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Retinoic Acid Receptors Interfere with the TGF-β/Smad Signaling Pathway in a Ligand-Specific Manner

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Abbreviations: ATRA, *all-trans* retinoic acid; FCS, fetal calf serum; RA, retinoic acid; RAR,

retinoic acid receptor; TGF-β, transforming growth factor-β

†: deceased

SUMMARY

Transforming growth factor-β (TGF-β) and retinoic acid (RA) are important regulators of cell growth and differentiation. The TGF-β receptors utilize Smad proteins to transduce signals intracellularly and regulate transcription of target genes, either directly or in combination with other sequence-specific transcription factors. Two classes of nuclear receptors, the retinoic acid receptors (RARs) and the retinoic X receptors are involved in mediating transcriptional responses to RA. Given the known interactions between the TGF-β and RAR pathways, we have investigated the role played by RAR ligands in modulating functional interactions between Smad3 and RARs. Using transient cell transfection experiments with an artificial Smad3/4 dependent reporter construct, we demonstrate that RARs overexpression enhances Smad-driven transactivation, an effect that requires both Smad3 and Smad4. We provide evidence that RAR effect on Smad3/4-driven transcription is prevented by natural and synthetic RAR agonists, and potentiated by synthetic RAR antagonists. The activity of two TGF-β-responsive human gene promoter constructs was regulated in a parallel fashion. Using both mammalian two-hybrid and immunoprecipitation/Western methods, we demonstrate a direct interaction between the region DEF of RARγ and the MH2 domain of Smad3, inhibited by RAR agonists and enhanced by their antagonists. We propose that RARs may function as co-activators of the Smad pathway in the absence of RAR agonists or in the presence of their antagonists, a phenomenon that contrasts with their known role as agonist-activated transcriptional regulators of RA-dependent genes.

INTRODUCTION

The retinoid receptors belong to a large superfamily of ligand-inducible transcription factors that include the steroid, vitamin D, and thyroid hormone receptors, the peroxisome proliferator-activated receptor, the insect edysteroid receptor, and a number of orphan receptors whose ligands are unknown (Chambon, 1996; Jetten et al., 2001). All nuclear receptors share several well-characterized structural domains, including a conserved DNA-binding domain, and a ligand-binding domain at the carboxyl terminus of the receptor (Kumar & Thompson, 1999; Pemrick et al., 1994). Nuclear retinoic acid receptors, the retinoic acid receptors (RARs) and retinoic X receptors (RXRs) classes, are each composed of α, β, and γ subtypes with more than one isoform for each receptor subtype (Chambon, 1996). RARs associate with RXRs to form RAR-RXR heterodimers which bind target DNA sequences, called retinoic acid response elements (RAREs), consisting of directly repeated hexameric half-sites with the consensus sequence 5'-PuG(G/A)(T/A)CA-3', separated by one to five nucleotides, depending on the target gene. Members of the RAR family recognize two natural stereoisomers of RA, all-*trans* RA (ATRA) and 9-*cis* RA, whereas the RXR family exclusively recognizes 9-*cis* RA. It has been suggested that individual receptor subtypes may control distinct gene expression patterns important for cell growth and differentiation (Chambon, 1996).

Cellular signaling from the TGF-β family of growth factors (e.g. activins, bone morphogenic proteins, and the TGF-βs) is initiated by binding of the ligand to transmembrane receptor serine/threonine kinases, TβRI and TβRII (Derynck & Feng, 1997). Following ligand activation, signaling from the receptors to the nucleus occurs predominantly by phosphorylation of cytoplasmic mediators belonging to the Smad family (Attisano & Wrana, 2002; Datto & Wang, 2000). The receptor-associated Smads (R-Smads), such as Smad1,

Smad2, Smad3, and Smad5, interact directly with, and are phosphorylated by, activated type I receptors of the TGF-β superfamily. Activation of the R-Smads is ligand-specific, but each then forms, upon phosphorylation, heteromeric complexes with Smad4, a common mediator for all receptor-activated Smad pathways. R-Smad/Smad4 complexes are then translocated into the nucleus where they function as transcription factors, either directly or in association with other DNA binding factors (Massagué & Wotton, 2000). Activated TβRI may phosphorylate Smad2, which drives activin-like transcriptional responses through association with transcription factors of the FAST family, or Smad3, which drives TGF-β-specific transcriptional responses, directly binding specific *cis*-elements which most often contain the nucleotidic sequence CAGACA. Another group of Smad proteins, the inhibitory Smads, including Smad6 or Smad7, prevent phosphorylation and/or nuclear translocation of R-Smad/Smad4 heterocomplexes (Attisano & Wrana, 2002; Datto & Wang, 2000; Massagué & Wotton, 2000).

Several recent reports have provided novel insights regarding the interactions of nuclear receptors with the Smad signaling pathway. In the case of vitamin D, which controls transcription of target genes through the vitamin D receptor (VDR), Smad3 acts as a co-activator specific for ligand-induced VDR-dependent transactivation by forming a complex with a member of the steroid receptor coactivator-1 protein family (SRC-1) in the nucleus (Yanagisawa et al., 1999). The estrogen receptor (ER) suppresses TGF-β-induced Smad3 activity when, at the mean time, ER-mediated transcription is enhanced by TGF-β signaling (Matsuda et al., 2001). Direct association of Smad3 with the androgen receptor has also been described, leading to suppression of Smad3/DNA association and resulting Smad3-dependent gene transactivation downstream of TGF-β (Chipuk et al., 2001). Peroxisome proliferator-activated receptor γ has been shown to inhibit TGF-β-induced connective tissue growth factor expression by interfering with Smad3 (Fu et al., 2001).

Cooperation of TGF-β with RA occurs both in physiological and pathological situations (Roberts & Sporn, 1992). An explanation may be that RA increases the production of active TGF-β and its receptors by a variety of cell types, a mechanism responsible for the growth inhibition of human keratinocytes and U937 leukemia cells, for example (Borger et al., 2000; Defacque et al., 1997; Nunes et al., 1996). Most recently, it was shown that ATRA is able to decrease the levels of phosphorylated Smad2/3 in response to TGF-β, possibly via a RARα-mediated activation of a nuclear serine-threonine phosphatase that remains to be identified (Cao et al., 2003). Another report demonstrated intracellular interactions between the TGF-β and RA pathways, whereby RA stimulates, and fusion proteins of retinoid receptors antagonize, TGF-β-induced growth inhibition of lung epithelial cells (La et al., 2003). In the latter case, direct interactions between RARα and the fusion protein PLZF-RARα with Smad3 were described, that correlate with increased or decreased $p15^{INK4b}$ expression, respectively.

In this report, we have studied the modulation of Smad3/4-dependent gene transcription by RAR agonists and antagonists. Our data demonstrate RAR ligand-dependent modulation of TGF-β response, whereby RAR agonists repress and RAR antagonists potentiate Smad-driven transcription via opposite modulation of RAR-Smad3 interactions. Details are provided herein.

RESULTS

RAR ligands alter TGF-β-induced Smad3/4-specific transcription-As a first approach to examine the possible interference of RAR signaling with the TGF-β/Smad pathway, we determined the effect of RAR agonists and antagonists on TGF-β-induced, Smad-driven, gene transactivation in WI-26 lung fibroblast cultures transfected with the artificial $(CAGA)_9$-lux reporter construct. The latter, which consists of nine copies of the nucleotidic motif CAGA, known to bind Smad3 and Smad4 with high affinity, cloned upstream of the adenovirus major late promoter, to control the expression of the luciferase reporter gene (Dennler et al., 1998), allows to monitor Smad3/4-specific transcription in response to TGF-β. As expected, TGF-β alone dramatically elevated $(CAGA)_9$-lux activity (Fig. 1A). ATRA and CD2043, a synthetic RAR agonist, had a dose-dependent inhibitory effect on TGF-β-induced transactivation, with maximal repression of 35% and 55% respectively, at a concentration of 10^{-6}M. At each concentration tested (10^{-10}, 10^{-8} and 10^{-6} M), CD2043 was consistently more potent than ATRA to repress Smad-dependent transcription. Inversely, CD3106, a synthetic RAR antagonist, dose-dependently potentiated TGF-β-induced, Smad3/4-specific, transcriptional response (Fig. 1B), with a maximal effect, 3-fold above levels achieved with TGF-β alone, at a concentration of 10^{-6}M. RAR agonists CD2043 and ATRA (not shown) added simultaneously with CD3106 at a 1/10 ratio (10^{-7}M CD2043 or ATRA, 10^{-6}M CD3106) neutralized each other in the Smad transactivation assay (Fig. 1C), consistent with RAR agonist/antagonist effects exerted at the level of endogenous RARs.

RAR and RXR overexpression enhances TGF-β-induced Smad3/4-specific transcription-
We next tried to determine whether overexpression of RARs and RXR would affect TGF-β response. As shown in Fig. 2A, transfection of expression vectors for RARα, RARβ and

RARγ resulted in the upregulation of Smad3/4-dependent transactivation of (CAGA)$_9$-lux in response to TGF-β. Reproducibly, RARγ was the most potent among RARs to enhance the TGF-β response. As an alternative to exogenous TGF-β, when Smad3 was overexpressed ectopically, co-expression of RARs resulted in a significant potentiation of the transactivation of (CAGA)$_9$-lux exerted by Smad3 (Fig. 2B). Similar to what we observed with exogenous addition of TGF-β, RARγ was the most potent of all RARs in this assay. Interestingly, identical results were obtained when experiments were performed in medium complemented with lipid-free serum (not shown), indicating that the RAR effect is not due to RA present in the serum.

Since RARs function as heterodimers with RXRs to drive RARE-dependent transcription, we next examined whether RXRα had similar effects as RARs on Smad3-specific transcription, and whether RXRα would cooperate with RARs to activate Smad3-driven gene expression. As shown in Fig. 2C, RXRα overexpression potentiated Smad3-driven (CAGA)$_9$-lux transactivation. Co-expression of RXRα together with RARs α, β and γ resulted in a strong potentiation of RARα effect on Smad3-specific transcription, whereas little augmentation of RARγ effect was observed, the latter likely masked by the strong effect of RARγ alone. Little effect of RARβ was observed, without or with RXRα.

The current model for Smad-dependent transcription in response to TGF-β implies heterodimerization of phosphorylated Smad3 with Smad4, nuclear translocation and subsequent DNA binding of Smad3/4 heterocomplexes (Attisano & Wrana, 2002; Datto & Wang, 2000; Massagué & Wotton, 2000). However, recent evidence suggests that nuclear transport of Smad3 in response to TGF-β does not require Smad4, although the latter is essential for Smad3-dependent transcriptional response (Fink et al., 2003). Two distinct approaches were taken to determine whether the effects of RARs described above were

strictly dependent on a fully functional Smad3/4 pathway or if RARs could replace one of the normal constituants of the Smad complex bound to its cognate DNA sequence. Firstly, expression of a dominant-negative form of Smad3, Smad3ΔMH2, not only prevented TGF-β effect on (CAGA)$_9$-lux transactivation in WI26 fibroblasts, but also abolished further activation by RARs (Fig. 3A). This result suggests that direct protein-protein interactions with Smad3 bound to DNA via its MH1 domain are necessary for RAR effect. Secondly, in the Smad4-deficient MDA-MB-468 human breast carcinoma cell line transfected with (CAGA)$_9$-lux and a RARγ expression vector, there was no activation of the construct by TGF-β, consistent with previous observations (Vindevoghel et al., 1998), whether in the absence or presence of RARγ (Fig. 3B). In the latter cell type, ectopic expression of Smad4 restored a "normal" TGF-β response, as well as the potentiating effect of RARγ on the latter. These data support the notion that proper Smad3/4-driven promoter transactivation in response to TGF-β, i.e. concomitant presence of Smad3 and Smad4 in the nucleus bound to their cognate DNA element, is required for RARs to exert their effect. Also, our data indicate that RARγ cannot replace Smad4 to drive Smad3-specific gene expression in a context devoid of Smad4.

RARγ interacts with the MH2 domain of Smad3 through its DEF region-Next, to better understand the molecular mechanisms underlying the functional cooperation of RARs with Smad3, protein-protein interactions between RARγ and Smad3 were examined by IP/Western approaches. For this purpose, COS-7 cells were transfected with Smad3-Flag and Gal4BD-RARγDEF expression vectors, together with a constitutively active form of TβRI. Immunoprecipitations of the cell lysates were performed with an anti-Gal$_4$BD antibody, followed by Western analysis with anti-Flag and anti-Gal$_4$BD antibodies. As shown in Fig. 4A, full-length Smad3 interacts with RARγ in solution, as evidenced by the co-precipitation of Smad3-Flag together with Gal4BD-RARγDEF. Similarly, the Myc-tagged MH2 domain of

Smad3 co-immunoprecipitated with RARγ (Fig. 4B), indicating that Smad3-RARγ interactions occur through the MH2 domain of Smad3.

We next used a mammalian two-hybrid transactivation assay aimed at measuring Smad3/RARγ interactions. In such system, the Gal4-lux reporter construct is co-transfected with an expression vector for the Gal4BD-RARγDEF fusion protein, in the absence or presence of an expression vector for VP16AD-Smad3. When Gal4BD-RARγDEF is bound to the Gal4 binding sites of the Gal4-lux construct, Smad3-RARγ interaction is expected drive luciferase activity higher, due to the strong VP16 transactivation domain fused to Smad3. Results of a representative experiment are presented in Fig. 4C: Gal4-RARγDEF expression resulted in detectable transactivation of Gal4-lux, likely representative of the activity of the AF-2 transactivation region within the E module of RARγ (Chambon, 1996). Co-expression of VP16AD-Smad3 strongly enhanced Gal4BD-RARγDEF-mediated transactivation of Gal4-lux, a phenomenon reflecting direct RARγ-Smad3 interaction. No effect of either empty pVP16AD or VP16AD-Smad3 alone was observed. When VP16AD-Smad3(1-146), lacking the Smad3 MH2 domain, was expressed instead of the full-length Smad3 fusion protein, no transactivation of Gal4-lux was observed, indicating that the MH2 domain of Smad3 is not dispensable in allowing RARγ-Smad3 interaction. These results are consistent with those obtained by IP/Western and presented in Fig. 4B, and with the known role of the MH2 domain of R-Smads in mediating protein-protein interactions, such as with Smad4, activated TβRI, Fast or Jun proteins, for example (Liberati et al., 1999; Nagarajan et al., 1999; Shi, 2001; Verrecchia et al., 2001b).

Ligand-dependent modulation of RARγ-Smad3 interactions and of Smad3-dependent transcription-To gain insights into the involvement of RARs in the modulation of Smad3/4-dependent transcription, we examined the role of synthetic RAR agonists and antagonists on

the functional interactions between Smad3 and RARγ. As shown in Fig. 5A, the RAR antagonist CD3106 dramatically potentiated the effect of RARγ on Smad3-driven transcription (over 4-fold above the transactivation level achieved with the RAR□/Smad3 combination), whereas the RAR agonist CD2043 and ATRA abolished the effect of RARγ on Smad3-driven (CAGA)$_9$-lux transactivation.

We next examined the influence of RAR ligands, agonists and antagonists, on RARγ/Smad3 interactions, in IP/western experiments. COS-7 cells were transfected with Flag-tagged full-length Smad3 and Gal4BD-RARγDEF expression vectors, either alone or in combination, in the absence (Fig. 5B, lanes 1-3) or presence or either CD2043 (lanes 4-6) or CD3106 (lanes 7-9). Expression of all proteins from their transfected expression vectors was verified in each sample prior to IP (Fig. 5B, lower panels). The lower migrating band in lanes 2, 5 and 8 obtained with the anti-Gal4BD antibody represents the Gal4BD itself, not fused to RARγ, properly expressed by the "empty" Gal4BD vector used as a DNA filler in these samples. Following IP with an anti-Gal4BD antibody (upper panel), Western analysis with a Flag antibody revealed the co-immunoprecipitation of Smad3 with RARγ in lane 3, as expected from our data presented in Fig. 4. The amount of RARγ-bound Smad3 was severely reduced by CD2043 (lane 6) whereas it was significantly increased by CD3106 (lane 9), suggesting that RARγ-Smad3 interactions are altered by RAR agonists and antagonists, in a fashion compatible with the modulation of Smad3-driven transcription exerted by RAR ligands. Specifically, the RAR agonist CD2043, which prevents the stimulatory activity of RARs on Smad-dependent transcription, also reduces Smad3-RARγ interactions, whereas, the RAR antagonist CD3106, which potentiates RARγ effect on Smad-dependent transcription, augments Smad3-RARγ physical interactions.

RAR ligands modulate TGF-β-induced transcription of natural promoter sequences.

The effects of RAR ligands on TGF-β-induced transcription were next tested in a genomic

DNA context. Specifically, we examined the effects of CD2043 and CD3106 regulation of

TGF-β-induced transactivation of the human type VII collagen (*COL7A1*) and plasminogen

activator inhibitor-1 (*PAI-1*) gene promoters. Both *COL7A1* and *PAI-1* promoter fragments

are defined Smad3/4 targets downstream of TGF-β (Dennler et al., 1998; Vindevoghel et al.,

1998). As shown in Fig. 6A, CD3106 strongly potentiated while CD2043 inhibited the

transactivation of the *COL7A1* promoter induced by TGF-β, in perfect parallel with the data

obtained with the artificial, yet highly specific, Smad3/4-dependent construct (CAGA)$_9$-lux

(see above Fig. 5A). Similar modulation of TGF-β response of the PAI-1 promoter by RAR

agonists was observed (Fig. 6B). Of note, significant regulation of both *COL7A1* and PAI-1

promoter activities by RAR ligands alone was observed, suggesting that specific RAR

responsive elements may exist within these promoters, and raising the possibility that, in

addition to acting via the Smad response elements as suspected from our current data, RARs

may also cooperate with Smad3/4 through the use of distinct regulatory sequences.

DISCUSSION

Until recently, although functional interactions between TGF-β and retinoids had been observed in a variety of settings (Roberts & Sporn, 1992), little was known about the precise mechanisms underlying the intracellular interplay between their respective signaling pathways. In this report, we have identified a new molecular mechanism by which RARs are able to modulate Smad-dependent transcription in a ligand-dependent fashion. We demonstrate that RARs potentiate Smad3/4-dependent transcriptional responses downstream of TGF-β, and that RAR agonists oppose while RAR antagonists favor Smad3-RAR interactions, resulting in inhibition or potentiation of TGF-β signaling, respectively. Although a role for of RAR conformation for RAR-Smad3 may only be ascertained by X-ray crystallography, our results are compatible with RAR conformation-dependent RAR-Smad3 interactions whereby a RAR in the presence of its antagonist, i.e. in a conformation that is transcriptionally inactive in the context of RAR responsive *cis*-elements (RARE) acts as a co-activator of Smad3/4-specific transcription. Inversely, RAR agonists, that render RARs transcriptionally competent when bound to their specific DNA recognition sites, diminish both RAR-Smad3 interactions and Smad3/4-dependent transcription downstream of the TGF-β receptors. Potential therapeutic implications of such findings remain to be identified.

Escape of epithelial cells from TGF-β growth control is a hallmark of many cancers (de Caestecker et al., 2000; Gold, 1999; Reiss, 1999). The capacity of TGF-β signaling to function as a tumor suppressor pathway in early carcinogenesis is best illustrated by the presence of inactivating mutations in genes encoding TGF-β receptors and Smads in human carcinomas. However, contrasting with its tumor-suppressor activities that control epithelial cell growth and maintain genetic stability, TGF-β may become pro-oncogenic at later stages of carcinogenesis. Indeed, not only are high levels of TGF-β expression correlated with the

advanced clinical stage of a tumor, but TGF-β is also known to exacerbate the malignant phenotype of transformed and tumor-derived cells. Furthermore, tumor derived TGF-β could contribute to tumor growth indirectly by suppressing immunosurveillance or by stimulating the production of pro-angiogenic factors, or by inducing an epithelial to mesenchymal transition in various tumor cells, leading to increased invasiveness and metastatic activity.

Given the complexity of TGF-β actions during carcinogenesis, identifying and understanding the mechanisms underlying cellular signaling crosstalks that modify TGF-β responses is critical for the development of efficient therapeutic agents used for chemoprevention or for the treatment of advanced cancers. Moreover, since TGF-β plays opposite roles on malignant progression depending on the stage of carcinogenesis, therapeutic approaches targeting the TGF-β pathway would have to be context-specific. Theoretically, chemoprevention would aim at enhancing TGF-β signaling in order to promote its tumor suppressor activities, whereas treatment of advanced cancer would require strategies aimed at inactivating TGF-β or its downstream signals.

Retinoids are widely used as chemopreventive drugs in the context on non-melanoma skin cancers or acute promyelocytic leukemia, for example. Interestingly, it has been suggested that their efficacy may result, to some extent, from their ability to induce the production of TGF-β by a variety of cell types (de Caestecker et al., 2000; Gold, 1999; Reiss, 1999). It is however not a generalized phenomenon as, for example, in a study comparing the effect of ATRA on melanoma growth and migration, it was shown that although proliferation and chemotaxis were inhibited by ATRA in all the cell lines tested, cell lines established from metastases were significantly more sensitive with respect to inhibition of invasion by ATRA. The most striking difference between the cell lines was a strong downregulation of TGF-β expression in cell lines derived from metastases when treated with ATRA in contrast to cell lines from primary melanomas (Jacob et al., 1998). Of note, we previously reported

constitutive Smad-dependent transcription in human melanoma cells, regardless of their proliferative response to exogenous TGF-β (Rodeck et al., 1999). It will be interesting to determine whether the results from Jacob et al. (see above) result from a direct interference fro ATRA with Smad signaling, and whether such interference is modified by the stage of the tumor.

In another study, Ro 41-5253, a RARα-selective antagonist that binds RARα but does not induce transcriptional activation and does not influence RAR/RXR heterodimerization and DNA binding, was shown to inhibit proliferation and induces apoptosis in MCF-7 and ZR-75.1 estrogen- receptor-positive breast-carcinoma cells (Toma et al., 1998). The authors suggested that the anti-proliferative effect was probably mediated by anti-AP-1 activity, a mechanism known to be implicated in the action of several retinoids (Allenby, 1995; Chen et al., 1995). However, the authors reported that induction of apoptosis by Ro 41-5253 correlated with an increased production of TGF-β1 protein by the tumor cells. In view of our current data, it cannot be excluded that Ro 41-5253 may also be potentiating TGF-β anti-proliferative action through activation of the Smad pathway, the latter leading to the expression of of p15^{INK4B} (Feng et al., 2000; Seoane et al., 2001), and p21$^{WAF1/CIP1}$ (Datto et al., 1995; Moustakas & Kardassis, 1998), two cyclin-dependent kinase inhibitors, known effectors of TGF-β-induced cell cycle arrest. These data, together with our results herein demonstrating the cooperative activity of antagonist-bound RARs with Smad3/4, require to further examine the exact mechanisms for the anti-tumor activity of RAR antagonists. It is particularly important because it was clearly shown that Ro 41-5253 is a compound that exerts its anti-tumor activity without inducing the toxic side effects of retinoids, and might therefore be considered as a candidate for cancer therapy (Toma et al., 1998).

To conclude, we provide novel insights on how retinoids may affect TGF-β signaling. Specifically, this work represents the first demonstration for RAR-ligand controlled RAR-

Smad3 interactions. Most surprisingly, we have identified RAR antagonists as activators of Smad3/4-dependent transcription, correlated with their capacity to increase RAR-Smad3 physical interactions. Work is in progress to determine whether RAR antagonists are able to restore TGF-β-induced growth inhibition in tumor cells that have escaped from TGF-β control and do not exhibit mutations in the genes encoding TGF-β signaling molecules (e.g. receptors or Smads) but rather, reduced signaling.

MATERIAL AND METHODS

Cell Cultures—Human lung fibroblasts (WI-26), MDA-MB-468 human breast adenocarcinoma cells, a cell line derived from a patient with breast carcinoma and bearing a homozygous deletion of the complete SMAD4 coding region (Schutte et al., 1996), and simian COS-7 cells were grown in Dulbecco 's modified Eagle 's medium supplemented with 10% heat inactivated fetal calf serum (FCS), 2mM glutamine and antibiotics (100 units/ml penicillin, 50 µg/ml steptomycin G and 0.25 µg/ml Fungizone^{TM}). Cells were maintained at 37°C in a 5% CO_2 - 95% air atmosphere. In some experiments, charcoal-stripped FCS was used to alleviate the potential influence of FCS-contained lipids (e.g. retinoids). No difference in the outcome of the experiments was noted when using lipid-free serum instead of whole serum (not shown). Human recombinant TGF-β1, referred to as TGF-β throughout the text, was purchased from R&D Systems Inc. (Minneapolis, MN). All-*trans* retinoic acid (all-*trans* RA) was purchased from Sigma (St-Louis, MO). Synthetic RA agonist (CD2043) and antagonists (CD3106, CD3581 and CD2848) were from Galderma R&D (Sophia-Antipolis, France).

Plasmid constructs—Human RARα□ □β□ □γ , RXRα, and□ GAL4BD-RARγDEF expression vectors, kind gifts from Drs. C. Rochette-Egly and P. Chambon, Strasbourg, France, have been described previously (Allenby et al., 1993). RARγ deletion expression vectors in pGL3 (Promega Corp.) were generated by PCR. (CAGA)$_9$-lux, a gift from Drs. S. Dennler and J.-M. Gauthier (Glaxo-Wellcome, Les Ulis, France, consisting of nine tandem repeats of the motif CAGA binding Smad3 and Smad4, was used as a Smad3/4-specific reporter (Dennler et al., 1998). (ARE)$_3$-lux, a prototypic Smad2-specific reporter construct consisting of three repeats of the activin response element (ARE) of the *Xenopus mix.2* gene

(Chen et al., 1997), was co-transfected with a Fast-1 expression vector (Germain et al., 2000), kind gift from Dr. C.S. Hill (Imperial Cancer Research Fund, London, U.K.). N-terminally Flag- and Myc-tagged Smad3, dominant-negative Smad3ΔMH2, and Vp16AD-Smad3 expression vectors, gifts from A. Atfi, Paris, France have been described previously (Verrecchia et al., 2001a; Vindevoghel et al., 1998). Smad3/4 responsiveness of the human *COL7A1* and *PAI-1* promoter constructs –527*COL7A1*-lux and p800-lux has been described previously (Vindevoghel et al., 1998; Dennler et al., 1998).

Transient Cell Transfections and Reporter Assays—Transient cell transfections were performed with the calcium phosphate/DNA co-precipitation procedure using a commercial assay kit (Promega Corp., Madison, WI). After glycerol shock, cultures were placed in DMEM containing 1% FCS. In some experiments, TGF-β and/or retinoids were added three hours later. pCMV-β-galactosidase was co-transfected in every experiment to monitor transfection efficiencies. Reporter activities were determined with commercial kits (Promega).

Immunoprecipitations and Western blotting—COS-7 cells were transfected with tagged Smad3 and RARγ expression vectors, together with pCMV-β□ galactosidase, the latter serving as a control for transfection efficiency. 24 h later, cells were washed twice with cold PBS, scraped, and solubilized in a buffer containing 20 mM Tris -HCl, pH8, 150 mM NaCl, 5 mM $MgCl_2$, 0, 5% NP-40, 10% glycerol, 1 mM orthovanadate, 1 mM PMSF, 20 μg/ml aprotinine and 20 μg/ml leupeptine. For Gal4-tagged protein immunoprecipitation, cell lysates were cleared of debris by centrifugation and incubated with anti-Gal4 antibody (Santa-Cruz Biotech, Santa Cruz, CA) overnight at 4°C, followed by incubation with protein G-Sepharose beads at 4°C for 1 h (Amersham Pharmacia Biotech, Uppsala, Sweden). After 5 washes with solubilization buffer, immunoprecipitates were eluted by boiling for 3 min in SDS sample

buffer (100 mM Tris-HCl, pH 8, 8, 0, 01% bromophenol blue, 36% glycerol, 4% SDS) and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then electrotransferred to nitrocellulose filters, immunoblotted with anti-Myc-HRP (Roche Diagnostics, Indianapolis, IN), anti-Flag-HRP (Sigma) or anti-Gal4 (Santa-Cruz Biotech, Santa Cruz, CA) antibodies, and revealed using a chemiluminescence detection system (ECL$^+$, Amersham-Pharmacia Biotech).

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LEGENDS TO FIGURES

FIG. 1: **Effect of RAR ligands on Smad3/4-dependent transcription downstream of TGF-β in WI-26 human lung fibroblasts**. *A*, Sub-confluent WI-26 fibroblast cultures were transfected with (CAGA)$_9$-lux. Following glycerol shock, DMEM containing 1% FCS was added. Six hours later, several concentrations of either ATRA (shaded bars) or the synthetic RAR agonist CD2043 (striped bars) were added, without (-) or with (+) TGF-β (10 ng/ml). Incubations were continued for 24 h before reporter gene activity was determined. *B*, The same protocol as in panel *A* was followed, except that the synthetic RAR antagonist CD3106 was added instead of RAR agonists. *C*, Sub-confluent WI-26 fibroblast cultures were transfected with (CAGA)$_9$-lux. Six hours later, CD2043 (10^{-7}M) was added, in the absence or presence of CD3106 (10^{-6}M), without (-) or with (+) TGF-β. Incubations were continued for 24 h before reporter gene activity was determined. Results are mean $\pm$ S.D. of three independent experiments.

FIG. 2: **RAR overexpression potentiates TGF-β/Smad signaling**. *A*, Sub-confluent WI-26 fibroblast cultures were co-transfected with (CAGA)$_9$-lux together with expression vectors for RARα, β or γ, as indicated. Six hours after transfection, TGF-β (10 ng/ml) was added. *B*, Sub-confluent WI-26 fibroblast cultures were co-transfected with (CAGA)$_9$-lux together with RAR expression vectors, in the absence (-) or presence (+) of a Smad3 expression vector, as indicated. *C*, Sub-confluent WI-26 fibroblast cultures were co-transfected with (CAGA)$_9$-lux together with expression vectors for RARα, β or γ, as indicated, in the absence (-) or presence (+) of either Smad3 or RXRα expression vectors, alone or in combination. In each case, incubations were continued for 24 h before reporter gene activity was determined.

FIG. 3: **Smad3 and Smad4 are both required for RAR effect on (CAGA)$_9$-lux transactivation by TGF-β.** *A*, Sub-confluent WI-26 fibroblast cultures were co-transfected with (CAGA)$_9$-lux together with RAR expression vectors, in the absence (-) or presence (+) of a dominant-negative Smad3 expression vector (D/N Smad3), as indicated. Six hours after transfection, TGF-β was added (+) and incubations continued for 24 h before reporter gene activity was determined. *B*, Sub-confluent Smad4-deficient MDA-MB-468 human breast adenocarcinoma cell cultures were co-transfected with (CAGA)$_9$-lux together with expression vectors for RARγ, in the absence (-) or presence (+) of a Smad4 expression vector, as indicated. Six hours after transfection, TGF-β was added and incubations continued for 24 h before reporter gene activity was determined.

FIG. 4: **Physical interactions occur between Smad3 and RARγ.** *A*, COS-7 cells were transfected with Flag-tagged Smad3, in the absence or presence of Gal4-tagged RARγDEF, as indicated. Empty Gal4 expression vector (Gal4e) was used as a control. Cell lysates were subjected to immunoprecipitation (IP) with anti-Gal4 antibodies, and co-precipitating Smad3 was detected by Western blotting (WB) with anti-Flag antibodies (top panel). Expression of RAR☐ and Smad3 was confirmed using anti-Flag (middle panel) and anti-Gal4 antibodies (bottom panel). *B*, COS-7 cells were transfected with Myc-tagged Smad3 MH2 domain and Gal4-RARγDEF expression vectors, as indicated. 40 h later, cell extracts were immunoprecipitated with an anti-Gal4 antibody, and immunoblotted with anti-Myc antibodies (see legend). Expression of the proteins of interest was verified by WB prior to IP. *C*, Confluent WI-26 fibroblast cultures were co-transfected with Gal4-lux reporter construct Gal4-RARγDEF fusion protein expression vector, in the presence or absence of either pVP16Smad3FL of pVP16Smad3(1-146) expression vectors. Empty VP16 and Gal4 vecotrs

were used to maintain equal amounts of transfected DNA in each plate, where indicated. 40 h later, luciferase activity, representative of protein-protein interactions, was measured.

FIG. 5: **RAR ligands modulate the outcome or RARγ-Smad3 functional interaction and modify RARγ-Smad3 interactions.** *A*, Sub-confluent WI-26 fibroblast cultures were co-transfected with (CAGA)$_9$-lux together with expression vectors for RARγ, in the absence (-) or presence (+) of a Smad3 expression vector, as indicated. Six hours after transfection, the RAR antagonist CD3106 (10^{-6}M) or the RAR agonist CD2043 (10^{-7}M) were added, as indicated. Luciferase activity was determined 24 h later. *B*, COS-7 cells were transfected with Flag-tagged Smad3, in the absence or presence of Gal4-tagged RARγDEF, as indicated. Empty Gal4 expression vector (Gal4e) was used as a control. Six hours after transfection, CD2043 (10^{-7}M) or CD3106 (10^{-6}M) were added. Fourty hours later, cell lysates were subjected to immunoprecipitation (IP) with anti-Gal4 antibodies, and co-precipitating Smad3 was detected by immunoblotting (WB) with anti-Flag antibodies (top panel). Expression of RARγ and Smad3 was confirmed using anti-Flag (middle panel) and anti-Gal4 antibodies (bottom panel).

FIG. 6: **RAR ligands modulate the activity of Smad3/4-dependent target gene promoters.** Sub-confluent WI-26 fibroblast cultures were transfected with the human type VII collagen promoter construct –524COL7A1-lux (*A*) or the human plasminogen activator inhibitor-1 promoter construct p800-lux (*B*). Following glycerol shock, DMEM containing 1% FCS was added. Six hours later, the synthetic RAR antagonist CD3106 or the RAR agonist CD2043 were added, without (-) or with (+) TGF-β (10 ng/ml). Incubations were continued for 24 h before reporter gene activity was determined.

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