Transforming Growth Factor (TGF)-β-activated Kinase 1 Mimics and Mediates TGF-β-induced Stimulation of Type II Collagen Synthesis in Chondrocytes Independent of Col2a1 Transcription and Smad3 Signaling*

Bo Qiao‡†, Silvia R. Padilla‡, and Paul D. Benya‡§¶

From the Orthopaedic Hospital, Los Angeles, J. Vernon Luck, Sr., M.D. Research Center and UCLA-Orthopaedic Hospital Department of Orthopaedic Surgery, David Geffen School of Medicine at UCLA, University of California, Los Angeles, California 90095

Transforming growth factor (TGF)-β, bone morphogenetic protein (BMP), and interleukin-1β activate TGF-β-activated kinase 1 (TAK1), which lies upstream of the p38 MAPK, JNK, and NF-κB pathways. Our knowledge remains incomplete of TAK1 target genes, requirement for cooperative signaling, and capacity for shared or segregated ligand-dependent responses. We show that adenoviral overexpression of TAK1a in articular chondrocytes stimulated type II collagen protein synthesis 3–6-fold and mimicked the response to TGF-β1 and BMP2. Both factors activated endogenous TAK1 and its activating protein, TAB1, and the collagen response was inhibited by dominant-negative TAK1a. Isoform-specific antibodies to TGF-β stimulated the response to endogenous and exogenous TGF-β but not the response to TAK1a. Expression of Smad3 did not stimulate type II collagen synthesis or enhance that caused by TGF-β1 or TAK1a, in contrast to its effects on its endogenous targets, CTGF and plasminogen-activated inhibitor-1. TAK1a, overexpressed alone and immunoprecipitated, phosphorylated MKK6 and stimulated the plasminogen-activated inhibitor-1 promoter following transient transfection; both effects were enhanced by TAB1 coexpression, but type II collagen synthesis was not. Stimulation by TAK1a or TGF-β did not require increased Col2a1 mRNA, and TAK1 actually reduced Col2a1 mRNA in parallel with the cartilage markers, SRY-type HMG box 9 ( Sox9) and aggrecan. Thus, TAK1 increased target gene expression (Col2a1) by translational or posttranslational mechanisms as a Smad3-independent response shared by TGF-β1 and BMP2.

TGF-β1 superclass family members, including TGF-βs and BMPs, play important roles in skeletogenesis (1) during pattern formation (2–5), mesenchymal condensation (5–8), chondrogenesis (9–13), and endochondral ossification (14–16). In the adult, they maintain the extracellular matrix of articular cartilage (15, 17–19) and regulate fracture repair (20).

TGF-β and BMP exhibit both common and specific effects. Both induce chondrogenesis and associated Col2a1 expression in limb bud mesenchyme and mesenchymal stem cells (9, 11, 12, 21, 22). Conversely, TGF-β inhibits and BMP enhances chondrocyte maturation and type X collagen expression in the growth plate (14, 16, 19). In general, such specificity is mediated by different ligands, similar but distinct receptors with integral serine/threonine kinase activity, and recruitment and phosphorylation of distinct subsets of receptor Smads (23–25). TGF-β binding to the type II TGF-β receptor (TβRII) leads to recruitment, phosphorylation, and activation of the type I receptor (TβRI). The activated TβRI kinase phosphorylates Smad2 or -3, enabling association with the universal common Smad, Smad4, prior to nuclear translocation. Heteromeric Smads then activate or suppress transcription in cooperation with other transcription factors, coactivators, or repressors (1, 26, 27). Similarly, BMP signals through the kinase activity of its high affinity type I receptors, BMPR1a and BMPR1b, in combination with BMPRII. Receptor activation in this case leads to phosphorylation of a different subset of receptor Smads, Smad1, -5, and -8, before interaction with Smad4 (1, 26).

In contrast to the specificity of receptors and Smad activation, both TGF-β and BMP activate the MAPK kinase kinase, TGF-β-activated kinase 1 (TAK1) (28), through its endogenous binding to the type II TGF-β receptor (TβRII) (29). Thus, the TAK1 signaling cascade can mediate shared responses or cooperate with individual Smads to enhance or modulate TGF-β- and BMP-specific responses. Following activation of endogenous TAB1 in preformed TAK1-TAB1 complexes (30–32), TAK1 autophosphorylates conserved serine 192 (30) and threonine 187 (33) in the activation loop to enable its kinase activity. In contrast, overexpression of TAK1 and TAB1 together is sufficient to activate TAK1 kinase activity (30). Downstream signaling by TAK1 diverges into several MAPK pathways. TAK1 activates the p38 MAPK cascade by directly phosphorylating Smads, Sma and Mad (mothers against decapentaplegic) homologs; CTGF, connective tissue growth factor; PAI-1, plasminogen-activated inhibitor-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK3, -4, and -6, MAPK kinase 3, 4, and 6, respectively; EGR-1, early growth response-1 transcription factor; MOI, multiplicity of infection; RPA, ribonuclease protection assay; CMV, cytomegalovirus; DME, Dulbecco’s modified Eagle’s medium; IL, interleukin; HIA, heat-inactivated.

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† To whom correspondence should be addressed: Orthopaedic Hospital, 2400 S. Flower St., Los Angeles, CA 90007-2697. Tel.: 213-742-1362; Fax: 213-742-1365; E-mail: pbenya@LAOH.ucla.edu.
‡ Present address: Dumont-UCLA Transplant Center, Dept. of Surgery, UCLA School of Medicine, Los Angeles, CA 90095.
¶ The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; TAK1, transforming growth factor-β-activated kinase 1; TAK1a, the TAK1 splice variant overexpressed in this report; TAB1, TAK1-binding protein 1; TβRI and TβRII, TGF-β receptor I and II; hTAK1a, human TAK1a; hTAB1, human TAB1;
MKK3 or MKK6 (34, 35) and indirectly activates the c-Jun N-terminal kinase (JNK) pathway by direct phosphorylation of SEK1/MKK4 (36). Thus, p38 activation of the transcription factor, activating transcription factor 2 (ATF-2) (37), and JNK activation of Jun family members and API transcription factors (38) are demonstrated responses to TAK1 activation. In addition, TAK1 cooperates with Smads to enhance transactivation by activating ATF-2 in response to either TGF-β or BMP (37, 39, 40). Importantly, TAK1 is also activated by the catabolic cytokines IL-1β and tumor necrosis factor-α (41, 42), leading to a conflict between their signaling through NF-κB and p38 and the anabolic signals of TGF-β and BMP.

High level expression of type II collagen is a phenotypic marker for chondrogenesis and a hallmark of the load-bearing structure of adult articular cartilage. Col2a1 gene expression is developmentally regulated by the HMG domain transcription factor Sox9 (43) in cooperation with L-Sox5 and Sox6 (44) through binding to sequences in the first intron enhancer (45). Other functional regulatory sites are present in the Col2a1 promoter/enhancer (46); however, the Sox9 binding domain is sufficient for tissue-specific expression (45). Although TGF-β has been shown to have varied effects on collagen synthesis in chondrocyte culture (47, 48), Chadjichristos et al. (49) have recently shown that TGF-β suppresses expression driven by the Col2a1 proximal promoter, extending similar earlier conclusions using the first intron enhancer (50, 51).

Here we demonstrate, in rabbit articular chondrocytes, enhanced synthesis of type II collagen protein by TGF-β and BMP2. This stimulation activated endogenous TAK1, which was blocked by dominant-negative TAK1a, and was mimicked by adenosiviral overexpression of TAK1a or TAK1a and TAB1 together. The response to TGF-β or TAK1a did not increase Col2a1 message and was not replicated by adenosiviral expression of Smad3. Thus, stimulation of type II collagen synthesis represents a response shared by both TGF-β and BMP2 that does not require increased transcription or stability of Col2a1 message or cooperation with Smad3 signaling.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Inhibitors**—The following antibodies were used: horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technologies); horseradish peroxidase-conjugated anti-goat IgG (A5420; Sigma); goat anti-CTGF (sc-14939; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse monoclonal anti-PAI-1, anti-lamin A/C, and anti-β-actin (1:1,000; Sigma) antibodies; rabbit polyclonal anti-TGF-β1 (catalog number AF-101-NA; 100 ng/ml), goat anti-TGF-β (catalog number AF-101-NA; 100 ng/ml), goat anti-TGF-β3 (catalog number AF-243-NA; 2.5 μg/ml). These antibodies were mixed and used at the indicated concentrations, which were 5-fold higher than the lowest completely inhibitory dose (1:1000).

**Plasmids**—Plasmids were obtained from the following sources: pGEK-MKK6 (K. Davis) (52), p800LUC (D. Loskutoff) (53), pUC18 (ATCC), rabbit Col2a1 3 untranslated region (E.Vuorio) (54), and pNF-κB-Luc (Clontech). To create vectors for TAK1 and TAB1 expression, the adenosiviral cosmid AxCAwt (55) (TakaRa; containing the chicken β-actin promoter, first intron enhancer, and the CMV enhancer (56)) was cut with Sall and Ncol to remove adenosiviral sequences and the CMV enhancer prior to ligation with a Sall-NotI adapter. The first intron enhancer was then removed with Hinfl and ClaI and resealed with a Hinfl-NotI-ClaI adapter to produce pA3. A Sall site at the 3′ end of the CMV enhancer of pCMV-V6-X (Origene) was generated from a cryptic site by QuickChange site-directed mutagenesis (Stratagene). The Sall-NotI fragment was inserted in the same site in pA3 to produce pC containing only the CMV promoter. The 376-bp Sall-NotI fragment from AxCAwt containing the CMV enhancer was inserted in the same site in pA3 to generate pCEA3 containing the minimal β-actin promoter preceded by the CMV enhancer. An arrayed human heart cDNA library (Origene) was screened for full-length cDNA for TAK1a and TAB1 by PCR using primers designed from published sequences (29, 57). The 3′ untranslated region was truncated, and KpnI, XbaI, and NotI sites were inserted 3′ to the wild type stop codon in the library clone pCMV-V6-X-hTAK1a by Excite deletional mutagenesis (Stratagene). The resultant BspHI-Xba fragment was inserted into the unamplified library clone. Similarly, pCMV-V6-X-hTAK1a was shortened, and a FLAG epitope tag was inserted using the following primers: forward, 5′-TGCGGTATACACCTGATCTAGATTGGCGGCCGCGGTCATAGC-3′; reverse, 5′-GCTGGTACACCGATCPTCCGTCGATCCTGATATTCCTGTCGCTGTCCTCAGCCGCTCTG-3′. The PCR product was cut with KpnI and ligated prior to transfer of the RareII-XbaI fragment to the same site in the unamplified clone. The shortened TAK1a and TAB1 constructs were subcloned into NotI sites to generate expression vectors pC-TAK1a and pCEA3-hTAK1a. Cloning was monitored and verified by sequencing. A tandem vector was made by inserting the blunted Sall-Pmel pC-hTAK1a expression cassette into Pmel-cut pCEA3-hTAK1a. Dominant negative mutants of TAK1 were generated by QuikChange (Stratagene) mutagenesis using 40-bp complementary primers centered at the mutation site. Sequenced mutant fragments for each clone were inserted into the Kasw-MluI fragment to produce pC-hTAK1a-KA and pC-hTAK1a-KW. Similarly, the S192A fragment was replaced the RareII-MluI fragment to create pC-hTAK1a-SA. The double mutant pC-hTAK1a-KWSA was generated sequentially.

**Adenoviral Vector Construction**—The adenoviral vectors Ad-pCEA3, Ad-pCEA3pC, Ad-pC-hTAK1a, Ad-pCEA3-TAB-FL, and pCEA3-TAB-FL/pC-hTAK1a (Tandem) were constructed using components from Takara. Expression cassettes were excised with Sall and Pmel, blunted, and ligated to Swal-cut promoterless transfer cosmID pAcvX (55). Following λ packaging (Promega), recombinant cosmids were used to generate recombinant ΔE1 and ΔE3 adenoviruses by homologous recombination in 293 cells transfected with cosmID and restricted viral DNA-terminal protein complexes (58). Adenoviruses were isolated by plaque purification and two rounds of dilution to 0.5 multiplicity of infection using BM4000 dishes propagated in 10° C fetal calf serum or HIA-dialyzed fetal calf serum in DMEM (high glucose) for 10 days. Chondrocytes were plated in PBS and released by brief incubation with a film of 0.05% trypsin, 0.53 mM EDTA. Cells were treated with recombinant human TGF-β1 (R&D Systems) at 10 ng/ml, for 4 °C for 45 min, washed twice with 40% ethanol, digested with CNBr,
and mapped in two dimensions as previously described (61, 62).

Transfection and Luciferase Assays—Primary chondrocytes were released and plated beneath 5% HIA fetal calf serum/DMEM at 1.7 × 10^5 cells/well in 48-well plates. After 18–24 h, cells were washed with serum-free medium, fed with ITS+/DME, and transfected for 3 h with Lipofectamine Plus (Invitrogen). Each well contained 1 μl of Plus reagent, 0.5 μl of Lipofectamine, 25 ng of p800Luc or 12.5 ng of pNF-kB, and test plasmids, and the DNA content was adjusted to 200 ng/well with pUC18. Cells were washed with ITS+/DME and fed this medium until termination 48 h later. Substrate buffer (50 mM) was used for lysis, and 10 μl were transferred to 20 μl of 4 mM CaCl_2, 4 mM MgCl_2, 50 mM Heps, pH 7.5, followed by the addition of 10 μl of 2× Luclite substrate (Packard). Replicate wells (n = 4) were analyzed for luciferase activity using a scintillation counter and single photon counting (Beckman).

RNA Isolation and Ribonuclease Protection Assays (RPAs)—Total RNA was isolated using Trizol LS (Invitrogen) and stored in 100% formamide at −80 °C. Rabbit-specific probes were generated from chondrocyte or fibroblast RNA using Thermoscript reverse transcriptase (Invitrogen) at 55 °C. The resulting cDNA and plasmids were used as targets in PCRs using the following primers: Col2a1 (forward, 5'-GCACCATTGACATTGAGGGA-3', reverse, 5'-ATGGTTTAAAATACAGAG-3'), Aggre can (forward, 5'CTAGACCTCCTCTACTGC-3', reverse, 5'-TCTCTAGGCTCCCTAGTGC-3'), Sox9 (forward, 5'-CCGCACCATCAGACAGGAC-3', reverse, 5'-ATCTGGAATCCCAAGCCGCAG-3'), L32 (forward, 5'-GTAAGTGAATTCGAGTCATG-3', reverse, 5'-GTCATGACCTGCTGGTGTTC-3'), hTAK1a (forward, 5'-GTTGCAAGATTTGACCAAGG-3', reverse, 5'-GTTGCAAGATTGACCCAGG-3'), CTGF (forward, 5'-CTGGTACACGGTACGG-3', reverse, 5'-GTTCACTAGTCCAGCTTTCCC-3'), hTAK1a (forward, 5'-GTTGCAAGATTTGACCAAGG-3', reverse, 5'-GTTGCAAGATTGACCCAGG-3'), and ITAB1 (forward, 5'-CAGATGCTTGCTGTGACGAC-3', reverse, 5'-GTTCACTAGTCCAGCTTTCCC-3'). The products of PCR were sequenced and screened for homology to verify their expected identity. PCR products were ligated to T7 polymerase adapters and amplified with adapter primers to generate templates for synthesis of 32P-labeled antisense riboprobes (Lig'nScribe; Ambion). RPAs used RPA II kits (Ambion). Antisense riboprobes were labeled with T3 or T7 polymerase adapters and amplified with adapter primers to generate templates for synthesis of 32P-labeled antisense riboprobes using SuperSignal West Dura substrate (Pierce). Molecular weight markers for chemiluminescent detection (PA-Markers) were obtained from Chemicon.

RESULTS

TAK1a Stimulates PAI-1 Promoter Activity in Chondrocytes—Transient transfection of secondary cultures of differentiated chondrocytes with hTAK1a in combination with the TGF-β-responsive p800-PAI-1-Luc reporter stimulated this promoter as well as 5 ng/ml TGF-β (Fig. 1). This 10-fold stimulation was only reached at the highest concentrations of TAK1a, whereas a similar maximum stimulation was reached by constitutively active hTAK1a-ΔN at much lower concentrations. In both cases, the response was saturated, and only cotransfection with TAB1 exceeded this plateau. At TAK1a concentrations below 2 ng/well, PAI-1-Luc activity was similar to TAK1a alone or in combination with TAB1, and tandem expression of both TAK1a and TAB1. Consequently, TAK1a activated by deletion (ΔN) or TAB1 overexpression appear to exhibit similar specific activities. In contrast, the slower increase in PAI-1-Luc activity produced by TAK1a alone suggests that higher concentrations were required for TAK1a to activate itself or be activated by another kinase or molecular interaction. Fig. 1 defines an optimal ratio of expression for exogenous TAK1a and TAB1 using these constructs when TAK1a was increased against a constant TAB1 concentration. However, when an optimal concentration for TAK1a was evaluated with increasing TAB1, luciferase activity declined dramatically (data not shown). Both results demonstrate the importance of the TAK1a/TAB1 expression ratio for biological activity, which was not optimal in the tandem vector governed by the relative strength of the small dual promoters. A further 1.5–2-fold increase in each of the optimal activities for plasmids containing TAK1a or
TAK1-mediated Type II Collagen Synthesis

Adenoviral overexpression of TAK stimulates type II collagen synthesis without phenotypic change and mimics TGF-β1 on type II collagen synthesis at the final protein level. TAK1a and TAB1 was observed if the transfected cultures were infected with adenoviral expression vectors at an MOI of 20 for 24h and then cultured for 48 h in ITS-/DMEM in the presence or absence of 5 ng/ml TGF-β1. Cells were labeled with [3H]proline in the last 24 h, treated at low temperature with pepsin, and analyzed by SDS-PAGE. Digital image analysis was performed on the α(I)-chain (type II collagen) bands in fluorographs of triplicate wells. Error bars, S.E., n = 3. Ad-Tandem, Ad-pCEA3-hTAB1(pC-hTAK1a); Ad-pCEA3(pC), the corresponding promoter-only adenoviral vector. B, radiolabeled, pepsin-treated collagen was cleaved with cyanogen bromide and mapped in two dimensions. Peptides derived from type II collagen (representing the differentiated phenotype) are marked by parentheses and trace amounts of peptides from type I collagen are marked by the arrows. Samples were derived from those in A. ΔN, Ad-hTAK1a-ΔN.

TAK1a and TAB1 was observed if the transfected cultures were simultaneously treated with 5 ng/ml TGF-β1 (data not shown) and may parallel activation of Smad2/3 or other postreceptor signals.

Adenoviral Overexpression of TAK Stimulates Type II Collagen Synthesis—We have constructed adenoviral expression vectors for TAK1a and TAB1, because the transfection efficiency of chondrocytes is low. Infection with adenoviral vectors produced transduction efficiencies of >90%, based on β-galactosidase expression, and permitted the use of conventional [3H]proline labeling and SDS-PAGE to monitor the effects of TAK1a on type II collagen synthesis at the final protein level rather than relying on mRNA levels or reporter constructs.

In secondary chondrocytes plated at confluent density, TGF-β1 stimulated type II collagen synthesis 6-fold, whereas infection with TAK1a and TAK1a-ΔN adenoviral vectors produced 4–5-fold stimulation (Fig. 2A). Thus, TAK1a mimicked TGF-β1 in this response. Infection with the tandem vector, expressing both TAK1a and TAB1, produced the same response as TAK1a alone. This suggests that the interaction with TAB1 is not necessary under conditions of overexpression but may still be required for endogenous TAK1 activation. Simultaneous treatment with TAK1a and TGF-β1 produced little change relative to TAK1a alone, suggesting minimal involvement by parallel signaling pathways downstream of TβRI. Collagen produced by TAK1a-ΔN treatment was predominantly α(I), as judged by two-dimensional CNBr peptide mapping (Fig. 2B). This indicates that the response to TAK1a overexpression was not due to a change to type I collagen (α(I)-chain) synthesis but rather reflects enhanced production of collagens of the differentiated phenotype, predominantly type II collagen.

Autocrine TGF-β and Smad3 Are Not Required for TAK1a-dependent Stimulation—TGF-β1 has been shown to activate its own promoter and establish a positive autocrine feedback loop (63). To test whether TAK1a mimicked this role of TGF-β1 and caused stimulation of type II collagen synthesis by inducing TGF-β production, chondrocytes were cultured in the presence and absence of a mixture of blocking antibodies directed against all three isoforms of TGF-β. Pretreatment of TGF-β1-containing medium with antibodies and their continued presence during the following 48-h culture period with TGF-β1 not only blocked the 4-fold stimulation of α(I)II) synthesis caused by TGF-β1 but reduced synthesis to below basal levels (Fig. 3A). Thus, basal synthesis was partially due to an autocrine response to endogenous TGF-β production, and the antibody mix effectively blocked signaling by both endogenous and exogenous TGF-β1. In contrast, the 3-fold stimulation produced by Ad-pC-hTAK1a or ΔN was not blocked by the antibody mix but rather stimulated by it. TAK1a-dependent stimulation, therefore, did not require endogenous TGF-β or its postreceptor signals, including Smad activation. The cause of the antibody-dependent enhancement is not known, but it may be due to release from effects of inhibitory Smads.

The requirement for Smad signaling in TAK1a or TGF-β1 stimulation of type II collagen synthesis was addressed directly using an adenoviral vector expressing Smad3 (Fig. 3B). TGF-β1 or TAK1a alone (5–20 MOI) produced a 3-fold stimulation of collagen synthesis, whereas Smad3 had no effect at its maximally effective dose of 20 MOI (Fig. 3B, lane 7). Signaling by Smad3 did not cooperate with TAK1a, since coinfection did not elevate the response above that of TAK1a alone but actually completely blocked the effects of TAK1a (lanes 8 and 9) when TAK1a expression was still within the effective range (compare TAK1 expression in lanes 4 and 5 with lanes 8 and 9). Similarly, overexpressed Smad3 also blocked stimulation by TGF-β1, whereas Smad1 did not (lanes 10 and 11). This inhibitory effect of Smad3 was still present when both TAK1a and TGF-β1 were part of the treatment (lanes 12 and 13).

The expected functionality of Ad-Smad3 in this experiment was verified by monitoring expression of Smad3-responsive endogenous targets, PAI-1 and CTGF, by Western bloting (Fig. 3B). TGF-β1 enhanced PAI-1 expression 9-fold, whereas TAK1a alone and Smad3 alone exceeded this level, producing 110- and 83-fold stimulation, respectively. In addition, Smad3 cooperated with TAK1a, generating a 350-fold increase, with TGF-β1 (340-fold increase), and with both to generate a 2600-fold stimulation. CTGF expression was increased 25-fold by TGF-β1 and 13- and 17-fold by TAK1a and Smad3, respectively. The combined response was additive and was only slightly increased by costimulation with TGF-β1. Thus, substantial responses of these marker genes to Smad3 alone or in combination with TAK1a or TGF-β1 distinguish PAI-1 and
CTGF signaling mechanisms from those involved with stimulating type II collagen synthesis.

**TAK1α Mediates the Stimulation of Type II Collagen Synthesis by TGF-β and BMP2**—The involvement of TAK1 in TGF-β-dependent stimulation of α1(II) synthesis was addressed directly by treating chondrocytes with adenoviral vectors expressing dominant negative TAK1α-K63W or K63A before exposure to TGF-β1 (Fig. 4A). Both constructs provided dose-dependent inhibition of α1(II) synthesis induced by TGF-β1; TAK1α-K63W provided maximal inhibition, reducing synthesis to basal levels. In the absence of added TGF-β, both TAK1α-K63W and K63A also suppressed basal synthesis, again supporting participation of endogenous TGF-β in basal synthesis. Because BMP also has been shown to activate TAK1 (28), we evaluated its capacity to stimulate type II collagen synthesis by a TAK1-dependent mechanism. BMP2 produced a 5-fold stimulation of synthesis that was reduced by 60% in the presence of TAK1α-K63A. Thus, two members of the TGF-β superfamily, TGF-β1 and BMP2, share the capacity for TAK1-mediated stimulation of type II collagen synthesis.

**Kinase Activity of Overexpressed TAK1**—The kinase activity of endogenous and recombinant TAK1 was determined by immune complex kinase assays using a TAK1α C-terminal anti-peptide antibody and MKK6 as substrate. Both adenovirally expressed TAK1α and TAK1α/TAB1, expressed from the tandem vector, produced TAK1α autophosphorylation and MKK6 phosphorylation, whereas the double mutant, TAK1α-KWSA, produced neither (Fig. 4B, upper panels). When immunoprecipitates were exposed to kinase conditions in the presence of only unlabelled ATP, washed, and then submitted to the radiolabeled kinase assay (Fig. 4B, lower panels), TAK1α expressed alone lost the capacity to phosphorylate MKK6 as well as itself. In contrast, coexpression of TAK1α and TAB1 maintained a strong capacity to phosphorylate MKK6, whereas autophosphorylation was lost due to prior incorporation of unlabeled ATP. These results and those from Fig. 4C demonstrate that only 10% of TAK1α overexpressed alone is activated to participate in downstream signaling in the absence of exogenous TAB1 and (b) such activation apparently does not utilize TAB1 or does not utilize TAB1 through the usual mechanism that provides stabilization of kinase activity in vitro.

**Activation of Endogenous TAK1 and TAB1**—The capacity of dominant-negative TAK1α to block collagen synthesis by TGF-β and BMP2 strongly supports mediation by TAK1. This role for TAK1 also requires activation of endogenous TAK1 by these ligands. Immune complex kinase assays based on the TAK-ct antibody did not detect activation of TAK1 kinase activity or autophosphorylation (Fig. 4B) of endogenous TAK1 from control cells and those treated for 10 min with TGF-β1 and IL-1β. In subsequent studies, immunoprecipitation with TAB-m or antibodies raised to the whole TAK1 molecule also failed to detect TAK1 kinase activity. However, only small amounts of endogenous TAK1 were present in immunoprecipitates when compared by Western blotting to direct cell lysates (data not shown). Thus, the absence of TAK1 kinase activity may have been due to the lack of immunoprecipitation of activated TAK caused by interaction with scaffold proteins and/or signaling partners. Activation was therefore addressed directly by Western blotting of whole cell lysates (Fig. 4C). Treatment with 100 ng/ml BMP2 for 10 min increased the intensity of endogenous TAK1 and generated two new forms of TAK1 with retarded mobility characteristic of phosphorylation-dependent activation (30). TAB1 also exhibited a BMP-dependent phosphorylation pattern. TGF-β treatment (20 ng/ml) did not induce phosphorylation after 10 min, but after 30 min, the slower migrating phosphorylated form of TAK1 was readily detected. The same amount of this phosphorylated form of TAK1 was also present when TAK1α or TAK1α and TAB1 were overexpressed (compare Fig. 4C, lanes 4–6), indicating that generation of this form is controlled by a limiting endogenous cofactor(s). Following 30 min of TGF-β1 stimulation, the endogenous unstimulated form of TAB1 was completely replaced by two slower migrating phosphorylated forms. Thus, a TGF-β-depend-
ent endogenous TAB1-activating kinase, possibly TAK1, has access to all cellular TAB1, regardless of the possible participation of TAB1 in different ligand-specified complexes.

Adenoviral transduction with TAK1a and TAB1 together for 48 h completely modified all overexpressed TAK1a but only generated phosphorylated TAK1a with intermediate mobility (with the exception above). Thus, TAB1 binding to TAK1a was not sufficient to generate the most highly phosphorylated form of TAK1a in this cellular system. Similarly, only the intermediate phosphorylated form of TAB1 was produced by tandem expression. Endogenous TAB1 was completely resistant to activation by TAK1a overexpressed alone (Fig. 4C, lane 5), despite the substantial kinase activity of TAK1a (Fig. 4B). Such resistance is consistent with the activity of a proposed inhibitor of activation of endogenous TAK1/TAB1 complexes (30).

**FIG. 4.** TAK1a mediates the stimulation of type II collagen synthesis by TGF-β and BMP2 and exhibits TAB1-independent kinase activity. A, chondrocytes were infected 24 h after plating with dominant-negative TAK1 adenoviral vectors, Ad-pC-hTAK1a-K63A, and Ad-pC-hTAK1a-K63W. Seventy-two hours after initiating infection, cells were treated for 24 h with 5 ng/ml TGF-β1 (T) or 100 ng/ml BMP2 (B) in the presence of [3H]proline. Samples from triplicate wells were pooled before SDS-PAGE. B, for immune complex kinase assays, chondrocytes were infected with Ad-pC-hTAK1a (TK), kinase-negative (KN) Ad-pC-hTAK1a-KWSA, and the tandem vector (TK, TB) pCEA3-hTAB1(pC-hTAK1a). Forty-eight hours after beginning infection, cells were treated with TGF-β1 (40 ng/ml; T) or IL-1β (40 ng/ml; IL) for 10 min prior to lysis, immunoprecipitation with TAK-ct antibody, and TAK1a immune complex kinase assay using bacterially expressed GST-MKK6 as substrate. Lysates were equally divided prior to immunoprecipitation to permit direct kinase assays in the presence of [γ-32P]ATP (upper panels) or kinase assay after pretreatment with cold ATP (lower panels). The right panels are 7-fold shorter exposures of the last two lanes. C, Western blots (IB) demonstrating activation of endogenous TAK1 and TAB1 and their overexpressed counterparts. Chondrocytes were treated for 10 or 30 min with TGF-β1 (5 ng/ml) or BMP2 (100 ng/ml) and lysed with SDS sample buffer at the same time as cells exposed to 48 h of adenoviral expression of TAK1a or coexpressed TAK1a and TAB1 (tandem construct). TAK-ct and TAB-m primary antibodies were used for detection. Long dashes, unmodified TAK1 or TAB1 bands; short dashes, activated/phosphorylated bands. Right panel, shorter exposures of lanes 5 and 6.

**FIG. 5.** RNA levels do not reflect the stimulation of type II collagen synthesis by TGF-β and decline in response to adenoviral expression of TAK1a. A, chondrocytes were treated with TGF-β1 (5 ng/ml) or infected at different MOI with adenoviral vectors expressing TAK1a constructs using the protocol in Fig. 2A. At termination of labeling for collagen synthesis, RNA was isolated from parallel cultures and submitted to RPA analysis with correction for L32 expression. Triplicate cultures were separately analyzed for collagen synthesis to provide the quantitative data, and a physical pool was used for the fluorograph presented. The arrows indicate a change in lane labels for RPA analysis. Kinase-negative TAK1 (KN-TK), Ad-pC-hTAK1a-KWSA. B, the change in efficiency of type II collagen generation relative to the amount of specific mRNA was calculated from the data in A by dividing the -fold stimulation for type II collagen by the relative mRNA content. The black bars indicate treatment for 48 h with 5 ng/ml TGF-β1.
pressed together from separate plasmids or from the tandem plasmid increased activation to 20-fold (Fig. 6). In contrast, TGF-β1 partially suppressed basal and all TAK1α-dependent increases in activation of NF-κB. Thus, exogenous TAK1α, but not TAK1α activated by TGF-β1, activated NF-κB and mimicked the suppressive effects of IL-1β on cartilage-specific mRNAs. Importantly, both sources of TAK1 stimulate type II collagen synthesis at the protein level.

**DISCUSSION**

Regulation of type II collagen synthesis is important for normal skeletogenesis as well as maintenance of the structural and functional properties of growth plate and articular cartilage. Here we describe a TAK1-dependent mechanism that is responsible for enhanced production of type II collagen in adult, differentiated chondrocytes and does not require increased transcription of the Col2a1 gene. TAK1 mimics both TGF-β and BMP2 in this regard, and its effects are not dependent on Smad3 signaling. Enhanced production thus occurs in an environment of established type II collagen synthesis and should be distinguished from Col2a1 expression induced during development or activation of chondrogenic precursor cells. The latter are transcriptional processes mediated by Sox9 and relevant Smads. They are also likely to involve TAK1 signaling, because Smad transactivation utilizes DNA-dependent and protein-protein interactions with other transcription factors (1, 26, 27) that can be activated by kinases downstream of TAK1.

We have shown that TAK1 is predominately responsible for stimulation of type II collagen synthesis in differentiated chondrocytes in response to TGF-β and BMP2 by (a) blocking their effects with dominant negative TAK1-K63W and TAK1-K63A, (b) demonstrating their activation of endogenous TAK1 and TAB1, and (c) mimicking their effects using adenoviral overexpression of wild type TAK1α. In the latter case, we have shown that overexpressed TAK1α can signal and yield a response on its own without simultaneous overexpression of its activating protein TAB1. Similarly, TAK1 can act alone at high concentrations to stimulate the PAI-1 promoter. This agrees with the report of Li et al. (65) that overexpressed TAK1 alone can activate MKK4 and JNK in HEK293 cells but contrasts with the original and common observation that overexpressed TAB1 is required for TAK1 activation and signaling (29, 30, 36). Such apparent TAB1 independence may result from TAK1 saturation of endogenous regulatory proteins and its subsequent self-association and autoactivation by intermolecular phosphorylation (33). Similarly, overexpression of TAK1 may lead to its exposure to kinases that usually have no access to it. Alternatively, ceramide (36) or Sef (similar expression to fibroblast growth factor genes) (66) can activate TAK1 and induce JNK activation; however, the involvement of phosphorylation or association with TAB1 as activating mechanisms in these situations remains unclear.

The nature of the TAK1 activated state appears different among overexpressed TAK1α alone, TAK1α overexpressed with TAB1, and TGF-β-activated endogenous TAK1. This was most clearly demonstrated by the complete loss of kinase activity when TAK1α, overexpressed alone, was preincubated under kinase conditions with unlabeled ATP before kinase assay. Under the same conditions, TAK1α overexpressed with TAB1 retained its kinase activity (Fig. 4B). Thus, even TAK1α that initially exhibits autophosphorylation and transphosphorylation activity in vitro and biological activity in vivo (TAK1α alone) is not stable in vitro without interaction with TAB1. The lack of stability also suggests that in vivo activation of exogenous TAK1α alone is not mediated by interaction with endogenous TAB1, a conclusion consistent with the absence of endogenous TAB1 activation detected by Western blotting (Fig. 6).

**FIG. 6. TAK1α overexpression activates NF-κB, and TGF-β suppresses this activation.** The NF-κB-responsive reporter, pNF-κB-Luc, was cotransfected with promoterless pUC18 (NP), promoter-only plasmids (pC and pCEA3(pC)), or TAK1α plasmids, alone or in combination with TAB1. Both separate and tandem expression vectors were used. Luciferase assays were performed 48 h after transfaction and TGF-β1 addition (black bars). Error bars, S.E.; n = 4.
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4C, lane 5). TAK1a overexpressed alone also must be activated in vivo in such a way that autophosphorylation sites remain open for subsequent incorporation of label during the initial kinase assay. The presence of two functional sites (30, 33) meets this requirement. In addition, an activated conformation or associated factor must be lost during immunoprecipitation or assay so that TAK1 is incapable of reactivating itself by autophosphorylation. Denaturation in the absence of TAB1 during the kinase assay or inactivation by an associated phosphatase could yield this result.

Two phosphatases, PP2Cβ-1 (67) and PP2Ax (65), have been shown to dephosphorylate and inactivate active TAK1 or TAK1-TAB1 complexes both in vitro and in vivo. PP2Ax binds to both inactive and active TAK1 (65), and thus a ternary complex is expected both before and after ligand-dependent TAK1 activation. The results from the immune complex kinase assays (Fig. 4B) are most consistent with the absence of phosphatase or its inhibition in the immunoprecipitates derived from TAK1-TAB1-infected cells. Continuous phosphatase activity during pretreatment with unlabeled ATP should have reduced substrate phosphorylation in the subsequent kinase assay. In addition, phosphatase activity balanced by TAB1-dependent reactivation of TAK1a should have allowed TAK1a autophosphorylation in the final kinase assay. Neither of these results was observed. In contrast, phosphatase activity could explain the loss of both autophosphorylation by TAK1a alone following incubation with cold ATP, because TAK1a reactivation in this TAB1-deficient environment is unlikely.

Activation of TAK1 overexpressed in the presence of TAB1 is mediated by intramolecular autophosphorylation of Ser192 (30) and/or intermolecular autophosphorylation of Thr1197 (33) in the activation loop. Both sites participate in the capacity of TAK1 for transphosphorylation and activation of the NF-κB pathway, and both lead to phosphorylation of associated TAB1 and the appearance of multiple phosphorylated forms of both TAK1 and TAB1 that exhibit retarded mobility on Western blots (30, 33). In agreement with these data, we showed that stimulation of chondrocytes by TGF-β or BMP generates two retarded, presumably phosphorylated, forms of both endogenous TAK1 and TAB1 that exhibit retarded mobility on Western blots (30, 33). However, when overexpressed with TAB1, TAK1a migrates almost entirely as the intermediate form, which is greatly reduced or absent when TAK1a is overexpressed alone. This pattern of abundance of the intermediate form matches that of TAK1a kinase activity (Fig. 4B) and suggests that this is a catalytically active form of TAK1a. However, the biological activity of TAK1a in stimulating type II collagen synthesis does not require the high level of kinase activity produced by tandem expression. TAK1a alone and TGF-β1 produce the same level of stimulation with minimal presence of the intermediate form. Alternatively, the slowest migrating, most phosphorylated form of TAK1 may be responsible for its effects on collagen synthesis. TGF-β1, TAK1a overexpressed alone, and tandem expression of TAK1a and TAB1 (Fig. 4C, lanes 4–6) generate very similar amounts of this form, suggesting that its formation is controlled by limited quantities of other endogenous interaction partners. Importantly, it can be induced by overexpressed TAK1a without TGF-β1 treatment and without the apparent involvement of TAB1, since no slowly migrating, activated forms of endogenous TAB1 were detected.

Conversely, TGF-β treatment phosphorylated all cellular TAB1 to its intermediate or slowest form at the same time that only a fraction of endogenous TAK1 was activated. It is uncertain whether this result is due to endogenous TAB1 content that is insufficient to bind all TAK1 or to the existence of distinct TAK1-TAB1 complexes that permit TAB1 phosphorylation without prior or subsequent phosphorylation of TAK1. The latter possibility gains support from the detection of a kinase in TAK1 immune complexes that phosphorylates TAB1 without IL-β stimulation (Fig. 7 in Ref. 32). Regardless of the mechanism, complete phosphorylation of TAB1 by a single cytokine (i.e. TGF-β1) suggests that selective signaling by TAK1 in response to this and other cytokines requires the function of additional components of TAK1 signaling complexes. Models of endogenous TAK1 activation/signaling based on the IL-1β pathway (including tumor necrosis factor receptor-associated factor 6, TAB1, -2, and -3, and polyubiquitination) (31, 32, 68, 69), the Wnt pathway (70, 71), and the Sef pathway (66) are complex but likely to be conceptually similar to TAK1 activation in response to TGF-β and BMP2.

In addition to the role of TAB1 in activating and stabilizing TAK1 kinase activity, TAB1 appears to exhibit a targeting function. This was most clearly demonstrated by the fact that after stimulation at a low dose, increased levels of constitutively active TAK1a-ΔN did not produce increased expression of the PAI1-luciferase reporter, whereas coexpression with TAB1 established a new higher level of expression shared by coexpression of wild type TAK1a and TAB1 (Fig. 1). Thus, more abundant active TAK1 could not substitute for the association of TAK1a with TAB1. This may be due to the potential of TAB1 to increase substrate binding and turnover or to a capacity to recruit and activate binding partners that subsequently facilitate downstream signaling, as seen with TAB2 and TAB3 in IL-1β signaling (68). Similarly, TAB1 enhanced TAK1a activation of NF-κB signaling but failed to increase TAK1 stimulation of type II collagen synthesis, perhaps characterizing two different types of TAK1 signaling.

Smad2, -3, and -4 are the principal transcriptional mediators of TGF-β signaling (25), whereas Smad1, -4, -5, and -8 mediate the effects of BMP (1). We have used overexpressed Smad3 to demonstrate the absence of Smad3 involvement in TGF-β1- and TAK1a-mediated stimulation of type II collagen synthesis (Fig. 3B). Overexpressed Smad3 was unable to stimulate synthesis on its own or enhance stimulation by TAK1a or TGF-β1. In addition, no Smad2 or Smad3 signals are expected in the presence of effective TGF-β1-neutralizing antibodies (Fig. 3A), a situation where stimulation by TAK1a remained. This is consistent with the lack of Smad3 binding to the proximal promoter of Col2a1 (49). Independence from receptor Smads is also supported by (a) the similar stimulation of type II collagen synthesis caused by both TGF-β1 and BMP2, which signal through completely different sets of receptor Smads, (b) activation of TAK1a by both TGF-β1 and BMP2 (28) (Fig. 4C), and (c) the parallel, nearly complete inhibition of collagen synthesis by dominant negative TAK1a (Fig. 4A). Despite this evidence, the involvement of receptor Smads other than Smad3 cannot be ruled out, since overexpressed Smad3 blocked collagen synthesis induced by both TAK1a and TGF-β1 (Fig. 4B). The simplest interpretation of this result is that Smad3 competitively displaced another necessary Smad from its binding site in the L45 loop of TβRI or altered the function of the Smad adaptor, SARA (72, 73). Such displacement by Smad3 has been used with dominant negative Smad2/3 to effectively distinguish Smad3 and Smad2 effects (74).

Horton et al. (51) and Chadjichristos et al. (49) have demonstrated TGF-β-dependent reduction of Col2a1 transcription mediated by the enhancer (51) or Sp1/Sp3 binding elements in the 63-bp proximal promoter (49). In contrast, the results presented here from a variety of protocols demonstrate substantial TGF-β-induced increases in type II collagen synthesis without
alteration of Col2a1 mRNA and are in agreement with the stimulation of type II collagen synthesis reported in rabbit chondrocytes following adenosine expression of TGF-β or treatment with recombinant protein (47). These differences may result from changes in chondrocyte phenotype during culture (75). During this process, cell-shape and density-dependent signaling and responsiveness may change (49, 60, 62, 76) with or without overt modulation of the collagen phenotype. The present studies also utilized only the endogenous promoter in its chromosomal genmic context.

TAK1 stimulation of type II collagen synthesis and its concurrent sharp reduction in Col2a1 mRNA are entirely consistent with the capacity of TAK1 to activate NF-κB following TAK1 overexpression or IL-1/β tumor necrosis factor-α treatment (41, 42) and the capacity of NF-κB to inhibit Col2a1 transcription by reducing Sox9 expression and its binding to the Col2a1 first intron enhancer (64). We have verified TAK1-dependent activation of NF-κB in this chondrocyte system and demonstrated that, although TGF-β1 activates TAK1, TGF-β1 inhibits NF-κB activation due to overexpressed TAK1. This may represent a mechanism for the antagonism of IL-1β by TGF-β. The down-regulation of Col2a1 mRNA by TAK1 also demonstrates that TAK1 signaling is sufficient for this response and does not require support from upstream participants in the IL-1β pathway. Tan et al. (77) have reported a rapid, IL-1β-dependent, EGR-1-mediated down-regulation of constitutive transcription from the Col2a1 proximal promoter. This response utilized both EGR-1 activation and transcription, did not alter Sox9 expression, and did not require the intron enhancer. Since Murakami et al. (64) reported no IL-1β effect using a similar 89-bp proximal promoter, these reports are difficult to reconcile. However, both the NF-κB and EGR-1 effects could be mediated by TAK1, since (a) TAK1 is essential for IL-1β-dependent NF-κB signaling (42), (b) p38 MAPK has been implicated in IL-1β-dependent suppression of Col2a1 expression (78), (c) EGR-1 is activated by p38 (78), and (d) p38 is activated indirectly by TAK1. Despite this potential for shared mediation by TAK1, the present data most closely match the NF-κB/Sox9 pathway, because TAK1 decreased both Sox9 and aggrecan mRNA in parallel with Col2a1 mRNA, and aggrecan and Col2a1 expression share Sox9 dependence during development (43).

Enhanced efficiency of the translational apparatus through increased initiation, elongation, or message recycling provides a rapid mechanism for regulating protein expression without changes in transcription (80). This is a possible explanation for the TGF-β1-dependent increase in type II collagen synthesis that occurs without increased Col2a1 mRNA. That such increased synthesis occurs following TAK1 overexpression, in the face of decreased message levels, further reinforces the absence of a Col2a1 transcriptional mechanism. The increased efficiency of message utilization depicted in Fig. 6 also could be due to changes in the complex posttranslational processing and export of procollagen. The TAK1 effects reported here may result directly from phosphorylation of its substrates or those of its effector kinases or indirectly through transcriptional regulation of targets other then Col2a1 that influence translation or downstream processes. In either case, TAK1-dependent mechanisms may significantly contribute to the functions of TGF-β and BMP in chondrogenesis and maintenance of adult articular cartilage attributed to these factors by genetic studies using dominantly negative TgRIRI (19) and knock-out or conditional knock-out of essential expression of Bmpr1b (4) and Bmpr1a (17).

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