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Peroxisome Proliferator-Activated Receptor-γ-Independent Repression of Collagenase Gene Expression by 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic Acid and Prostaglandin 15-Deoxy-Δ(12,14) J_2: A Role for Smad Signaling

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ABSTRACT

Matrix metalloproteinases (MMPs) degrade extracellular matrix components, and overexpression of these enzymes contributes to tissue destruction in arthritis. Of particular importance are the collagenases, MMP-1 and MMP-13, which have high activity against the interstitial collagens in cartilage. In this study, we address the mechanisms of two inhibitors of collagenase gene expression, the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and 15-deoxy-Δ(12,14)-prostaglandin J_2 (15-dPGJ$_2$). Although both inhibitors are ligands for the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPAR-γ), a connection between PPAR-γ and collagenase gene expression has yet to be established. Here, we test the hypothesis that CDDO and 15-dPGJ$_2$ use PPAR-γ to repress MMP gene expression. Our findings with the PPAR-γ antagonist 2-[4-[2-[3-(2,4-difluorophenyl)-1-heptylureido]ethyl]rsqb]-phenylsulfanyl]-2-methylpropionic acid (GW9662) and mouse embryonic fibroblasts lacking PPAR-γ demonstrate that CDDO and 15-dPGJ$_2$ use PPAR-γ-independent mechanisms to inhibit collagenase gene expression. To address a potential PPAR-γ-independent mechanism leading to the repression of MMPs by CDDO, we tested the effect of CDDO on the transforming growth factor-β (TGF-β) signaling pathway. We found that CDDO requires Smads (transcription factors activated by TGF-β) for the repression of MMP-1. Specifically, MMP-1 is inhibited neither by CDDO in the absence of TGF-β receptor-activated Smad3 nor when a negative regulator, Smad7, attenuates TGF-β signaling. We conclude that CDDO represses MMP gene expression through a novel PPAR-γ-independent mechanism that requires Smad signaling.

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a nuclear hormone receptor that regulates the expression of genes involved in insulin sensitivity and adipogenesis (Forman et al., 1996). Recent studies have proposed a distinct role for this nuclear receptor as an anti-inflammatory mediator activated by PPAR-γ ligands (Ricote et al., 1998; Kawahito et al., 2000; Chawla et al., 2001). Although it is clear these ligands act directly through PPAR-γ to regulate insulin sensitivity and adipogenesis, it is unclear whether a similar mechanism regulates genes involved in inflammation. PPAR-γ-independent mechanisms have been described for some of these ligands (Straus et al., 2000; Chawla et al., 2001; Ward et al., 2002), indicating diverse roles for PPAR-γ in the regulation of different target genes. Thus, it is essential to determine the requirement for PPAR-γ in the regulation of multiple target genes and to define receptor-independent mechanisms used by PPAR-γ ligands.

The pathologies of rheumatoid arthritis and osteoarthritis are associated with inflammation that is mediated partly by the action of inflammatory cytokines secreted from macrophages within affected joints (Mengshol et al., 2002). These cytokines [i.e., interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α)] induce the expression of matrix metalloproteinases (MMPs) in chondrocytes and synovial fibroblasts

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ABBREVIATIONS: PPAR, peroxisome proliferator- activated receptor; IL, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; 15-dPGJ$_2$, 15-deoxy-Δ(12,14) prostaglandin J_2; CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; TGF, transforming growth factor; DMEM, Dulbecco's modified Eagle's medium; MEF, mouse embryonic fibroblast; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPRE, PPAR-γ response element; RSV, Rous sarcoma virus; RLU, relative luciferase unit; AP, activator protein.

(Mengshol et al., 2002). In turn, the enzymatic action of these MMPs contributes to the destruction of extracellular matrix components in bone, cartilage, and tendons (Mengshol et al., 2002). Several studies have suggested that PPAR-γ ligands may modulate the process of joint destruction, either by reducing the expression of inflammatory cytokines (Ricote et al., 1998; Chawla et al., 2001) or MMPs (Shu et al., 2000; Fahmi et al., 2001, 2002; Sabatini et al., 2002). Furthermore, although not all cells express PPAR-γ, its expression has been noted in chondrocytes and synovial fibroblasts (Bordji et al., 2000; Fahmi et al., 2001, 2002; Sabatini et al., 2002), suggesting that PPAR-γ may have a function within joints.

An endogenous ligand for PPAR-γ, the cyclopentone prostaglandin 15-dPGJ$_2$ binds to PPAR-γ with a K_i of 1.2×10^{-9} M (Kliewer et al., 1995). This prostaglandin suppresses adjuvant-induced arthritis in rats (Kawahito et al., 2000), reduces the expression of inflammatory cytokines involved in arthritis (Ricote et al., 1998; Chawla et al., 2001), and inhibits the expression of MMP-1 and MMP-13 produced by synovial fibroblasts and chondrocytes (Fahmi et al., 2001, 2002). MMP-1 and MMP-13 are chief mediators of joint destruction because these enzymes are able to degrade type II collagen, the most abundant collagen within cartilage (Mengshol et al., 2002). However, some of the anti-inflammatory effects of 15-dPGJ$_2$ have been attributed to PPAR-γ-independent mechanisms (Straus et al., 2000; Chawla et al., 2001; Ward et al., 2002), and the role of PPAR-γ in the inhibition of MMP-1 and MMP-13 by 15-dPGJ$_2$ has not been elucidated.

We have described another compound with anti-inflammatory activity, the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) (Suh et al., 1999), which inhibits the synthesis of inflammatory mediators inducible nitric-oxide synthase and cyclooxygenase-2 (Suh et al., 1999) and blocks MMP-1 and MMP-13 gene expression in human SW-1353 chondrosarcoma cells (Mix et al., 2001) and primary osteoarthritic chondrocytes (Elliott et al., 2003). Although the chemical structures of CDDO and 15-dPGJ$_2$ are distinct, both compounds contain an α,β-unsaturated ketone group (Kliewer et al., 1995; Honda et al., 1998), potentially explaining some of their similar effects. Interestingly, CDDO binds to PPAR-γ with a K_i between 10^{-8} and 10^{-7} M and, like other PPAR-γ ligands, mediates adipogenic differentiation by activating PPAR-γ (Wang et al., 2000). CDDO inhibits cell proliferation and induces differentiation of leukemia cells independent of PPAR-γ (Place et al., 2003). Thus, like 15-dPGJ$_2$, CDDO exerts its effects through PPAR-γ-dependent and -independent mechanisms. Here too, however, the role of PPAR-γ in the inhibition of MMP-1 and MMP-13 expression by CDDO is unclear. Given the potential benefits of using PPAR-γ ligands as inhibitors of MMP gene expression, addressing the role of PPAR-γ in the repression of MMPs by CDDO and 15-dPGJ$_2$ is critical.

Many of the cellular activities of CDDO parallel the effects of the pleiotropic growth factor known as transforming growth factor-β (TGF-β). For example, TGF-β can regulate differentiation, suppress cell proliferation, and, importantly, modulate the expression of MMPs (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002). TGF-β mediates many of its effects via TGF-β receptor-activated transcription factors known as Smads (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002). A recent study indicates that synthetic triterpenoids CDDO and CDDO-imidazolide target the TGF-β pathway in epithelial and leukemia cells (Suh et al., 2003), thus potentially affecting numerous cellular processes. However, the connection between CDDO and TGF-β has not been elucidated in other cell types, and this elucidation is critical given the tissue-specific effects of TGF-β (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002). Furthermore, the impact of CDDO on TGF-β signaling has not been addressed as a mechanism contributing to MMP repression.

In this study, we test the hypothesis that PPAR-γ mediates the inhibition of MMP-1 and MMP-13 gene expression by 15-dPGJ$_2$ and CDDO. We use SW-1353 human chondrosarcoma cells stimulated with IL-1β as a model for osteoarthritic chondrocytes. Our findings indicate that CDDO and 15-dPGJ$_2$ use PPAR-γ-independent mechanisms to inhibit MMP-1 and MMP-13 gene expression. We further characterize the mechanism of CDDO by addressing its effect on the TGF-β signaling pathway, and we identify a novel mechanism for CDDO that uses Smad signaling for the repression of MMPs.

Materials and Methods

Cell Culture. Human SW-1353 chondrosarcoma cells (American Type Culture Collection, Manassas, VA) and wild-type and Smad3 −/− dermal fibroblasts (gift from Kathleen Flanders, National Cancer Institute) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and penicillin/streptomycin at 37°C, 5% CO_2. PPAR-γ −/− and +/− mouse embryonic fibroblasts (MEFs) (Rosen et al., 2002) were cultured in DMEM containing 10% fetal calf serum and penicillin/streptomycin at 37°C, and 10% CO_2. PPAR-γ status of the MEFs was confirmed by RT-PCR (data not shown). Cells were grown to confluence, washed with Hanks' balanced salt solution, and placed in serum-free DMEM containing 0.2% lactalbumin hydrolysate for experiments. CDDO was synthesized (Honda et al., 1998) and provided for use by Drs. Tadashi Honda and Gordon Gribble (Dartmouth College, Hanover, NH). As described previously (Mix et al., 2001), cells were treated with 300 nM CDDO for 24 h before the addition of 10 ng/ml IL-1β (Promega, Madison, WI) for an additional 24 h. 15-dPGJ$_2$ at 1 or 5 μM (Cayman Chemical, Ann Arbor, MI) was added to cells for 24 h before the addition of IL-1β. Where indicated, 10 μM GW9662 (gift from Timothy Willson, GlaxoSmithKline, Uxbridge, Middlesex, UK) was added to cells for 1 h before the CDDO or 15-dPGJ$_2$ pretreatment. Rosiglitazone (gift from Timothy Willson, GlaxoSmithKline) was used at a concentration of 1 μM. Where indicated, 10 ng/ml TGF-β (R&D Systems, Minneapolis, MN) was added to cells simultaneously with IL-1β.

Northern Blotting. RNA was isolated using the TRIzol protocol (Invitrogen, Carlsbad, CA). RNA (10 μg) was separated on a 1% agarose formaldehyde-formamide gel and transferred to gene-screen membranes (PerkinElmer Life and Analytical Sciences, Boston, MA). Blots were hybridized with MMP-1, MMP-13, and GAPDH cDNA labeled with [α-^{32}P]dCTP, followed by autoradiography. NIH Image software was used to quantitate band intensities. Data shown represent at least three experiments.

Western Blotting. Nuclear proteins were separated on a 10% SDS polyacrylamide gel and transferred to an Immobilon membrane. PPAR-γ protein was detected using a polyclonal antibody (Santa Cruz Biochemicals, Santa Cruz, CA), peroxidase-conjugated secondary antibody (Santa Cruz Biochemicals), and enhanced chemiluminescence (Amersham Biosciences Inc., Piscataway, NJ).

RT-PCR. RNA was treated with DNA-free reagents (Ambion, Austin, TX), and 2 μg were reverse-transcribed using an oligo(dT) primer and Moloney murine leukemia virus-RT (Invitrogen). End-point PCR (30 cycles for PPAR-γ, MMP-13 and 20 cycles for GAPDH, β-actin) was conducted on 200 ng of RT product using Platinum Taq

(Invitrogen). PCR products were separated on an acrylamide gel and stained with ethidium bromide. PPAR-γ primers, TCTCTCCGTAATGGAAGACC and GCATTATGAGACATCCCCAC (Fahmi et al., 2001); β-actin primers, GGGACCTGACCGACTACCTC and GGGCGATGATCTTGATCTTC (Mengshol et al., 2000); GAPDH primers, ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA (Fahmi et al., 2001); and murine MMP-13 primers, CCATTTTGATGATGATGAAAC and GTGCAGGCGCCAGAAGAATCT. Quantitative real-time PCR was conducted on reverse-transcribed RNA [MMPs (500 ng) and GAPDH (100 ng)] using SyberGreen Master Mix (Applied Biosystems, Foster City, CA). MMP-1 primers, AGCTAGCTCAGGATGACATTGATG and GCCGATGGGCTGGACAG (Wyatt et al., 2002); MMP-13 primers, TGGCATTGCTGACATCATGA and GCCAGAGGGCCCATCAA; GAPDH primers, CGACAGTCAGCCGCATCTT and CCCCATGGTGTCTGAGCG (Wyatt et al., 2002). Triplicate reactions were run in the Opticon DNA Engine (MJ Research, Watertown, MA), and transcript quantitation was based on standard curves with plasmids containing MMP and GAPDH cDNA. MMP expression was normalized to GAPDH, and data are presented as a percentage of the control IL-1 treatment. Data shown represent the mean of at least three experiments ± standard deviations.

Transfections. Cells were transiently transfected in 6-well plates using GenePORTER I or II reagents and protocols (Gene Therapy Systems Inc., San Diego, CA). Constructs used in transfections were PPAR-γ response element (PPRE)-tk-luciferase (Kliewer et al., 1992), 4.3-kb human MMP-1 promoter-luciferase (Rutter et al., 1997), pcDNA3-PPAR-γ, pcDNA3-PPAR-γ-DN (Barroso et al., 1999), $CAGA_{12}$-luc (Dennler et al.), and pcDNA3-Smad7 (Suh et al.). RSV-empty and cytomegalovirus-green fluorescent protein were used as empty vectors. Twenty-four hours after transfection, cells were washed with Hanks' balanced salt solution and 2 ml of lactalbumin hydrolysate medium containing the indicated treatment was added for 24 h. Cell lysates were harvested as described by Rutter et al. (1997), and luciferase activity was measured in a luminometer as relative luciferase units (RLUs). Different luminometers were used during the course of this study, accounting for variation in RLU values. Data shown represent the mean of at least three experiments, each conducted in triplicate ± standard deviations.

Statistics. Student's paired *t* test was used for statistical analysis of transfection experiments (http://www.physics.csbsju.edu/stats/). *P* values <0.05 were considered statistically significant).

Results

CDDO and 15-dPGJ$_2$ Inhibit MMP-1 and MMP-13 Gene Expression. SW-1353 human chondrosarcoma cells were used to study the inhibition of MMPs by CDDO and 15-dPGJ$_2$. Based on our previous studies, these cells are a suitable model of MMP regulation in chondrocytic cells (Mengshol et al., 2000; Mix et al., 2001). In the absence of IL-1β, SW-1353 cells express very low levels of MMP-1 or MMP-13; however, stimulation with IL-1β induces the transcription of both of these genes (Mengshol et al., 2000; Mix et al., 2001). Consistent with our previous findings (Mix et al., 2001), Northern blot analysis demonstrates that CDDO (300 nM) antagonizes IL-1β induction of MMP-1 by 40% and MMP-13 by 60% (Fig. 1A). Quantitative real-time RT-PCR indicates 100- and 50-fold inductions of MMP-1 and MMP-13 mRNA levels by IL-1β in 24 h, respectively (data not shown), and the prostaglandin 15-dPGJ$_2$ (5 μM) inhibits the induction of both genes by 50% (Fig. 1B). Importantly, in agreement with other studies (Fahmi et al., 2001, 2002), we observed no cellular toxicity at this concentration of 15-dPGJ$_2$ (data not shown). In contrast with the effects of CDDO and 15-dPGJ$_2$, another PPAR-γ ligand, rosiglitazone (Forman et al., 1995) (0.1–50 μM), does not effect the expression of MMP-1 or MMP-13 in SW-1353 cells (data not shown), indicating that not all PPAR-γ ligands repress these genes.

PPAR-γ Activation by CDDO and 15-dPGJ$_2$. Because CDDO and 15-dPGJ$_2$ have been described as ligands for PPAR-γ (Forman et al., 1995; Kliewer et al., 1995; Wang et al., 2000), we were interested in the potential connection between PPAR-γ and the inhibition of MMPs. To begin addressing this potential connection, we documented constitutive expression of PPAR-γ mRNA and protein and found that expression is not altered by CDDO or IL-1β (Fig. 2A).

The MMP-1 and MMP-13 promoters do not contain PPREs; therefore, to test the functional activity of endogenous PPAR-γ, SW-1353 cells were transfected with a PPRE from the rat acyl-CoA promoter fused to a luciferase reporter gene (Kliewer et al., 1992). Treatment with 300 nM CDDO, a concentration sufficient to inhibit MMP-1 and MMP-13 gene expression in these cells (Fig. 1A) (Mix et al., 2001), results in a 2-fold increase in activity from the PPRE reporter (Fig. 2B; $p = 0.02$). Importantly, this level of induction is consistent with a previous study

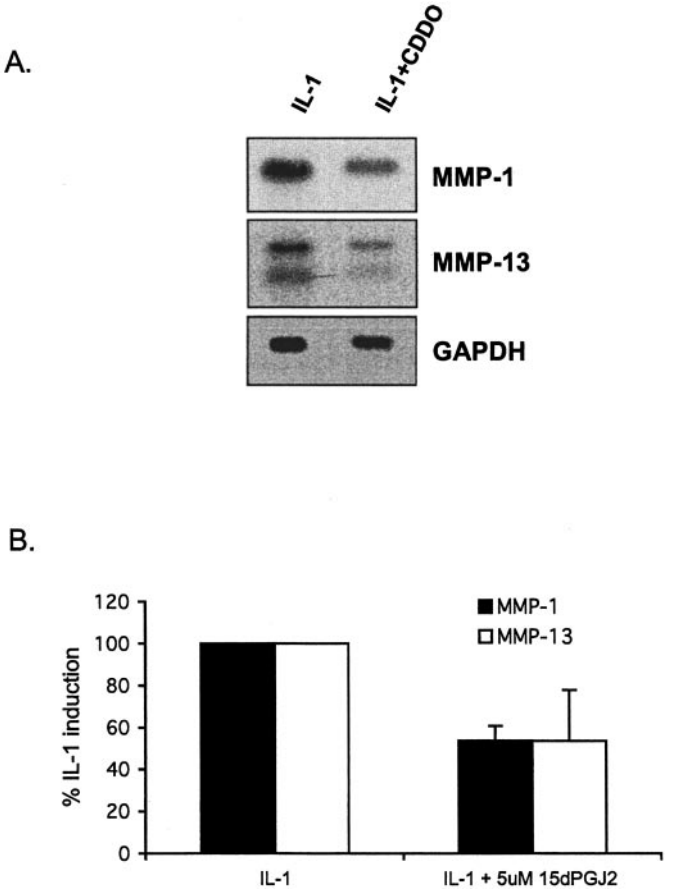

Fig. 1. IL-1 induction of MMP-1 and MMP-13 is reduced by CDDO and 15-dPGJ$_2$. A, SW-1353 cells were left untreated or pretreated for 24 h with 300 nM CDDO, followed by treatment with IL-1β (10 ng/ml) for an additional 24 h. MMP-1, MMP-13, and GAPDH mRNA levels were analyzed by Northern blotting. B, SW-1353 cells were left untreated or pretreated for 24 h with 5 μM 15-dPGJ$_2$, followed by treatment with IL-1β for an additional 24 h. MMP-1, MMP-13, and GAPDH levels were measured by quantitative real-time RT-PCR. MMP values were normalized to GAPDH and the data are presented as the average percentage of IL-1 induction in three independent experiments.

describing CDDO as a PPAR-γ ligand (Wang et al., 2000). Thus, the endogenous PPAR-γ protein in SW-1353 cells is transcriptionally active, and CDDO can function as a PPAR-γ agonist in these cells. Furthermore, this transcriptional response is dependent on endogenous PPAR-γ, because cotransfection of a PPAR-γ dominant-negative receptor that lacks the ability to transactivate (Barroso et al., 1999) abrogates this response to CDDO (Fig. 2B). Basal PPRE promoter activity is also reduced by this dominant-negative receptor, suggesting that an endogenous PPAR-γ ligand may regulate this promoter. In addition, consistent with the activation of PPAR-γ by 15-dPGJ$_2$ in primary human chondrocytes (Fahmi et al., 2001), we demonstrate that 15-dPGJ$_2$ transactivates the PPRE promoter in SW-1353 cells (Fig. 2C; $p = 0.02$). Thus, we have determined that CDDO and 15-dPGJ$_2$ function as PPAR-γ agonists in SW-1353 cells and, importantly, that PPAR-γ transactivation occurs at concentrations of these ligands that can also inhibit MMP gene expression.

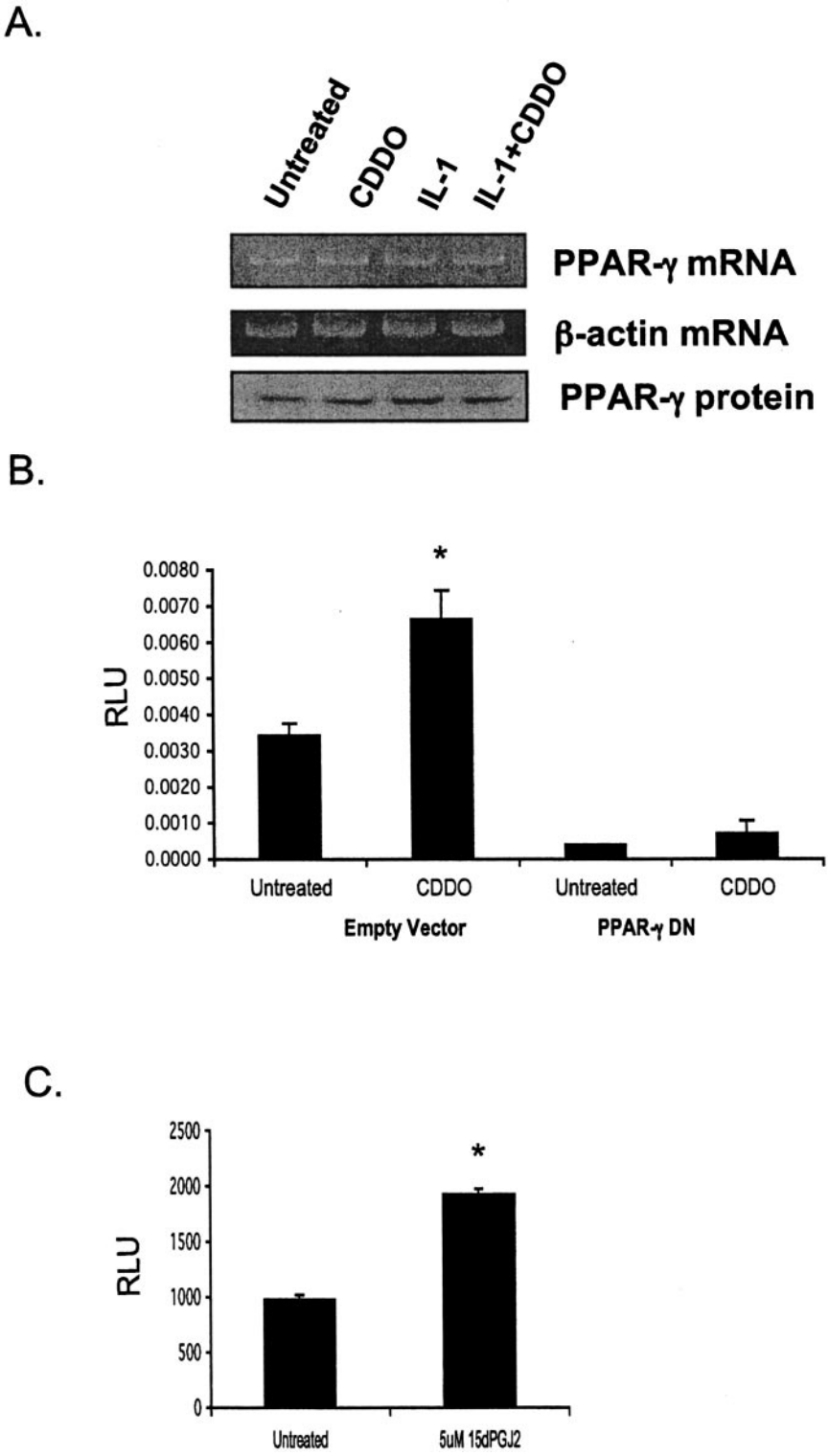

Fig. 2. PPAR-γ expression and activity in SW-1353 cells. A, SW-1353 cells were left untreated or treated with 300 nM CDDO for 24 h, followed by the addition of 10 ng/ml IL-1β for an additional 24 h, as indicated. PPAR-γ and β-actin mRNA were amplified by RT-PCR, and products were visualized on an acrylamide gel stained with ethidium bromide. PPAR-γ protein was measured by Western blotting. B, SW-1353 cells were cotransfected in triplicate with 1 μg of PPRE-tk-luciferase reporter construct and either 1 μg of RSV-empty vector or 1 μg of pcDNA3-PPAR-γ dominant- negative (DN) expression construct, as indicated. Twenty-four hours after transfection, cells were washed and transferred to serum-free medium alone or medium containing CDDO (300 nM). Luciferase activity was recorded as RLUs after 24 h of treatment. Different luminometers were used in B and C. Empty vector, untreated versus CDDO; ⋆, $p = 0.02$. C, SW-1353 cells were cotransfected with 1 μg of PPRE-tk-luc and 1 μg of RSV-PPAR-γ, followed by treatment with 15-dPGJ$_2$ (5 μM) in serum-free medium for 24 h; ⋆, $p = 0.02$.

PPAR-γ Independent Inhibition of MMP-1 and MMP-13 Gene Expression. To test the role of PPAR-γ in the inhibition of MMP expression by CDDO and 15-dPGJ$_2$, we employed the PPAR-γ antagonist GW9662 (Fu et al., 2001). This compound blocks PPAR-γ transactivation by covalently binding to the ligand-binding domain of the receptor and precluding binding of other ligands to this site (Fu et al., 2001). To verify the activity of GW9662 in SW-1353 cells, we transfected cells with the PPRE reporter gene. In addition, PPAR-γ was overexpressed in this experiment to measure the ability of GW9662 to antagonize maximum levels of PPAR-γ. Treatment with either CDDO or the potent PPAR-γ agonist rosiglitazone (Forman et al., 1995) induces PPRE reporter activity (Fig. 3A, black bars). However, pretreatment with 10 μM GW9662 for 1 h completely blocks the transactivation observed with these PPAR-γ agonists (Fig. 3A, black versus white bars; $p = 0.01$). Thus, GW9662 inhibits the activation of PPAR-γ in SW-1353 cells.

Next, we used GW9662 to determine whether PPAR-γ is required for the inhibition of MMP expression by CDDO or 15-dPGJ$_2$. SW-1353 cells were pretreated with GW9662 followed by treatment with either CDDO or 15-dPGJ$_2$ and IL-1β. Importantly, GW9662 does not antagonize the inhibition of MMP-1 or MMP-13 mRNA by CDDO (Fig. 3B), demonstrating that functional PPAR-γ is not required for the inhibition of these genes by CDDO. Similarly, as measured by real-time RT-PCR, 15-dPGJ$_2$ reduces MMP-1 and MMP-13 mRNA levels, even in the presence of GW9662 (Fig. 3C). Treatment with GW9662 alone inhibits MMP-13 expression (Fig. 3, B and C); although the mechanism of this inhibition is unclear, additive repression is observed with GW9662 and 15-dPGJ$_2$ together (Fig. 3C). Nonetheless, in the presence of a pharmacological inhibitor of PPAR-γ, both CDDO and 15-dPGJ$_2$ effectively reduce MMP-1 and MMP-13 gene expression, demonstrating that these compounds can affect MMPs independently of PPAR-γ.

To further test the requirement for PPAR-γ in the inhibition of MMPs by CDDO and 15-dPGJ$_2$, MEFs deficient in PPAR-γ were used (Rosen et al., 2002). MMP-13 is the only collagenase expressed in mice (Balbin et al., 1996), and MMP-13 mRNA is induced by IL-1β in both PPAR-γ +/− and −/− MEFs (Fig. 4A). We did not detect a significant difference in the level of IL-1β induction of MMP-13 between PPAR-γ +/− and −/− MEFs using quantitative real-time RT-PCR (data not shown). Treatment with CDDO inhibits IL-1β induction of MMP-13, regardless of the PPAR-γ status of the MEFs (Fig. 4A). Real-time RT-PCR supports this finding and indicates 50% repression of MMP-13 by both CDDO (300 nM) and 15-dPGJ$_2$ (1 μM) in the PPAR-γ −/− MEFs (Fig. 4B). Importantly, this level of repression is consistent with that seen in SW-1353 cells (Fig. 1), PPAR-γ +/− MEFs (Fig. 4A; data not shown), and simian virus 40-transformed MEFs (data not shown), suggesting that MMP-13 repression is not affected by PPAR-γ dosage. In summary, our results with the PPAR-γ antagonist GW9662 and the PPAR-γ −/− MEFs demonstrate that CDDO and 15-dPGJ$_2$ inhibit MMP expression independently of PPAR-γ, suggesting that these compounds use alternative mechanisms to inhibit MMPs.

TGF-β Inhibits MMP-1 and MMP-13 Expression. PPAR-γ-independent mechanisms have been described for some of the anti-inflammatory effects of 15-dPGJ$_2$ (Straus et al., 2000; Chawla et al., 2001; Ward et al., 2002), and these mechanisms may also contribute to the suppression of MMPs. Thus, we focused on elucidating PPAR-γ- independent mechanisms of CDDO that may lead to MMP repression. Because CDDO can target the TGF-β signaling pathway (Suh et al., 2003), and because TGF-β regulates the expression of MMPs (Verrecchia and Mauviel, 2002), we hypothesized that CDDO may inhibit MMP gene expression by modulating TGF-β signaling. To begin addressing this possibility, we examined the effects of TGF-β on the expression of MMP mRNAs in SW-1353 cells. We found that TGF-β inhibits IL-1β induction of MMP-1 and MMP-13 by approximately 50% (Fig. 5). However, combining CDDO with TGF-β yielded no further inhibition of either gene (data not shown).

The similar effects of CDDO and TGF-β led us to test the possibility that CDDO might induce TGF-β expression, subsequently contributing to the inhibition of MMPs. We found that SW-1353 cells (2×10^5) secrete 500 pg/ml of latent TGF-β_1 during a 24-h period, as measured by enzyme-linked immunosorbent assay (data not shown). Active TGF-β was not detectable in the conditioned medium. Heat-activated conditioned medium from these cells suppresses the proliferation of mink lung epithelial cells by 90% (data not shown), a hallmark of TGF-β activity (Jennings et al., 1988). However, CDDO affects neither the level of TGF-β produced by these cells nor the ability of TGF-β to suppress cell proliferation (data not shown), indicating that CDDO is not modulating TGF-β synthesis or activity.

CDDO Antagonizes Smad-Mediated Transcription. The downstream transcriptional effects of TGF-β are mediated largely by TGF-β receptor-activated transcription factors known as Smad proteins (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002). Smads function as both activators and repressors of transcription, and their effects are elicited in a promoter-specific manner through either direct DNA binding or interactions with other transcription factors (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002). To investigate the effects of CDDO on the TGF-β pathway, we tested the effect of CDDO on Smad-mediated transcription. SW-1353 cells were transfected with a reporter gene containing multiple copies of a TGF-β response element from the PAI-1 promoter, CAGA$_{12}$-luciferase (Dennler et al., 1998). Smad3 and Smad4 bind to this element in the PAI-1 promoter and mediate transcriptional activation of this gene in response to TGF-β (Dennler et al., 1998).

We found that in the absence of exogenous TGF-β, transcriptional activity from CAGA$_{12}$-luciferase is low and CDDO has no effect (Fig. 6, inset). As expected, treatment with TGF-β results in a dose-dependent increase in promoter activity (Fig. 6, black bars), indicating that endogenous Smads are functional in these cells. However, cotreatment with 300 nM CDDO antagonizes this induction by approximately 30% at each concentration of TGF-β (Fig. 6, black bars versus white bars; $p = 0.01$). Thus, CDDO antagonizes Smad-mediated induction of this reporter, suggesting a mechanistic connection between Smads and CDDO.

Smads Are Involved in the Inhibition of MMP-1 by CDDO. Next, we wanted to determine whether the interaction between CDDO and Smads is relevant to the inhibition of MMPs. Smad3, a TGF-β receptor-activated Smad, is a key transcription factor mediating the regulation of MMP-1 and MMP-13 gene expression by TGF-β (Uria et al., 1998; Tardif et al., 2001; Yuan and Varga, 2001; Leivonen et al., 2002).

Thus, we examined the role of Smad3 in the repression of MMP-1 by CDDO. We transfected 4.3 kb of the human MMP-1 promoter fused to the luciferase reporter into wild-type and Smad3 −/− murine dermal fibroblasts. IL-1β induced MMP-1 promoter activity in wild-type cells by approximately 6-fold, and CDDO (300 nM) and TGF-β (10 ng/ml)

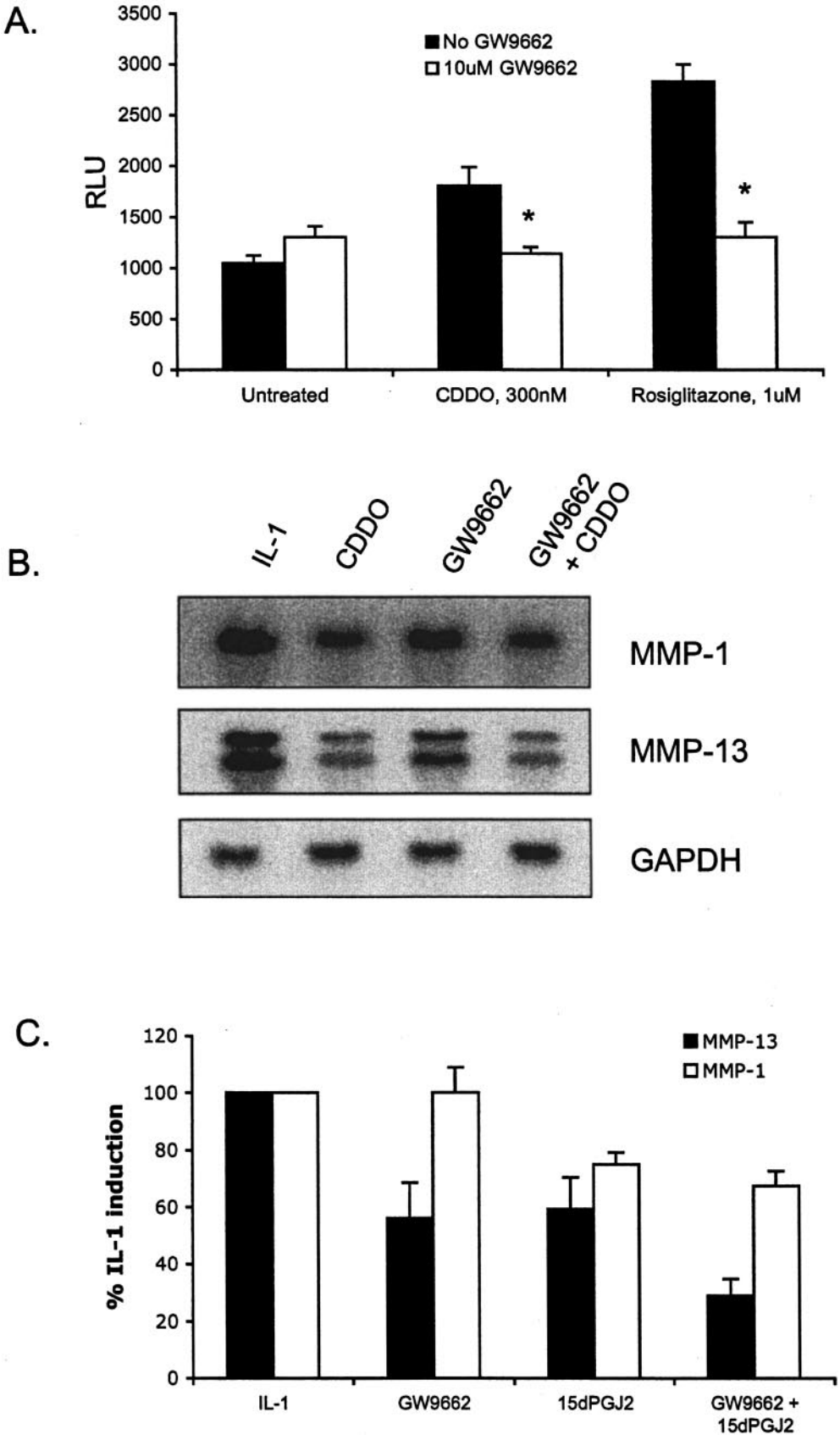

Fig. 3. GW9662 antagonizes PPAR-γ transactivation but not MMP inhibition by CDDO or 15-dPGJ$_2$. A, SW-1353 cells were cotransfected in triplicate with 1 μg of PPRE-tk-luciferase reporter construct and 1 μg of RSV-PPAR-γ expression construct. Cells were pretreated with GW9662 (10 μM) for 1 h, followed by treatment with CDDO (300 nM) or rosiglitazone (1 μM) for 24 h, as indicated. Luciferase activity was recorded as RLUs. CDDO versus GW9662 + CDDO; $\star$, $p = 0.01$; rosiglitazone versus GW9662 + rosiglitazone; $\star$, $p = 0.01$. B, SW-1353 cells were pretreated with GW9662 (10 μM) for 1 h, followed by the addition of CDDO (300 nM) for 24 h, as indicated. All cells were treated with IL-1β (10 ng/ml) for an additional 24 h. MMP-1, MMP-13, and GAPDH were measured by Northern blotting. C, cells were pretreated with GW9662 (10 μM) for 1 h, followed by the addition of 15-dPGJ$_2$ (5uM) for 24 h, as indicated. All cells were treated with IL-1β (10 ng/ml) for an additional 24 h. Real-time RT-PCR was conducted on MMP-1, MMP-13, and GAPDH. Normalized data are presented as the average percentage of IL-1 induction from triplicate treatments.

antagonized this induction by 50 and 75%, respectively (Fig. 7A, $p = 0.01$). Importantly, CDDO and TGF-β also repress endogenous MMP-1 expression in human dermal fibroblasts by 75% (data not shown), indicating similar regulation of the transfected human MMP-1 promoter in mouse dermal fibroblasts.

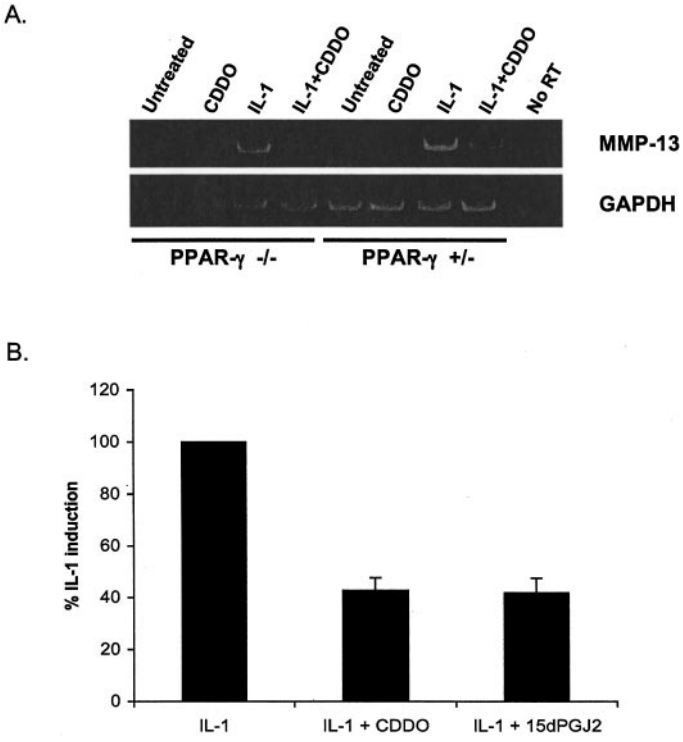

Fig. 4. CDDO and 15-dPGJ$_2$ inhibit MMP-13 induction in PPAR-γ $-/-$ MEFs. A, MEFs were either left untreated or pretreated with CDDO (300 nM) for 24 h, followed by addition of IL-1β (10 ng/ml) for an additional 6 h, as indicated. MMP-13 and GAPDH mRNA were amplified by RT-PCR. Reverse transcriptase (no RT) was omitted, as indicated. Products were visualized on an acrylamide gel stained with ethidium bromide. B, MEFs were either left untreated or pretreated with CDDO (300 nM) for 24 h, followed by the addition of IL-1β (10 ng/ml) and 15-dPGJ$_2$ (1 μM), where indicated, for an additional 6 h. Real-time RT-PCR was conducted on MMP-13 and GAPDH. Data are normalized to GAPDH and reported as an average percentage of IL-1β induction in three independent experiments.

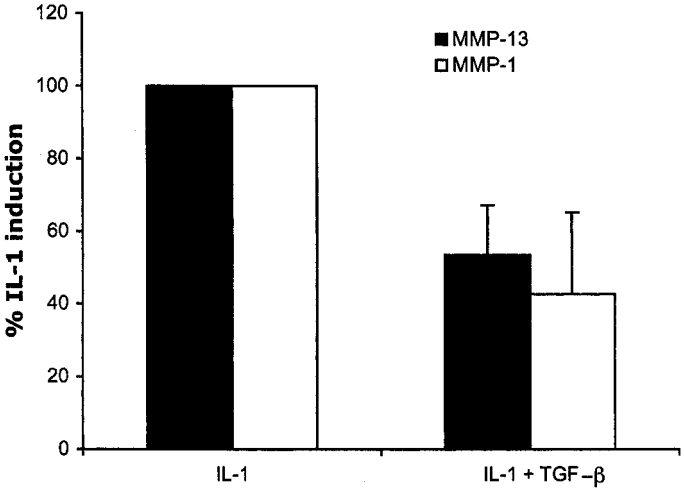

Fig. 5. TGF-β inhibits MMP-1 and MMP-13 gene expression. SW-1353 cells were treated with IL-1β (10 ng/ml) or IL-1β + TGF-β (10 ng/ml) for 24 h. RNA was harvested and MMP levels were measured by real-time RT-PCR. Data are normalized to GAPDH and reported as an average percentage of IL-1β induction in three independent experiments.

In Smad3 $-/-$ dermal fibroblasts, IL-1β induces a 2-fold increase in MMP-1 promoter activity (Fig. 7B). AP-1 proteins are required for MMP-1 induction, and Smad3 $-/-$ cells have been reported to lack the AP-1 protein c-fos (Piek et al., 2001). Thus, the lower induction of MMP-1 in the Smad3$-/-$ dermal fibroblasts may be caused by the absence of a critical factor, such as c-fos. Nonetheless, TGF-β represses IL-1β-induced MMP-1 promoter activity in the Smad3 $-/-$ dermal fibroblasts (Fig. 7B, $p = 0.01$), suggesting that TGF-β uses an alternative mechanism in the absence of Smad3. In contrast, CDDO does not block MMP-1 transcription in the absence of Smad3 (Fig. 7B), suggesting that Smad3 is either mediating MMP-1 repression by CDDO directly or, alternatively, that another factor regulated by Smad3 is required for this repression.

TGF-β signaling is suppressed by inhibitory Smads, such as Smad7, that function by binding to the TGF-β receptor and subsequently blocking the nuclear translocation of Smad transcriptional complexes (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002). Endogenous Smad7 functions as part of a negative feedback mechanism to suppress TGF-β signaling (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002). Thus, overexpression of exogenous Smad7 can attenuate Smad signaling and block the effects of TGF-β (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002). Importantly, Smad7 overexpression blocks the inhibition of MMP-1 by TGF-β (Yuan and Varga, 2001). Thus, to further address the role of Smads in the repression of MMP-1 by CDDO, we used Smad7 to antagonize Smad signaling.

Mouse embryonic fibroblasts were cotransfected with the human MMP-1 promoter and either a Smad7 expression construct or an equivalent amount of empty vector. IL-1β induces MMP-1 promoter activity by 6-fold, and both CDDO (300 nM) and TGF-β (10 ng/ml) completely block this induction (Fig. 8, black bars; $p = 0.001$). We found that overexpression of Smad7 has no effect on basal- or IL-1β-induced MMP-1 promoter activity; however, Smad7 partially rescues the inhibition of MMP-1 by TGF-β (Fig. 8, black versus white bars; $p = 0.01$). This result is consistent with previous studies (Yuan and Varga, 2001) and confirms that receptor-activated Smads contribute to the repression of MMP-1 by TGF-β. The incomplete rescue by Smad7 in this experiment (Fig. 8) is consistent with the partial suppression of MMP-1 promoter activity observed in the absence of Smad3 (Fig. 7B), suggesting that additional pathways may mediate MMP-1 repression by TGF-β.

Most importantly, Smad7 overexpression rescues the inhibition of MMP-1 promoter activity by CDDO (Fig. 8, black bars versus white bars; $p = 0.01$), indicating that endogenous Smad activity is required for CDDO to function. We consistently observe a 50% decrease in repression by CDDO in the presence of the Smad7 expression construct with quantities ranging from 0.1 to 1 μg (data not shown). Whereas the MEFs used in these experiments are heterozygous for PPAR-γ (Fig. 4A), overexpression of wild-type or dominant-negative PPAR-γ does not influence these results (data not shown), consistent with our findings that PPAR-γ does not regulate MMP expression (Figs. 3 and 4). Additionally, Smad7 attenuates the inhibition of MMP-1 promoter activity by CDDO in wild-type murine dermal fibroblasts (data not shown). Thus, CDDO uses Smads to inhibit MMP-1 because

Smad7, a suppressor of Smad signaling, antagonizes the function of CDDO.

Discussion

Previous studies with PPAR-γ ligands have implicated a role for PPAR-γ in collagenase gene expression (Fahmi et al., 2001; Fahmi et al., 2002). However, it has been unclear whether PPAR-γ ligands mediate MMP-1 and MMP-13 inhibition through their cognate receptors directly or use alternative mechanisms. Our study investigates the role of PPAR-γ in the suppression of MMP-1 and MMP-13 gene expression by using two known PPAR-γ ligands, the synthetic triterpenoid CDDO and the prostaglandin 15-dPGJ$_2$. Although these ligands exhibit distinct chemical structures, both bind with high affinity to PPAR-γ (Kliewer et al., 1995; Wang et al., 2000).

We document the parallel activation of PPAR-γ and the suppression of MMP-1 and MMP-13 gene expression by CDDO and 15-dPGJ$_2$, indicating that PPAR-γ is functional in these cells and suggesting that activated PPAR-γ may target MMP promoters (Figs. 1 and 2). However, our studies with the PPAR-γ antagonist GW9662 (Fig. 3) and MEFs lacking PPAR-γ (Fig. 4) indicate that these ligands are acting independent of PPAR-γ to repress MMPs. Although another nuclear receptor may be functionally compensating for PPAR-γ, it seems unlikely because 1) GW9662 completely antagonizes transactivation of the PPRE reporter construct (Fig. 3), demonstrating that another receptor cannot activate this reporter when PPAR-γ is inactivated, and 2) CDDO does not transactivate the PPRE reporter in PPAR-γ deficient MEFs unless PPAR-γ is overexpressed (data not shown). Thus, we have revealed a dichotomy in which CDDO and 15-dPGJ$_2$ can activate PPAR-γ but MMP gene expression is repressed independently of this receptor. Our findings support the growing body of evidence indicating that CDDO and 15-dPGJ$_2$ are promiscuous ligands that are likely to exert their effects through multiple mechanisms (Straus et al., 2000; Chawla et al., 2001; Ward et al., 2002; Place et al., 2003; Suh et al., 2003). That the MMP-1 and MMP-13 promoters lack PPREs and an additional PPAR-γ agonist, rosiglitazone, does not repress these genes (data not shown) also argue against the direct involvement of PPAR-γ.

PPAR-γ-independent mechanisms have been described for 15-dPGJ$_2$ and may also account for the ability of this prostaglandin to inhibit MMP expression (Straus et al., 2000; Chawla et al., 2001; Ward et al., 2002). Specifically, 15-dPGJ$_2$ can bind directly to IκB kinase and interfere with nuclear factor-κB activation (Straus et al., 2000; Boyault et al., 2001). Nuclear factor-κB is required for the induction of MMP-1 and MMP-13 by IL-1β (Mengshol et al., 2000; Vincenti and Brinckerhoff, 2002); thus, antagonizing this critical transcription factor with 15-dPGJ$_2$ could contribute to the inhibition of these genes. In addition, this prostaglandin can modulate AP-1, a transcription factor critical for MMP regulation (Boyault et al., 2001; Straus et al., 2000).

So far, PPAR-γ is the only protein known to bind to CDDO, and this interaction leads to the differentiation of 3T3-L1 fibroblasts into adipocytes (Wang et al., 2000). However, CDDO inhibits cell proliferation and induces differentiation of leukemia cells independently of PPAR-γ (Place et al., 2003); thus, similar to 15-dPGJ$_2$, CDDO uses both PPAR-γ-dependent and -independent mechanisms to exert its effects.

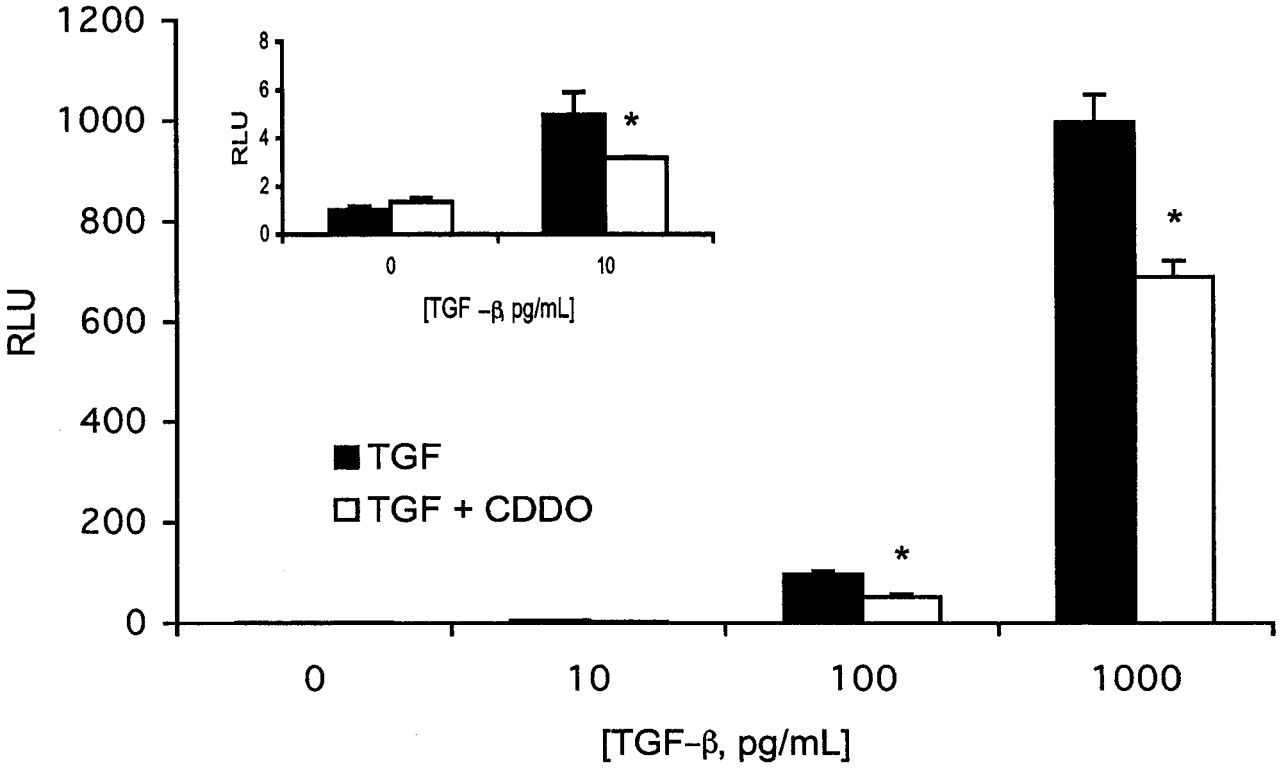

Fig. 6. CDDO antagonizes Smad-dependent transcription. SW-1353 cells were transfected in triplicate with CAGA$_{12}$-luciferase and left untreated in serum-free medium, treated with TGF-β alone (10–1000 pg/ml), or treated simultaneously with CDDO (300 nM) and TGF-β. Luciferase activity was recorded as RLUs after 24 h of treatment; ⋆, $p = 0.01$.

We have documented an additional PPAR-γ-independent action of CDDO, the repression of MMP gene expression, and our findings suggest that CDDO must use alternative mechanisms for the regulation of these target genes.

Interestingly, many of the effects of CDDO parallel the pleiotropic effects of TGF-β (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002). Furthermore, both CDDO and CDDO-imidazolide target the TGF-β signaling pathway in epithelial and leukemia cells (Suh et al., 2003). To define a mechanism contributing to the repression of MMPs by CDDO, we examined the effects of CDDO on the TGF-β pathway in our system. We found that TGF-β inhibits the expression of MMP-1 and MMP-13 (Fig. 5), and Smads are known to mediate the downstream effects of TGF-β on target MMP promoters (Uria et al., 1998; Selvamurugan and Partridge, 2000; Tardif et al., 2001; Yuan and Varga, 2001; Leivonen et al., 2002). To test for possible interactions between CDDO and Smads, we used the consensus Smad reporter $CAGA_{12}$-luciferase as a measure of Smad activity. Interestingly, we found that CDDO antagonized the activation of this reporter by TGF-β (Fig. 6), suggesting a connection between Smad signaling and CDDO. Because Smads are transcriptional activators in the context of this CAGA reporter, CDDO may convert activating Smad complexes into repressive complexes.

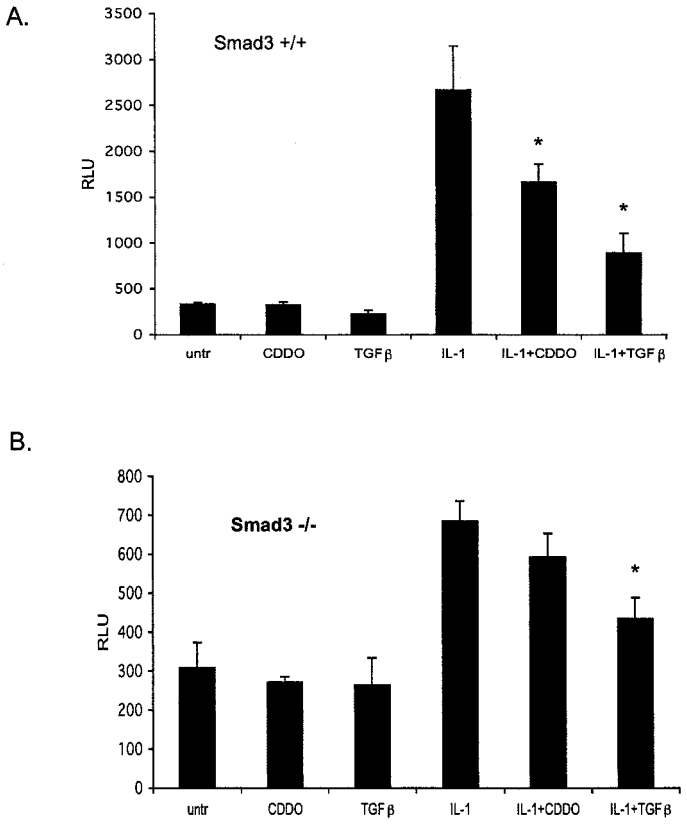

Fig. 7. Smad3-dependent inhibition of MMP-1 promoter by CDDO. Wild-type (A) or Smad3 −/− (B) dermal fibroblasts were transfected in triplicate with 1ug of the human MMP-1 promoter (4.3 kb) and treated as indicated. Luciferase activity was recorded as RLUs after 24 h of treatment; ⋆, $p = 0.01$.

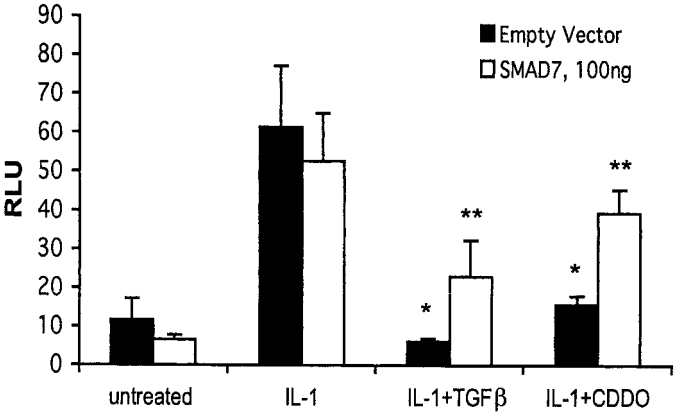

Fig. 8. Smad7 antagonizes repression of the MMP-1 promoter. PPAR-γ +/− MEFs were cotransfected with 1ug of the human MMP-1 promoter (4.3 kb) and either 100 ng of Smad7 expression construct or 100 ng of empty vector, and treated with IL-1β (10 ng/ml), IL-1β + TGF-β (10 ng/ml), or IL-1β + CDDO (300 nM), as indicated. Luciferase activity was recorded as RLUs after 24 h of treatment; ⋆, $p = 0.001$; ⋆⋆, $p = 0.01$.

While observing repression of this Smad-dependent reporter by CDDO in chondrocytic cells, we noticed marked synergy between TGF-β and CDDO-imidazolide in epithelial cells (Suh et al., 2003), suggesting that these synthetic triterpenoids may have tissue-specific effects on the Smad pathway. Consistent with this observation, TGF-β is known to mediate many of its effects in a tissue-specific manner (Massague and Wotton, 2000; Verrecchia and Mauviel, 2002).

We have documented a connection between CDDO and the TGF-β pathway in chondrocytic cells and have determined that this interaction contributes to the repression of MMPs. Our study extends the previous description of CDDO targeting the TGF-β pathway in epithelial and leukemia cells (Suh et al., 2003) and further reveals the complexity of CDDO-Smad interactions. The connection between CDDO and Smads was extended to MMP-1 because we determined that Smads are required for the inhibition of this promoter by CDDO. The MMP-1 promoter is not inhibited by CDDO in the absence of Smad3 (Fig. 7), implicating this transcription factor as a key effector of CDDO. In addition, attenuating endogenous Smad signaling by overexpressing the negative regulator Smad7 abrogated the ability of CDDO to repress MMP-1 (Fig. 8). These results are complimentary because Smad7 functions to suppress TGF-β signaling and thereby blocks the activation of Smad3 (Verrecchia and Mauviel, 2002).

CDDO may interact with a component(s) of the Smad pathway, either directly or through another nuclear protein that may have affinity for CDDO. Interestingly, the Smad consensus CAGA site derived from the PAI-1 promoter (Dennler et al., 1998) (Fig. 6) is found in multiple copies throughout the MMP-1 and MMP-13 promoters (Rutter et al., 1997; Tardif et al., 1997). Because CDDO antagonizes transcriptional activity of the CAGA reporter gene (Fig. 6), it is possible that CDDO uses a similar mechanism targeting CAGA sites in the MMP promoters to repress these genes. Alternatively, Smads can repress transcription by interacting with AP-1 proteins bound to promoter sequences (Tardif et al., 2001; Verrecchia et al., 2001); thus, CDDO may influence Smad/AP-1 interactions within the MMP promoters.

We have determined that CDDO and 15-$dPGJ_2$ do not use PPAR-γ to antagonize MMP gene expression. The significance of these findings extends beyond these PPAR-γ ligands and challenges the involvement of PPAR-γ in the inhibition of MMPs by other ligands. Furthermore, clarifying PPAR-γ-independent mechanisms of MMP repression may reveal

novel therapeutic targets. As demonstrated in this study, compounds that can modulate specific components of the TGF-β pathway may have therapeutic promise as inhibitors of MMP gene expression.

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