de Caestecker *et al*., 1998) or inactivated SMAD signaling by cytoplasmic retention of R-SMADs (Kretzschmar *et al*., 1997; Kretzschmar *et al*., 1999).

Whereas TGFβ inhibits inflammatory and immune responses and reduces stem cell cycle activity (Fortunel *et al*., 2000b), IL-1β is the prototype of a proinflammatory cytokine, involved in inflammation and host defense (O'Neill, 2000). On activation of the cell surface type I IL-1β receptor by IL-1β, a cascade of signaling events is initiated, leading to c-Jun NH_2-terminal kinase (JNK) and nuclear factor-κB (NF-κB) activation, ultimately resulting in transcriptional activation of proinflammatory genes (O'Neill, 2000). Recently, the mitogen-activated protein kinase kinase kinase homologue TGFβ activated kinase 1 (TAK1), which was originally identified as a mediator of TGFβ (Yamaguchi *et al*., 1995) and bone morphogenetic protein (BMP) (Shibuya *et al*., 1998) signaling, also has been shown to function as a key intermediate in the IL-1β cascade, providing a link between TRAF6 and downstream effectors NF-κB and JNK-1(Ninomiya-Tsuji *et al*., 1999; Takaesu *et al*., 2000; Jiang *et al*., 2002).

Inhibition of TGFβ signaling by the proinflammatory cytokines interferon-γ, tumor necrosis factor (TNF)-α, and IL-1β has been described, which all inhibit TGFβ signaling by up-regulating expression of the inhibitory SMAD7 gene (Topper *et al*., 1997; Ulloa *et al*., 1999; Bitzer *et al*., 2000). In human umbilical cord vein cells, however, SMAD7 gene expression is not induced by either TGFβ, TNF-α, or IL-1β, indicating that the SMAD7 transcriptional response to these

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secreted factors is subject to cell type-dependent constraints (Topper *et al*., 1997). Inhibitory interactions between IL-1β and TGFβ also have been described; for example, IL-1β-induced production of cytokines involved in hematopoietic cell proliferation is inhibited by TGFβ (Ruscetti *et al*., 1992) and similar results have been described in T lymphocytes (Espevik *et al*., 1987; Chantry *et al*., 1989).

Here, we provide evidence for a direct, SMAD7-independent, inhibitory interaction between the IL-1β and TGFβ signaling cascades. IL-1β stimulation inhibits SMAD3 transcriptional activity as a result of IL-1β–induced complex formation between TAK1 and the MAD homology (MH) 2 domain of SMAD3. In addition we show that IL-1β inhibits TGFβ-induced target gene expression and that IL-1β neutralizes the inhibitory effect of TGFβ on in vitro myeloid colony formation. The results presented in this manuscript describe a molecular mechanism underlying IL-1β inhibition of TGFβ signaling that is SMAD7 independent and indicate that this cross-talk has implications for TGFβ target gene expression and cellular responses of hematopoietic progenitor cells.

MATERIALS AND METHODS

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from A549 and HepG2 cells stimulated with TGFβ (1 ng/ml; R&D Systems, Minneapolis, MN), IL-1β (200 U/ml; Roche Diagnostics, Almere, The Netherlands) or TNF-α (500 U/ml; Boehringer Ingelheim, Vienna, Austria) by using TRIzol, according to the supplied protocol (Invitrogen, Carlsbad, CA). When both IL-1β and TGFβ were added, IL-1β was added 30 min before TGFβ. RNA (3 μg) was reverse transcribed with Moloney-murine leukemia virus (Invitrogen) by using random hexamers and subjected to PCR analysis (*Taq* polymerase; Invitrogen). Real-time PCR analyses were performed on serially diluted cDNA samples with a Sybr Green kit and a Lightcycler (Roche Diagnostics). The specificity of the PCR reactions was verified by generation of a melting curve and by agarose gel electrophoresis of the amplified products.

The sequences of the PCR primers used were SMAD7: forward (F), GCCTCGGACAGCTCAATTCG and reverse (R), CGTCCACGGCTGCTGCATAA; SKI: F, CTCATCCGAGACAGCTTCTA and R, AGGACAAGGAGGAGGTGAAT; MMP-2: F, GGCCCTGTCACTCCTGAGAT and R, GGCATCCAGGTTATCGGGGA; and PAI-1: F, AGACCTTGGCCTCTCCTTGG and R, TGGCAGGCAGTACAAGAGTG.

Cell Lines and Transfections

A549 (ATTC CCL-185) cells were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS), and HepG2 (ATCC HB-8065) cells were maintained in DMEM containing 10% FCS and 1× minimal essential medium nonessential amino acids. Both media were further supplemented with 100 IU/ml penicillin, 1 mg/ml streptomycin, and 2 mM L-glutamine (Invitrogen). For transient transfections, 100,000 cells were seeded per 35-mm dish and transfected using either the calcium phosphate coprecipitation method (HepG2) or FuGENE (Roche Diagnostics) when A549 cells were used. The following day, the media were changed, and 48 h after transfection the cells were harvested in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was determined using the Promega luciferase assay system. In all transfections, a β-galactosidase expression plasmid (pDM2LacZ; Boer *et al*., 1990) was included to normalize luciferase activities. β-Galactosidase activity was determined in 100 mM Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 100 mM 2-mercaptoethanol, and 0.67 mg/ml *O*-nitrophenylgalactopyranoside. All transfections were carried out in triplicate and repeated at least twice in independent experiments by using different batches of plasmid DNA.

Plasmids

The SBE-Luc reporter contains four copies of the JunB SMAD binding element, as described previously (Jonk *et al*., 1998). Deleting a 3′ *Pst*I fragment from the HA-TAK1 construct generated the HA-TAK1 (1-402) construct. SMAD2-3 chimeric constructs were generated using PCR and sequenced for integrity.

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Nuclear Fractionation

A549 nuclear extracts were prepared according to the "mini extracts" method described in Schreiber *et al*. (1989). Nuclear extracts were separated using SDS-PAGE and analyzed using Western blotting and enhanced chemiluminescence (ECL) (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from A549 cells as described in Dignam *et al*. (1983). Annealed oligonucleotides (forward, TCGAGAGCCAGACAAAAAGCCAGACATTTAGCCAGACAC and reverse, TCGAGTGTCTGGCTAAATGTCTGGCTTTTTGTCTGGCTC) were labeled using Klenow fragment 1 and [α-^{32}P]dATP and purified with Sephadex G-50 columns. Binding reactions were carried out for 30 min at room temperature in 10 mM Tris, pH 8.0, 1 mM EDTA pH 8.0, 2 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01% NP-40 containing 5 μg of extract, 1 μg of poly(dI-dC), 0.1 ng of probe, and where appropriate, 100-fold molar excess competitor oligos. Preincubation with SMAD3 antibodies was carried out at room temperature, 30 min before probe addition. Before loading onto 5% polyacrylamide gels (0.5× Tris borate-EDTA), 20% Ficoll was added to the reactions, and after electrophoresis gels were dried and autoradiographed.

Immunoprecipitation

For immunoprecipitations, cells were harvested, washed with ice-cold phosphate-buffered saline, and subsequently lysed in 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 2 mM PMSF, 1 μM pepstatin, and 1 mM dithiothreitol) for 15 min on ice. Cell lysates were incubated with α-myc agarose conjugate (9E10 sc-40 AC; Santa Cruz Biotechnology, Santa Cruz, CA) rotating O/N at 4°C. The immune complex was washed three times with lysis buffer and heated in sample buffer, separated by SDS-PAGE, and analyzed using western blotting and ECL.

In Vivo Labeling

A549 cells (1×10^6), seeded in 60-mm plates, were transiently transfected with myc-SMAD3, myc-SMAD3A3, and myc-SMAD2 expression plasmids by using FuGENE (Roche Diagnostics). Forty-eight hours after transfection, the cells were starved in serum- and phosphate-free DMEM for 4 h, [^{32}P]orthophosphate was added to the medium (0.5 Ci/ml) for 2 h, and the cells were stimulated with TGFβ or IL-1β for 20 min. Myc-tagged proteins were precipitated from cell lysates as described under "Immunoprecipitation." The precipitated proteins were resolved on 10% SDS-PAGE, blotted onto polyvinylidene difluoride membrane, and quantified using a PhosphorImager (STORM 860; Amersham Biosciences UK). After detection of radioactive proteins, the blots were rehydrated, and myc-tagged proteins were detected using α-myc antibodies, followed by ECL.

In Vitro Myeloid Colony Assay

Colony-forming unit granulocyte/macrophage assays were essentially performed as described previously (Vellenga *et al*., 1990). Bone marrow mononuclear cells were obtained from healthy controls undergoing cardiac surgery after informed consent. Mononuclear cells were isolated by discontinuous gradient centrifugation by using Lymphoprep (Nycomed, Asker, Norway). Cells (10^5) were plated in 1 ml of semisolid medium, consisting of 1.2% methylcellulose (Fluka, Buchs, Switzerland) in DMEM, supplemented with 20% FCS (Invitrogen), 1% deionized bovine serum albumin (Invitrogen), 0.001% α-thioglycerol, 10 ng/ml granulocyte/macrophage-colony stimulating factor (GM-CSF) (Genetics Institute, Cambridge, MA) and 10 ng/ml IL-3 (Genetics Institute). When appropriate, 200 U/ml IL-1β or 1 ng/ml TGFβ1 was added to the medium. Cell cultures were incubated in duplicate for 14 d at 37°C and 5% CO_2, after which the number of colonies was counted using an inverted microscope; only colonies consisting of >50 cells were scored.

RESULTS

Inhibition of SMAD-dependent TGFβ Signaling by IL-1β Is Mediated through TAK1

To investigate whether IL-1β targets SMAD-mediated TGFβ signaling, the effect of IL-1β on transcriptional activation of a SMAD-dependent reporter construct by TGFβ was determined. HepG2 and A549 cells were transfected with a SMAD-specific reporter, SBE-Luc, containing multimerized SMAD binding elements (SBEs) from the JunB gene, driving the expression of the luciferase gene (Jonk *et al*., 1998). TGFβ stimulation of HepG2 and A549 cells, transfected with SBE-Luc reporter constructs, resulted in a 7- (HepG2) to 18-fold

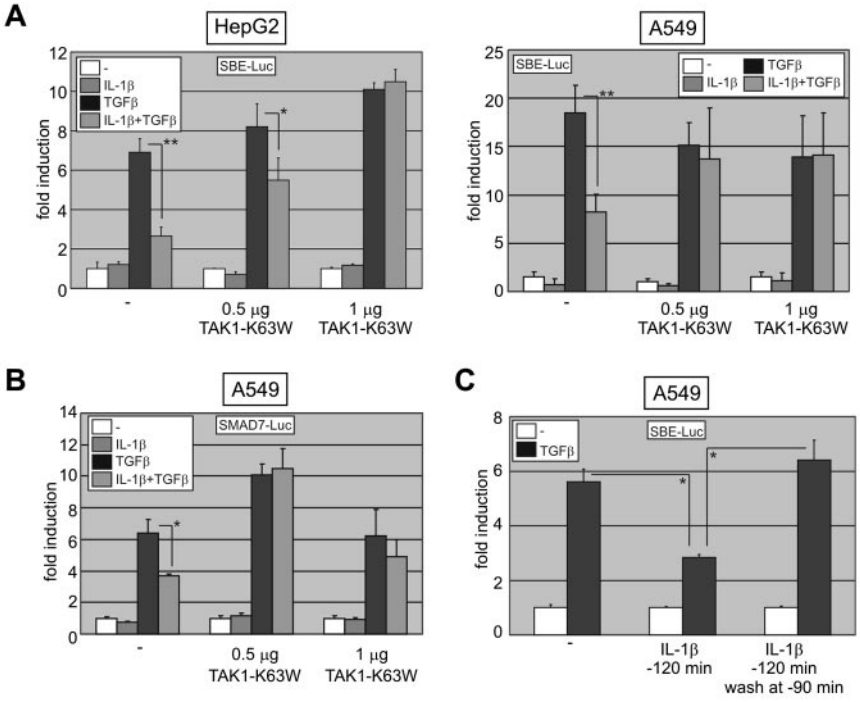

Figure 1. IL-1β inhibition of SMAD-mediated TGFβ signaling requires TAK1. (A) HepG2 and A549 cells were transfected with the indicated combinations of a reporter construct containing SMAD-responsive elements (SBE-Luc) and a dominant negative TAK1 (TAK1-K63W) expression plasmid. After 48 h, cells were treated with TGFβ and/or IL-1β for 8–12 h and harvested. Cells were either untreated (white bars) or treated with IL-1β (dark gray bars), TGFβ (black bars), or both (light gray bars). When both IL-1β and TGFβ were present, IL-1β was added 30 min before TGFβ. (B) HepG2 cells were transfected with a reporter construct containing the SMAD7 promoter. The inhibitory effect of IL-1β on SMAD7-Luc transactivation by TGFβ, in the presence of increasing amounts of TAK1-K63W expression plasmid, is depicted as IL-1β sensitivity. (C) A549 cells were transfected with a SBE reporter. The inhibitory effect of IL-1β on SBE-Luc transactivation by TGFβ, in the continuous (−120 min) or transient (−120 min/wash at −90 min) presence of IL-1β, is depicted as fold induction. In all transfections, a LacZ expression plasmid (pDM2-LacZ) was included as an internal standard and normalized luciferase activity is depicted as the mean with the SE of the mean. $^{**}p < 0.001$, $^{*}p < 0.05$.

(A549) increase in reporter activity. Although IL-1β treatment had little effect on basal SBE reporter activity, it reduced TGFβ-induced activity of the SBE-reporter by approximately two- to threefold (Figure 1A). Next, we asked whether TAK1 was involved in IL-1β inhibition of TGFβ signaling. A catalytically inactive TAK1 construct, TAK1-K63W, was overexpressed, and the effect on SBE-Luc reporter activity was determined. Cotransfection of SBE reporters with a TAK1-K63W expression plasmid restored TGFβ signaling in the presence of IL-1β, indicating that IL-1β inhibition of TGFβ signaling requires a functional TAK1 kinase (Figure 1A). Basal or TGFβ-induced SBE-Luc reporter activity was not affected by TAK1-K63W overexpression (Figure 1A). The same results were obtained using the promoter of the SMAD7 gene: overexpression of TAK1-K63W reduced the sensitivity of the SMAD7 promoter to IL-1β and restored TGFβ responsiveness (Figure 1B). These results indicate that a catalytically active TAK1 is required for the inhibitory effect of IL-1β on TGFβ signaling. To gain more insight into the kinetics of IL-1β inhibition of TGFβ signaling (also see Figure 6A), IL-1β was either added 120 min before TGFβ or added 120 min before TGFβ and removed after 30 min (90 min before TGFβ stimulation). If IL-1β was continuously present, SBE-Luc activation by TGFβ was reduced, but when IL-1β was removed 90 min before TGFβ stimulation, activation levels are completely restored, suggesting that reactivation of the IL-1β pathway occurs when IL-1β is continuously present (Figure 1C).

IL-1β Inhibits Target Gene Activation by TGFβ

To determine whether this inhibitory effect of IL-β on TGFβ-reporter gene activation also occurs at the level of endogenous gene activation, the effect of IL-1β on the transcriptional activation of target genes by TGFβ was determined in A549 cells by using quantitative RT-PCR (qPCR) analyses. A549 cells were treated with either TGFβ, IL-1β, or both, and SMAD7 (a SMAD3-specific TGFβ target gene), SKI (a TGFβ target gene activated by SMAD2 and/or SMAD3), MMP-2 (a SMAD2-specific TGFβ target gene), and PAI-1 (a SMAD3-dependent TGFβ target) mRNA expression levels were determined (Datto *et al*., 1999; Piek *et al*., 2001). SMAD7 gene expression was induced eightfold after 1 h of TGFβ stimulation, whereas after 3 h, induction levels were down to twofold and back to fourfold after 6 h. Pretreatment with IL-1β followed by TGFβ stimulation significantly reduced SMAD7 gene induction to five-, one- and twofold, respectively. IL-1β treatment alone did reduce SMAD7 mRNA baseline levels. (Figure 2). IL-1β also reduced TGFβ-mediated activation of the SKI gene, and this effect was most prominent after 6 h. IL-1β alone had a minor effect on SKI baseline mRNA levels (Figure 2). No significant effect of IL-1β on either TGFβ-induced MMP-2 or PAI-1 mRNA levels could be detected. In conclusion, these results indicate that IL-1β negatively interferes with TGFβ-induced target gene expression and most prominently with SMAD3-dependent TGFβ target genes such as the SMAD7 and SKI gene.

IL-1β and TAK1 Specifically Inhibit TGFβ Receptor-regulated SMAD3

To determine whether SMAD3 is specifically targeted by IL-1β/TAK1, as suggested by the qPCR analyses, HepG2 (and A549; our unpublished data) cells were transfected with various SMAD-responsive reporters in combination with the appropriate R-SMADs and SMAD4, TAK1, and TAK1 activating binding protein (TAB1) (Shibuya *et al*., 1996). Cotransfection of increasing amounts of TAK1/TAB1 with SMAD3/SMAD4 resulted in a progressive reduction of SBE and PAI-1 reporter activation (Figure 3A). Overexpression of the TAB1 expression plasmid alone had no effect on either basal or SMAD-induced reporter gene activity, and TAK1/TAB1 overexpression did not affect basal SBE-Luc and PAI-1-Luc activity (our unpublished data).

To determine whether the inhibitory effect of TAK1 is SMAD3 specific, we tested the effect of TAK1 on reporter activation by other R-SMADs. TAK1/TAB1 had no effect on SMAD2 activation of either an SBE-Luc reporter (Figure 3B)

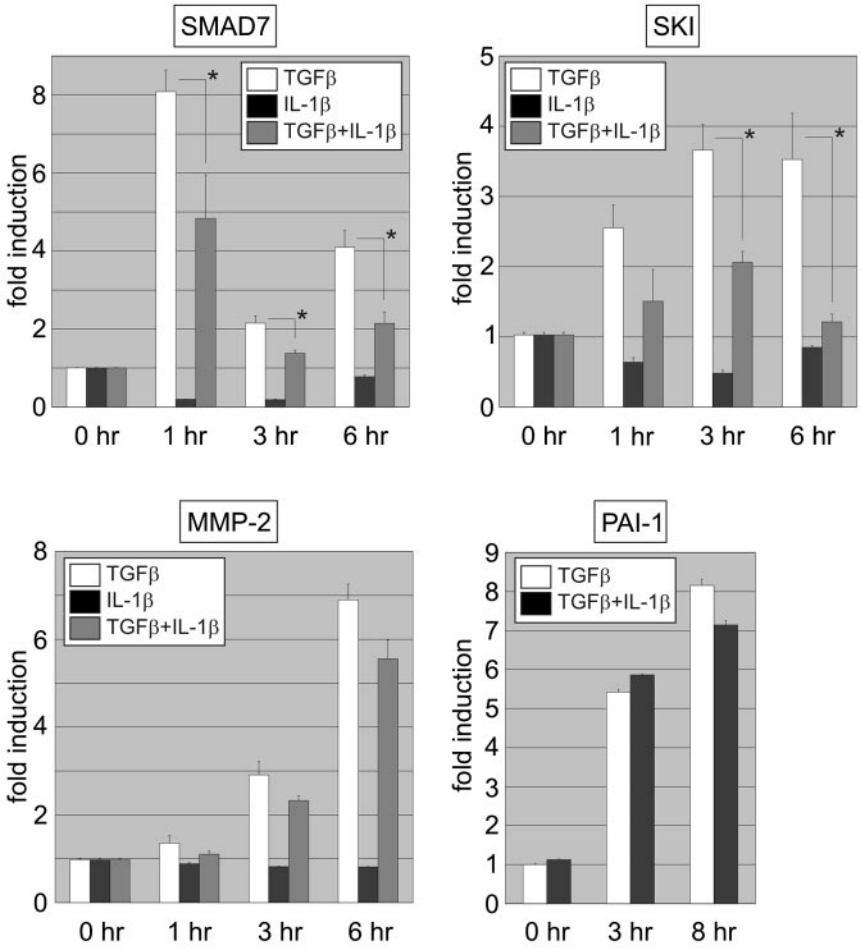

Figure 2. IL-1β inhibits endogenous TGFβ target gene expression. qPCR analysis of TGFβ target genes SMAD7, SKI, MMP-2, and PAI-1 expression in A549 cells after TGFβ and IL-1β treatment. In the SMAD7, MMP-2 and SKI qPCRs, cells were treated for 0, 1, 3, or 6 h with TGFβ (white bars), IL-1β (black bars), or both (gray bars). For the PAI-1 qPCR, cells were treated for 0, 3, and 8 h with TGFβ (white bars) or TGFβ and IL-1β (black bars). When TGFβ and IL-1β were both present, IL-1β was added 30 min before TGFβ. Expression levels of the analyzed genes are depicted as "fold induction" relative to the expression levels in the absence of TGFβ and IL-1β. qPCR analyses were at least repeated three times, a typical experiment is depicted. Student's *t* test analyses indicated that IL-1β inhibition of TGFβ target gene induction was significant for SMAD7 at all time points and SKI at 3 and 6 h. *$p < 0.05$.

or two Activin-specific reporters, ARE-Luc, and a goosecoid promoter construct, Gsc-Luc (Figure 3C). Next, the effect of TAK1 on SMAD1 reporter activation was determined using either the SBE-luc reporter or a BMP-specific reporter, BRE-Luc (Korchynskyi and ten Dijke, 2002). No inhibition of SMAD1-reporter activation was observed; in the BRE reporter, even a potentiation of promoter activity was observed (Figure 3, B and D). In conclusion, these data show that TAK1 specifically inhibits SMAD3-mediated TGFβ signaling.

TAK1 Sensitivity of SMAD3 Is Mainly Localized in the MH1 Domain

To determine the domains in SMAD3 that are targeted by TAK1, we made use of the observation that SMAD3 transcriptional activity was efficiently inhibited by TAK1, whereas SMAD2 activity was not affected, and generated SMAD2-3 chimeric constructs. The effect of TAK1 on transcriptional activation of SBE-Luc reporters by these SMAD2–3 chimeras was determined. The fold-repression of SMAD-mediated reporter gene activation by TAK1 is depicted as "TAK1 sensitivity" (Figure 4). SBE reporter activation by SMAD3 is inhibited by TAK1, a threefold reduction in activity, whereas transcriptional activation by SMAD2 is unaffected. Replacing the SMAD2-MH2 domain with the SMAD3-MH2 domain (compare the 2-2-2 and 2-2-3 constructs) had no effect on TAK1 sensitivity. Replacing the SMAD2 MH1 domain with a SMAD3 MH1 domain, however, increased TAK1 sensitivity by twofold (compare 2-2-2 with 3-2-2). The linker region of SMAD3 does not seem to be involved in TAK1 repression of SMAD3 transcriptional activity (compare the 2-2-3/2-3-3 and 3-3-2/3-2-2 constructs. It is clear that primarily the SMAD3 MH1 domain is targeted by TAK1; however, the observation that the sensitivity is highest in a 3-3-3 construct suggests that the SMAD3 linker and MH2 domains, at least in the context of a SMAD3 MH1 domain, contribute to TAK1 sensitivity of SMAD3.

IL-1β Does Not Affect TGFβ-induced Nuclear Translocation and DNA Binding of SMAD3

Transcriptional activation of target genes by SMADs requires a sequence of events that include 1) activation of R-SMADs by an activated receptor complex, 2) nuclear translocation of SMADs, 3) binding to target sequences, and 4) transcriptional activation of these target genes. In the following experiments, we determined whether IL-1β and TAK1 targets (one of) these steps. IL-1β does not interfere with the activation of the MMP-2 gene by TGFβ, indicating that IL-1β does not target the TGFβ signaling cascade at the level of the receptor. Furthermore, TAK1 specifically inhibits SMAD3, whereas SMAD2-mediated activation of the SBE-Luc reporter is not affected. Therefore, we decided to focus on downstream events. A549 and HepG2 cells were stimulated with TGFβ for various time points, and nuclear fractions were made and analyzed on Western blots. After 15 min of TGFβ treatment, a clear accumulation of SMAD3 in

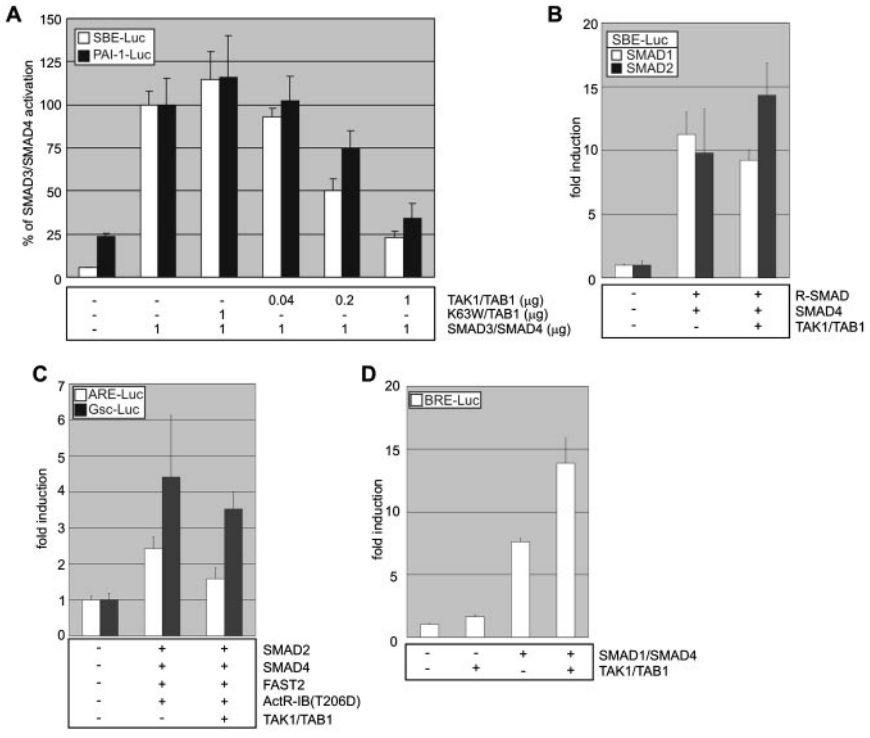

Figure 3. TAK1 specifically inhibits transcription activation by SMAD3. (A) HepG2 cells were transfected with the indicated combinations and amounts of a reporter construct containing either multiple SMAD binding elements (SBE-Luc) or the promoter of the PAI-1 gene (PAI-1-Luc) and TAK1, TAB1, TAK1-K63W, SMAD3, and SMAD4 expression plasmids. The activity of these reporters in the presence of SMAD3 and SMAD4 is set at 100% and depicted as the mean of triplicates and the SE of the mean. In all transfections, a LacZ expression plasmid (pDM2-LacZ) was included as an internal standard. (B) HepG2 cells were transfected with a reporter construct containing multiple SMAD binding elements (SBE-Luc) in combination with TAK1, TAB1, SMAD1, SMAD2, and SMAD4 expression plasmids as indicated. Reporter activity is depicted as fold induction of the unstimulated reporter with the SE of the mean. (C) HepG2 cells were transfected with a reporter construct containing either multiple Activin response elements (ARE-Luc) or the promoter of the zebrafish Goosecoid gene (Gsc-Luc) in combination with TAK1, TAB1, FAST2, ActR-IB(T206D), SMAD2, and SMAD4 expression plasmids. Reporter activity is depicted as fold induction of the unstimulated reporter with the SE of the mean. (D) HepG2 cells were transfected with a reporter construct containing either multiple BMP-responsive elements (BRE-Luc) of the promoter of the *Xenopus laevis* Vent-2 gene (Xvent2-Luc) together with TAK1, TAB1, TAK1-K63W, SMAD1, and SMAD4 expression plasmids. Reporter activity is depicted as fold induction of the unstimulated reporter with the SE of the mean.

the nucleus, compared with unstimulated cells, was observed (Figure 5A). Treatment of the cells with IL-1β before TGFβ stimulation had no effect on SMAD3 nuclear translocation, indicating that the inhibitory effect of IL-1β on SMAD3-TGFβ signaling occurs at a downstream step. Next, we investigated the effect of IL-1β on the ability of SMAD3 to bind DNA. A549 cells were untreated, stimulated with TGFβ, or pretreated with IL-1β before TGFβ stimulation. Nuclear extracts were generated and analyzed for SMAD3 DNA binding activity by using a radiolabeled double-stranded SBE oligo as a probe. TGFβ stimulation clearly resulted in the formation of complexes with decreased mobility (indicated with SMAD3 in Figure 5B). To validate that these complexes contain SMAD3, a supershift was performed using α-SMAD3 antibodies, which resulted in a further reduction in mobility of the observed complexes, verifying that these contained SMAD3 (indicated by s-SMAD3 in Figure 5B). To control for the specificity of the retarded complexes, 100× excess unlabeled competitor (self) or noncompetitor (nonself) oligos was added (Figure 5B, lanes 100× self and 100× nonself). Pretreatment with IL-1β had no effect on the ability of SMAD3 to bind DNA. In conclusion, these data show that IL-1β does not interfere with SMAD3 nuclear translocation or DNA binding, suggesting that IL-1β most likely interferes with the ability of SMAD3 to activate target gene transcription, as was observed in the qPCR analyses and reporter studies.

Association with TAK1 and Phosphorylation of SMAD3 in Response to IL-1β

To determine the level of interaction between the IL-1β/TAK1 and TGFβ/SMAD3 signaling cascades, HepG2 cells were transfected with a myc-tagged SMAD3 construct, and

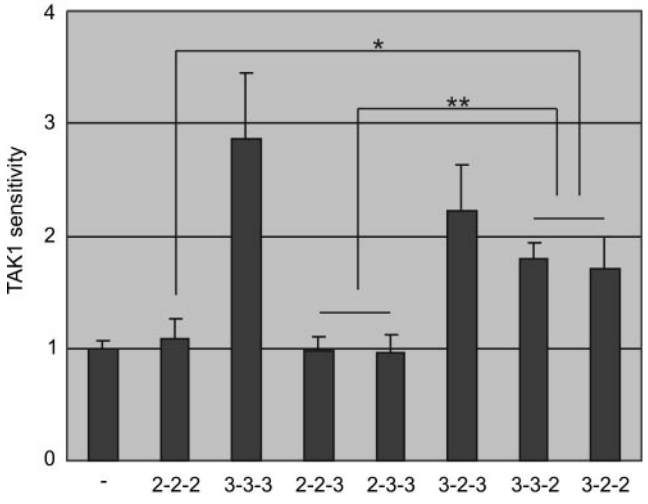

Figure 4. TAK1 sensitivity of SMAD3 is mainly localized in the MH1 domain. NIH-3T3 cells were transfected with a SMAD-responsive reporter (SBE-Luc), TAK1, TAB1, SMAD2, SMAD3, or SMAD2–3 chimeric expression plasmids. The inhibitory effect of TAK1 on SBE-Luc transactivation by the indicated SMAD constructs is depicted as TAK1 sensitivity. In all transfections, a LacZ expression plasmid (pDM2-LacZ) was included as an internal standard, and normalized luciferase activity is given as the mean (n = 6) with the SE of the mean. An analysis of variance followed by a Tukey post hoc test resulted in the significant differences indicated (3-3-3 and 3-2-3 are not significantly different, 3-2-3 does not differ from 3 to 3-2 and 3-2-2, but is different from 2 to 2-2, 2-2-3 and 2-3-3, not indicated in figure). *$p < 0.05$.

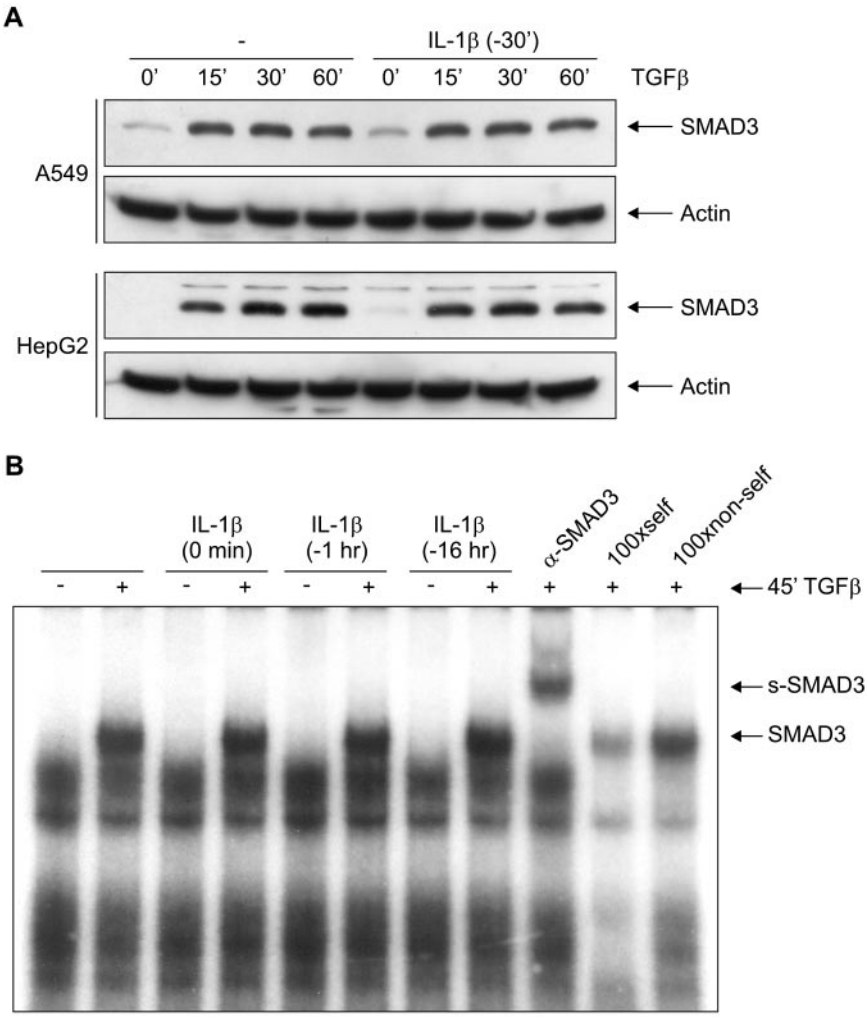

Figure 5. TAK1 does not affect nuclear translocation or DNA binding of SMAD3. A) A549 and HepG2 cells were stimulated with TGFβ and IL-1β as indicated. Nuclear fractions were prepared and subjected to SDS-PAGE and Western blotting using anti-SMAD3 and anti-actin antibodies. (B) A549 cells were stimulated with TGFβ and IL-1β as indicated, and nuclear extracts were prepared and used in EMSAs. The TGFβ-induced complex containing SMAD3 (SMAD3) as well as the SMAD3-containing supershifted complex (s-SMAD3) are indicated by arrows.

SMAD3-associated proteins were precipitated from untreated and IL-1β–treated HepG2 cells. Western analysis of the immunoprecipitates indicated that TAK1 coprecipitated with SMAD3 in response to IL-1β (Figure 6A). In a time-course experiment, we determined that complex formation between SMAD3 and TAK1 occurs within 2 min of IL-1β stimulation and can be detected up to 30 min, indicating that IL-1β stimulation results in rapid, transient SMAD3 and TAK1 complex formation (Figure 6A).

To identify the interacting domains in SMAD3 and TAK1, deletion constructs were generated and analyzed in coimmunoprecipitation experiments. The MH1-, linker-, and MH2-domains of SMAD3 were tested for IL-1β–induced interaction with TAK1 in coimmunoprecipitation experiments. TAK1 immunoreactivity was only detected in complexes precipitated from IL-1β–stimulated cells transfected with the SMAD3-MH2 domain (Figure 6B). To determine the domain in TAK1 that interacts with SMAD3 and to test whether an intact catalytic domain is required for complex formation with SMAD3, deletion constructs and a TAK1-K63W mutant were tested in coimmunoprecipitations. The carboxy-terminal 177 amino acids of TAK1 [HA-TAK1(1-402)] are not required and can be deleted without affecting interaction with SMAD3 (Figure 6C). A functional catalytic domain of TAK1 is also not required for SMAD3 interaction because mutation of the ATP-binding site of the TAK1 kinase domain (HA-TAK1-K63W) did not affect interaction with SMAD3 (Figure 6C). Furthermore, coimmunoprecipitation experiments indicated that IL-1β stimulation does not lead to complex formation of SMAD3 with either Erk-1, Erk-2, p38, or JNK-1, all MAPK positioned downstream of TAK1 (our unpublished data). These results further support the observation that inhibition of SMAD3-TGFβ signaling by IL-1β occurs at the level of TAK1 and is not mediated by downstream MAPK kinases or MAPKs.

To investigate whether IL-1β induces phosphorylation of SMAD3, A549 cells were transfected with SMAD2, SMAD3, or SMAD3AS (a mutant in which the C-terminal SSXS motif is mutated in AAXA to reduce SMAD3 phosphorylation levels) expression plasmids and cultured in the presence of inorganic ^{32}P. Next, cells were treated with either TGFβ or IL-1β, subjected to α-myc immunoprecipitations, SDS-PAGE, autoradiography, and Western analysis. TGFβ treatment resulted in a dramatic (30-fold) increase in SMAD3 phosphorylation. IL-1β stimulation also resulted in an increase in SMAD3 phosphorylation (1.5-fold) both in the SMAD3 and SMAD3A3 construct. SMAD2 phosphorylation levels were not altered in response to IL-1β (Figure 6D).

Inhibition of Myeloid Progenitor Proliferation by TGFβ Is Completely Restored by IL-1β

Previous studies demonstrated that TGFβ inhibits in vitro colony formation (Fortunel *et al*., 2000b). This was further

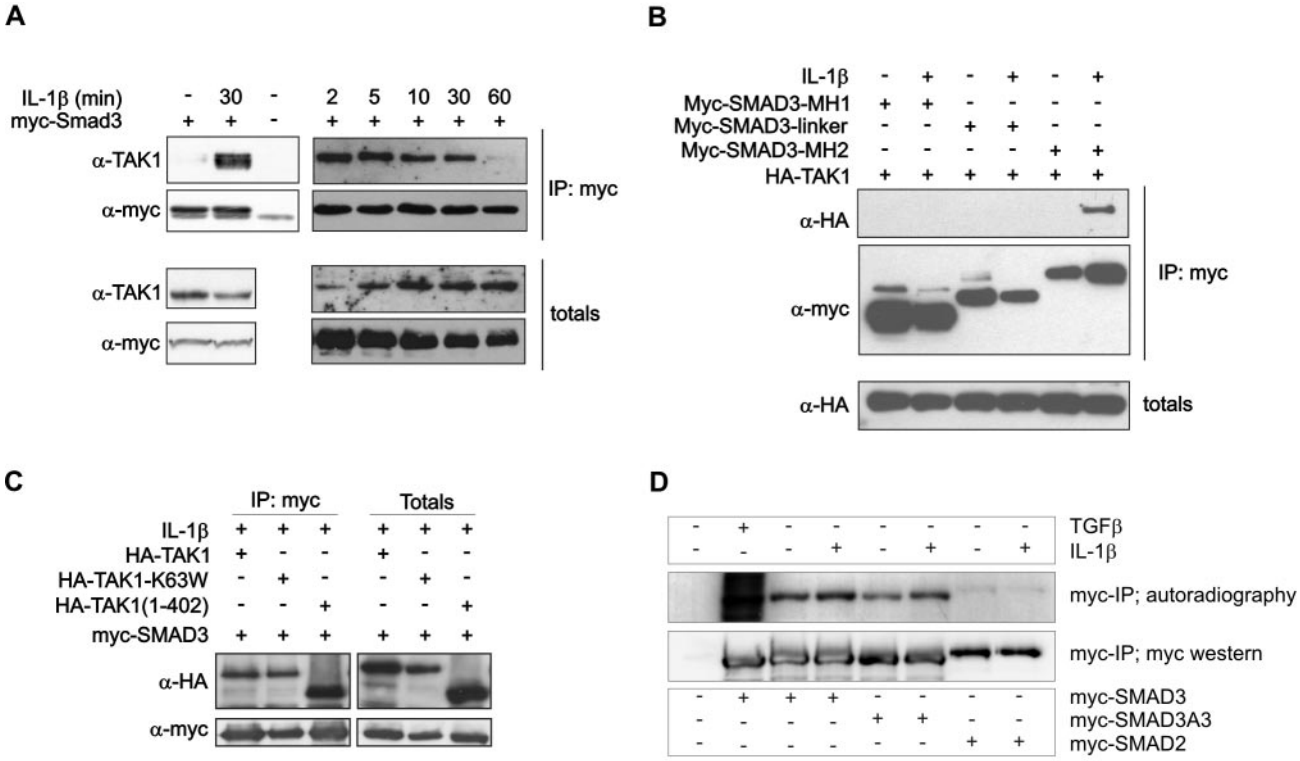

Figure 6. IL-1β induces association of TAK1 and SMAD3 and SMAD3 phosphorylation. (A–C) HepG2 cells were transfected with myc-SMAD3, myc-SMAD3-MH1, myc-SMAD3-linker, myc-SMAD3-MH2, HA-TAK1, HA-TAK1-K63W, and HA-TAK1-(1-402) expression plasmids and stimulated with IL-1β as indicated. Cell lysates were subjected to myc immunoprecipitation (IP) followed by α-myc, α-TAK1, or α-HA immunoblotting. Myc-SMAD3, HA-TAK1 (and mutants thereof), and endogenous TAK1 expression levels were confirmed in total cell extracts (totals panels). (D) HepG2 cells, transfected with either myc-SMAD3, myc-SMAD3-A3, or myc-SMAD2 expression plasmids were incubated in medium containing ^{32}P and stimulated with either IL-1β or TGFβ as indicated. Cell lysates were subjected to anti-myc immunoprecipitations and resolved using SDS-PAGE followed by autoradiography and Western analysis with α-myc antibodies.

illustrated in experiments in which TGFβ signaling was inhibited by either blocking antibodies (Fortunel *et al*., 2000a) or by antisense TGFβ oligonucleotides (Hatzfeld *et al*., 1991) where a (partial) loss of an autocrine TGFβ loop resulted in a release of primitive hematopoietic precursors from quiescence and stimulated in vitro colony formation.

In view of the observed inhibition of TGFβ signaling by IL-1β, we investigated the effects of IL-1β and TGFβ on in vitro colony formation by using human bone marrow cells. Myeloid colony formation was not affected if the cells were costimulated with IL-1β, whereas TGFβ treatment, as was reported previously, reduced colony formation by ~60%. This inhibitory effect of TGFβ, however, was completely alleviated by the addition of IL-1β (Figure 7). These findings clearly demonstrate that IL-1β can counteract the inhibitory effect of TGFβ on myeloid colony formation.

DISCUSSION

Convergence and integration of signaling pathways determines the biological response of cells and tissues to stimuli as hormones, ligands, or pathogens. The IL-1β and TGFβ signaling cascades are two pleiotropic signaling pathways that elicit a variety of biological responses. In the hematopoietic and immune system, these two signaling cascades essentially have opposite effects: IL-1β acts proinflammatory and stimulates (stem) cell cycling and cytokine production, whereas TGFβ basically acts anti-inflammatory and inhibits (stem) cell cycling and cytokine production (Ruscetti *et al*., 1992).

Here, we show that IL-1β negatively interferes with transcriptional activation of TGFβ target genes and that IL-1β can counteract the inhibitory effect of TGFβ on in vitro myeloid colony formation. Furthermore, we provide evidence for a direct, SMAD7-independent, inhibitory interaction between the IL-1β and TGFβ signaling cascades. We show that IL-1β induces the formation of a TAK1–SMAD3 complex and prevents transcriptional activation by SMAD3 in response to TGFβ.

The effect of IL-1β on TGFβ-induced target gene expression was analyzed using SMAD7, SKI, MMP-2, and PAI-1 as target genes. The inhibitory effect of IL-1β was the strongest on the rapidly TGFβ-induced SMAD7 and SKI genes (Figure 1). Because the interaction between TAK1 and SMAD3 is transient (Figure 6A), it is possible that IL-1β treatment does not result in a complete, long-term block in SMAD3 signaling. Combined with the different transcriptional activation characteristics of the SMAD7 and SKI genes in response to TGFβ (the transcriptional response of SKI is delayed and prolonged in comparison with SMAD7), this possibly explains the observed differences in IL-1β effectiveness in blocking TGFβ target gene activation. Previous reports have shown that TGFβ signaling can be inhibited by the cytokines interferon-γ, TNF-α, and IL-1β, all through up-regulation of SMAD7 gene expression (Topper *et al*., 1997; Ulloa *et al*., 1999; Bitzer *et al*., 2000). In the experiments depicted in

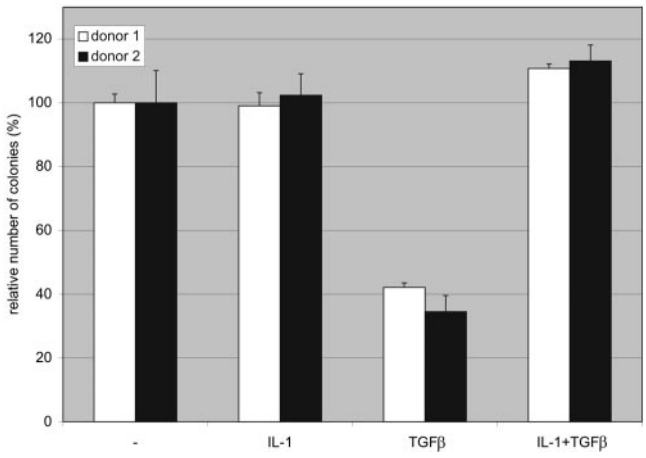

Figure 7. IL-1β neutralizes inhibition of myeloid colony formation by TGFβ. Human bone marrow mononuclear cells (n = 2) were cultured in an in vitro colony forming unit-granulocyte/macrophage assay in the presence of IL-3 and GM-CSF. IL-1β and TGFβ were added as indicated. The number of colonies formed in the absence of IL-1β and TGFβ was set at 100%.

Figure 1, up-regulation of SMAD7 gene expression in response to IL-1β (and TNF-α; our unpublished data) alone was not observed. Furthermore, RT-PCR analyses were performed 1, 3, and 6 h after TGFβ stimulation, a time scale in which it is very unlikely (at least at the first 2 time points) that transcription and translation of the SMAD7 gene occur, and a clear inhibitory effect of IL-1β on SKI gene activation by TGFβ was observed. These data indicate the existence of an alternative mechanism for IL-1β inhibition of TGFβ signaling.

TGFβ activation of the PAI-1 gene, shown by Datto and Piek (Datto *et al*., 1999; Piek *et al*., 2001) to be SMAD3 dependent, was not or only mildly inhibited by IL-1β (Figure 2). However, when the PAI-1 promoter was tested in transient transfection assays, SMAD3 activation of PAI-1 reporter was clearly inhibited by TAK1 (Figure 3). These data indicate that TAK1 inhibits SMAD3-mediated transcriptional activation but that IL-1β treatment does not result in reduced transcription of all TGFβ-SMAD3 target genes, i.e., PAI-1. The PAI-1 reporter and the endogenous PAI-1 gene seem to respond differently to IL-1β/TAK1. It is possible that the PAI-1 reporter (-800-Luc; Keeton *et al*., 1991) we used does not contain all the required sequence elements to completely mimic the transcriptional regulation of the endogenous gene. Alternatively, from different sets of experiments we have data showing that the PAI-1 promoter behaves differently as an episomal or as a stably integrated construct in terms of sensitivity to radiation, which also could explain the observed differences in responsiveness.

Studies in SMAD2- and SMAD3-deficient fibroblasts showed that TGFβ induction of SMAD7 gene expression relies on SMAD3 and that SMAD2 is indispensable for MMP-2 activation (Datto *et al*., 1999; Piek *et al*., 2001). This possibly explains the strong effect of IL-1β on TGFβ-induced SMAD7 mRNA levels. SMAD3 dependence of TGFβ-mediated transcriptional activation of the SKI gene has not been determined, so residual SMAD2-mediated TGFβ signaling could explain the inability of IL-1β to completely block TGFβ-induced SKI expression. IL-1β does not interfere with TGFβ-induced MMP-2 mRNA levels in A549 cells. MMP-2 is a SMAD2-specific TGFβ target gene and SMAD2 is not inhibited by IL1β/TAK1. These observations showed that IL-1β/TAK1 specifically targets SMAD3, an observation validated in transient transfection assays with different R-SMADs.

The proposed proinflammatory effect of this inhibitory interaction in terms of cell biological functions is in agreement with the phenotypes displayed by the SMAD2 and SMAD3 null mice. Targeted deletion of SMAD2 results in an early embryonic lethal phenotype, indicating that SMAD2 is critical for early embryonic development (Weinstein *et al*., 1998). SMAD3-deficient mice, however, survive up to 1–8 mo and eventually die of opportunistic infections due to a compromised immune system (Datto *et al*., 1999; Yang *et al*., 1999).

Besides a difference in biological function between SMAD2 and SMAD3, these SMADs also differ in their MH1 and MH2 domains and bind to different cofactors involved in transcriptional regulation by SMADs. Immunoprecipitations using SMAD3 deletion constructs and transfection assays using SMAD2–3 chimeric constructs indicated that TAK1 binds the SMAD3-MH2 domain and that both the MH1 and MH2 domains are involved in TAK1 repression of SMAD3 activity. TAK1 also binds the SMAD2-MH2 domain (Benus and Eggen, unpublished data), but transfection data using SMAD2-3 chimeras indicated that a 3-3-2 chimera is less TAK1 sensitive than SMAD3, indicating a difference in the SMAD2 and SMAD3-MH2 domains in terms of TAK1 repression. The most prominent inhibitory effect of TAK1 on SMAD3 can be allocated to the MH1 domain, a 3-2-2 chimera is 3 times more sensitive to TAK1 than SMAD2 and only twofold less sensitive than SMAD3. The linker region of R-SMADs has previously been shown to be a target for the MAPK extracellular signal-regulated kinase (Erk) to inhibit nuclear translocation (Kretzschmar *et al*., 1997, 1999). The SMAD3 linker does not seem to be involved in mediating TAK1 sensitivity because a 3-3-2 chimera is equally sensitive to TAK1 as a 3-2-2 chimera (Figure 4).

Several SMAD3-specific cofactors have been identified that bind to the MH1 and MH2 domains of SMAD3 (ATF2, AP-1 members, TFE3, VDR, and Evi-1; Moustakas *et al*., 2001), so it is possible that TAK1 perturbs the interaction with one of them by phosphorylating SMAD3 on a yet unknown residue(s). The hypothesis that TAK1 phosphorylates SMAD3 is supported by the observation that TAK1–SMAD3 interaction is transient and that a catalytically inactive TAK1 (TAK1-K63W) acts as a dominant negative TAK1.

In addition to IL-1β, TAK1 also has been positioned downstream of TGFβ and BMPs (Yamaguchi *et al*., 1995; Shibuya *et al*., 1998). In the experiments described here, TAK1 acts as an inhibitor of TGFβ signaling (downstream of IL-1β) and does not affect SMAD-mediated BMP signaling. It remains unclear how these cytokines exert (some of) their different biological effects by using the same mediator, TAK1. It could be context dependent in the sense that not all required components to link the cytokine to TAK1 activation are present in all cells. Alternatively, it is possible that TAK1 is localized in distinct signalosomes, resulting in ligand-specific activation of TAK1. A further understanding of how TGFβ signaling can both be partly mediated by TAK1 and also inhibited by TAK1 is at present unclear.

TAK1 has been positioned upstream of various MAPK cascades, but these seem not to be involved in IL-1β/TAK1-mediated inhibition of SMAD3-mediated TGFβ signaling. Interference with MAPK signaling by means of overexpression of dominant negative MKKs or use of chemical inhibitors did not affect inhibition of SMAD3 signaling by TAK1 (our unpublished data), further indicating that the TGFβ

and IL-1β signaling cascades interact at the level of TAK1-SMAD3.

The direct interaction between the IL-1β and TGFβ signaling cascades might have important biological implications, which is illustrated by the observation that IL-1β restores the proliferative potential of hematopoietic precursors in the presence of TGFβ in an in vitro myeloid colony formation assay. Although the role of TAK1 and SMAD3 in these assays remained elusive, these experiments demonstrated a clear biological effect of cross-talk between the IL-1β and TGFβ signaling cascades on the proliferative response of hematopoietic cells. In the microenvironment of the bone marrow stroma, variations in the local concentrations of cytokines that modulate progenitor cell renewal, proliferation, and differentiation determines the cellular response of these cells. IL-1β has been extensively studied as a cytokine leading to increased stem cell cycling, whereas TGFβ inhibits stem cell cycling (Ruscetti *et al*., 1992; Fortunel *et al*., 2000b). The observation that these two pathways converge provides novel insight in the mechanism of integration of these positively and negatively instructive signaling cascades at the intracellular level. The balance between IL-1β and TGFβ might act as a switch between a quiescent and cycling state of these cells. The observations that a loss of SMAD3-mediated TGFβ signaling by AML-Evi-1 (Kurokawa *et al*., 1998) or AML-ETO (Jakubowiak *et al*., 2000) translocations contribute to leukemogenesis and that spontaneous IL-1β secretion is observed in AML (Dokter *et al*., 1995) suggests that perturbations in the inhibitory interaction between the IL-1β and TGFβ cascades might promote uncontrolled cellular proliferation or even malignant transformation.

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