Cyclic adenosine 3',5'-monophosphate-elevating agents inhibit transforming growth factor-beta-induced SMAD3/4-dependent transcription via a protein kinase A-dependent mechanism.

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Cyclic Adenosine 3',5'-Monophosphate-Elevating Agents Inhibit Transforming Growth Factor-β-Induced SMAD3/4-Dependent Transcription Via a Protein Kinase A-Dependent Mechanism

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Abbreviations used are: AC, adenylate cyclase; CBP, CREB binding protein; CRE: cAMP Response element; CREB, CRE-binding protein; db-cAMP, dibutyryl-cyclic AMP; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; IBMX: isobutyl-methylxanthine; PAI-1, plasminogen activator inhibitor-1; PGE_2, prostaglandin E_2; PKA: protein kinase A; TGF-β, transforming growth factor- β; TNF-α, tumor necrosis factor-α.

ABSTRACT

TGF-β plays complex roles in carcinogenesis, as it may exert both tumor suppressor and pro-oncogenic activities depending on the stage of the tumor. SMAD proteins transduce signals from the TGF-β receptors to regulate the transcription of specific target genes. Cross-talks with other signaling pathways may contribute to the specificity of TGF-β effects. In this report, we have investigated the effects of cyclic adenosine 3',5'-monophosphate (cAMP), a key second messenger in the cellular response to various hormones, on SMAD-dependent signaling in human HaCaT keratinocytes. Using either an artificial SMAD3/4-dependent reporter construct or the natural TGF-β target, plasminogen activator inhibitor 1, we show that membrane-permeable dibutyryl cAMP, and other intracellular cAMP-elevating agents such as the phosphodiesterase inhibitor isobutyl-methylxanthine, the adenylate cyclase activator forskolin, or exogenous prostaglandin E_2 (PGE_2), interfere with TGF-β-induced SMAD-specific gene transactivation. Inhibition of protein kinase A (PKA), the main downstream effector of cAMP, with H-89, suppressed cAMP-dependent repression of SMAD-driven gene expression. Inversely, co-expression of either an active PKA catalytic subunit or that of the CRE-binding protein (CREB) blocked SMAD-driven gene transactivation. cAMP-elevating agents did not inhibit nuclear translocation and DNA binding of SMAD3/4 complexes but abolished the interactions of SMAD3 with the transcription co-activators CBP and p300 in a PKA-dependent manner. These results suggest that suppression of TGF-β/SMAD signaling and resulting gene transactivation by cAMP-inducing agents occurs via PKA-dependent, CREB-mediated, disruption of SMAD-CBP/p300 complexes.

INTRODUCTION

Cellular signaling from the Transforming Growth Factor-β (TGF-β) family of growth factors (e.g. activins, bone morphogenic proteins, and the TGF-β□) is initiated by binding of the ligands to transmembrane receptor serine/threonine kinases, TβRI and TβRII. Following receptor activation, signaling from the cell surface to the nucleus occurs predominantly by phosphorylation of cytoplasmic mediators of the SMAD family (Attisano & Wrana, 2000; Massagué & Wotton, 2000). Briefly, the receptor- associated SMADs (R-SMADs), such as SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8 interact directly with, and are phosphorylated by, activated type I receptors of the TGF-β superfamily. Activation of R-SMADs is ligand-specific, but each of them forms, upon phosphorylation, heteromeric complexes with SMAD4 which functions as a common mediator for all receptor-activated SMADs. R-SMAD/SMAD4 complexes are then translocated into the nucleus where they function as transcription factors, directly or in association with other DNA binding factors. Finally, inhibitory SMADs, such as SMAD6 or SMAD7, interfere with TGF-β signaling by preventing R-SMAD phosphorylation and subsequent nuclear translocation of R-SMAD/SMAD4 complexes, and inducing receptor degradation by recruiting E3 ubiquitine ligases of the Smurf family (Attisano & Wrana, 2000; Massagué & Wotton, 2000).

One of the first identified second messengers, cyclic adenosine 3',5'-monophosphate (cAMP) transmits signal from a variety of hormones acting at the cell surface via guanine-nucleotide-binding (G)-protein-coupled receptors to activate cAMP-dependent protein kinase A (PKA) (Montminy, 1997). The balance between adenylate cyclase (AC) and cyclic nucleotide phosphodiesterase activities determines intracellular cAMP levels. In the basal state PKA resides in the cytoplasm. cAMP induction liberates the catalytic subunits of PKA which then diffuse into the nucleus where they phosphorylate transcription factors, such as cAMP

Response Element Binding Protein (CREB) (Sassone-Corsi, 1998). PKA phosphorylates CREB at serine 133, thereby allowing its association with the transcriptional co-activators CBP and p300 (Chrivia et al., 1993; Kwok et al., 1994; Mayr & Montminy, 2001). CREB transactivates cAMP-responsive genes by binding as a dimer to a conserved, 8bp, palindromic cAMP response element (CRE), TGACGTCA. Over 100 genes with functional CREs have been identified thus far (Mayr & Montminy, 2001). Interestingly, prostaglandin E_2 (PGE_2), a product of the cyclooxygenase pathway produced in response to a variety of inflammatory signals, is able to activate AC, and several of the effects of prostaglandins are attributable to elevated cAMP levels and subsequent activation of PKA (Negishi et al., 1993).

In this study, we have examined the molecular bases for functional interactions between the cAMP/PKA pathway and TGF-β signaling in epithelial cells, using the human immortalized keratinocyte cell line HaCaT (Boukamp et al., 1988). We provide evidence that cAMP-elevating agents block SMAD3/4-specific transcription in response to TGF-β, as well as *PAI-1* gene expression at the transcriptional level. cAMP neither inhibits SMAD nuclear translocation nor DNA binding of SMAD3/4 signaling complexes to their specific target sequence. On the other hand, elevated cAMP levels interfere with SMAD-driven transcription via PKA-dependent disruption of the association of SMAD proteins with their transcriptional co-activators CBP and p300.

RESULTS

cAMP-elevating agents antagonize TGF-β-driven SMAD3/4-dependent gene expression via PKA/CREB activation

Several agents known to elevate intracellular cAMP levels were tested for their ability to affect TGF-β-driven transactivation of $(CAGA)_9$-lux, an artificial SMAD3/4-specific reporter construct consisting of nine repeats of the SMAD3/4-specific recognition sequence, CAGA, cloned upstream of the SV40 minimal promoter and driving the expression of the luciferase gene (Dennler et al., 1998). As shown in Fig. 1A, and as expected from previous observations, TGF-β efficiently transactivated $(CAGA)_9$-lux, up to 100-fold above control values. A 30-min pre-incubation of cultured HaCaT keratinocytes with increasing concentrations of either the membrane-permeable cAMP analog db-cAMP, the phosphodiesterase inhibitor iso-butyl-methylxanthine (IBMX), or the AC activator forskolin, resulted in a dose-dependent inhibition of TGF-β-induced SMAD3/4-specific transactivation of $(CAGA)_9$-lux. Inhibition of TGF-β-induced SMAD response by cAMP-elevating agents was not restricted to HaCaT keratinocytes, as similar results were obtained in WI-26 human lung fibroblasts and in the human metastatic melanoma cell line 1205Lu (not shown), suggesting that such suppressive effect of cAMP on TGF-β-induced SMAD signaling is a general phenomenon not dependent on a particular cell type.

We subsequently tried to determine whether expression of downstream effectors of the cAMP pathway were able to directly interfere with SMAD-dependent transcription induced by TGF-β. For this purpose, expression vectors for the catalytic subunit of PKA or the CREB transcription factor were co-transfected together with $(CAGA)_9$-lux before TGF-β stimulation of HaCaT cells. As shown in Fig. 1B, both PKA and CREB overexpression inhibited TGF-β-driven transactivation of the SMAD3/4-specific construct $(CAGA)_9$-lux. Furthermore, co-

expression of PKA and CREB resulted in additive inhibition of $(CAGA)_9$-lux induction by TGF-β□ □ □ □ □ □1□ □. CREB effect was dependent on PKA activity, as S133-CREB (Gonzalez & Montminy, 1989), a CREB mutant in which the PKA-specific phosphorylation site serine 133 has been substituted to an alanine and exerts a dominant-negative effect against PKA-induced cAMP-dependent transcription (Fig. 1C), did not antagonize TGF-β-induced SMAD3/4-dependent transactivation (Fig. 1D).

The ability of forskolin and db-cAMP to activate cAMP-dependent gene transactivation in HaCaT cells was verified in transient transfections using pCRE-lux. As shown in Fig. 2A, forskolin and db-cAMP efficiently transactivated the CRE-dependent construct, 12- to 16-fold above unstimulated control values. H-89, a specific PKA inhibitor, completely abolished CRE-dependent response to these cAMP-elevating agents. We next examined the capacity of db-cAMP to induce CREB phopshorylation, and the ability of H-89 to interfere with such process in HaCaT cells. Results are shown in Fig. 2B: Exposure of HaCaT keratinocytes to exogenous db-cAMP resulted in the rapid phosphorylation of CREB, which was totally abolished by H-89, consistent with our data obtained on CRE-dependent gene response to cAMP-elevating agents (see above Fig. 2A). Together, these results establish the functionality of the cAMP-PKA-CREB cascade in HaCaT keratinocytes and warrant further investigation of the interference of cAMP-elevating agents with the SMAD pathway in our experimental system.

We have previously shown that the pro-inflammatory cytokine Tumor Necrosis Factor-α (TNF-α) antagonizes TGF-β/SMAD signaling in a c-Jun-dependent manner (Verrecchia et al., 2000). To verify the stimulus specificity of PKA in antagonizing TGF-β signaling, HaCaT keratinocytes were transfected with $(CAGA)_9$-lux, then treated with either db-cAMP or TNF-α, in the absence or presence of H-89 at 20 μM. This concentration completely inhibits cAMP-induced, PKA-dependent, CREB phosphorylation (see Fig. 2B). As shown in Fig. 2C, both db-cAMP and TNF-α inhibited SMAD3/4-dependent transactivation by 47% and 66%,

respectively. H-89 alone strongly potentiated TGF-β effect. Interestingly, H-89 had little, if any, effect against TNF-α-driven inhibition of SMAD3/4-dependent transcription (-45% vs. –47%) whereas it completely prevented the inhibitory activity of db-cAMP (-11% vs. –66%). The enhancement of TGF-β response by H-89 is likely representative of an inhibitory effect of basal PKA activity against TGF-β/SMAD signaling, derepressed by H-89. Of note, a similar potentiation of TGF-β-driven SMAD-specific transactivation was observed when HaCaT cells were pretreated with 2',5'-dideoxyadenosine, a potent inhibitor of AC (data not shown). Together, these results indicate that basal AC and PKA activities play a negative regulatory role against TGF-β-induced SMAD signaling. On the other hand, it appears that PKA is not involved in the antagonistic activity exerted by TNF-α against SMAD signaling, consistent with our data identifying AP-1 as a critical component of TNF-α inhibitory effect (Verrecchia et al., 2000), requiring functional Jun-N-terminal kinase (JNK) activity (Verrecchia et al., 2003).

cAMP does not affect SMAD nuclear translocation and capacity to form complexes with DNA in response to TGF-β

Several approaches were taken to gain insight into the molecular mechanisms underlying the inhibitory activity of the cAMP pathway against SMAD3/4 signaling. First, we examined whether db-cAMP was able to interfere with the translocation of SMAD proteins induced by TGF-β. To this aim, HaCaT keratinocytes were or not pre-treated with db-cAMP before TGF-β was added for 30 min. An antibody directed against SMAD2 and SMAD3, the specific R-SMAD substrates for activated TβRI, was used to determine their cellular localization (Fig. 3A). Slides were counterstained with DAPI to localize cell nuclei precisely (right panels). As shown in the upper left panel, weak and diffuse signal specific for SMAD2/3 was present in both cytoplasm and nuclei from unstimulated HaCaT cells. TGF-β induced a rapid and

complete nuclear translocation of all SMAD2/3-specific immunoreactivity (middle left panel), which was not affected by a 30-min. pre-incubation with db-cAMP (lower left panel) otherwise sufficient to interfere with SMAD-dependent transcription (see Fig. 1).

We next tried to determine whether cAMP may interfere with SMAD/DNA complex formation. HaCaT keratinocytes were incubated with TGF-β for 30 min, an incubation time shown to be optimal for maximal SMAD/DNA complex formation and detection (Vindevoghel et al., 1998), without or with db-cAMP added for various time lapses before TGF-β. SMAD3/4-DNA complexes were detected in EMSA experiments using a radiolabeled 3xCAGA probe (Dennler et al., 1998). As shown in Fig. 3B (upper panel), consistent with previous observations (Verrecchia et al., 2000; Vindevoghel et al., 1998), a 30-min. incubation of HaCaT cells with TGF-β resulted in the formation of a unique protein/DNA complex (lane 2) that was supershifted with an anti-SMAD3 antibody (lower panel), attesting for its identity as a TGF-β-specific SMAD/DNA complex. Pre-incubation of the cells with db-cAMP for 2, 5 and 10 min. prior to TGF-β addition had no effect of SMAD/DNA complex formation (Fig. 3B, upper panel, lanes 4, 6 and 8 vs. lane 2). From these experiments, we conclude that elevated cAMP levels do not affect the nuclear translocation of SMADs in response to TGF-β, nor do they affect the formation of SMAD/DNA complexes.

CBP/p300 squelching by activated CREB is central to the interference of the cAMP cascade with SMAD signaling

Another possibility by which the cAMP pathway may interfere with SMAD-dependent transcription is at the level of recruitment of transcriptional co-activators. The role for CBP and p300 as essential co-activators for SMAD-driven gene expression has been well documented (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998; Shen et al., 1998; Topper et al., 1998). Competition for CBP/p300 has also been suggested to mediate some examples of

signal-induced transcriptional repression. Indeed, in the context of SMAD signaling, CBP/p300 squelching by c-Jun or Stat1 may explain, at least in part, the antagonism exerted by TNF-α and interferon-γ against SMAD signaling (Ghosh et al., 2001; Verrecchia et al., 2000).

As a first approach to determine whether the cAMP pathway could interfere with SMAD3-CBP or SMAD3-p300 interactions, we used a method based on the mammalian two-hybrid system. In a first set of experiments, HaCaT keratinocytes were transfected with a Gal4-lux reporter construct together with a Gal4 DNA binding domain-SMAD3 fusion protein (Gal4-SMAD3) expression vector, in the absence or presence of a VP16 activation domain-CBP (VP-CBP) fusion expression vector. As shown in Fig. 4A, Gal4-SMAD3 transactivated Gal4-lux in the presence of TGF-β, representative of the transactivation property of phosphorylated SMAD3. Co-expression of VP-CBP together with Gal4-SMAD3 strongly enhanced luciferase activity, representative of SMAD3-CBP interaction. The latter was efficiently abolished by exogenously added db-cAMP, as well as by forskolin (not shown). In another set of experiments, HaCaT keratinocytes were transfected with a Gal4-lux reporter construct together with a Gal4-p300 expression vector, in the absence or presence of a VP-SMAD3 fusion expression vector. Similar to the data described above on the association of SMAD3 with CBP, SMAD3-p300 interactions were also abolished by exogenous db-cAMP (Fig. 4B), suggesting that activation of the PKA pathway affects the association of SMAD3 with both transcriptional coactivators CBP and p300.

Because the amount of p300 is limiting within the cell nucleus (Hottiger et al., 1998), formation of CREB/p300 complexes may reduce the amount of p300 available to Smad3 for optimal transcription. If this scenario is correct, then, overexpression of p300 should overcome the inhibitory effect of cAMP on TGF-β/SMAD3 activation of the $(CAGA)_9$-lux construct. To test this hypothesis, we measured the effect of p300 overexpression on db-cAMP inhibition of $(CAGA)_9$-lux transactivation by TGF-β. As shown in Fig. 4C, p300 overexpression prevented

the inhibitory effect of db-cAMP. It should also be noted that p300 overexpression resulted in increased $(CAGA)_9$-lux activity in response to TGF-β stimulation, a result which is consistent with the concept of limited availability of p300, and confirms the role of p300 as a SMAD3 coactivator (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998; Shen et al., 1998; Topper et al., 1998).

From the data presented above, it appears that elevation of intracellular cAMP directly affects the interaction of SMAD3 with its transcriptional co-activators CBP and p300. This effect is probably due to p300/CBP squelching from SMAD complexes by CREB, resulting in the blockade of SMAD-specific transcription.

cAMP–elevating agents interfere with TGF-β-induced, SMAD-dependent, PAI-1 gene expression

The data presented above and obtained using artificial, pathway-specific reporter constructs led us to examine whether cAMP-elevating agents interfere with the activation of a natural, prototypic TGF-β/SMAD target. As a first approach to test this hypothesis, HaCaT cells were transfected with p800-lux, which consists of 800 bp of the human *PAI-1* promoter driving the expression of the luciferase gene (Keeton et al., 1991). As shown in Fig. 5A, db-cAMP dose-dependently inhibited TGF-β-driven p800-lux transactivation. In addition, both forskolin and IBMX efficiently antagonized TGF-β effect. Such antagonism of db-cAMP against TGF-β-driven p800-lux transactivation was blocked by the PKA inhibitor H-89 (Fig. 5B). In another set of experiments, p800-lux was co-transfected with an expression vector for the catalytic subunit of PKA. As shown in Fig. 5C, TGF-β-driven *PAI-1* promoter transactivation was blocked by overexpression of PKA. Together, these results demonstrate efficient transcriptional repression of TGF-β-induced *PAI-1* promoter transactivation by cAMP-

elevating agents in a PKA-dependent manner, similar to what is observed when using the SMAD3/4-specific $(CAGA)_9$-lux reporter construct.

To determine whether the regulation of endogenous *PAI-1* followed the same regulatory pattern as p800-lux, HaCaT keratinocytes were incubated for 24 to 48 h with TGF-β in the absence or presence of either db-cAMP or forskolin. PAI-1 production was then assessed by Western blotting. As shown in Fig. 5D (upper panel), TGF-β strongly elevated PAI-1 production by keratinocytes, but this effect was efficiently blocked by both db-cAMP and forskolin. In the mean time, actin levels were not altered by any of the treatments. Quantitative analysis of the blots with a phosphorimager revealed that db-cAMP and forskolin inhibited TGF-β effect by 70 to 80%.

PGE_2 represses TGF-β-induced SMAD-dependent gene transcription in a PKA-dependent manner

To validate our findings in the context of a natural cAMP-elevating agent, we examined the effects of exogenous PGE_2 on TGF-β-dependent SMAD-specific transcription and *PAI-1* gene regulation. In a first set of experiments, we first verified the ability of PGE_2 to activate CREB-dependent transcription, representative of the activation of the cAMP signaling cascade. HaCaT keratinocytes were transfected with pCRE-lux. As shown in Fig. 6A, efficient transactivation of pCRE-lux was observed in response to exogenous PGE_2, which could be totally prevented by the PKA inhibitor H-89. Next, HaCaT keratinocytes were transfected with $(CAGA)_9$-lux. As shown in Fig. 6B, PGE_2 antagonized TGF-β-driven SMAD3/4-dependent transactivation. In another series of experiments, HaCaT cells transfected with the *PAI-1* promoter/reporter construct p800-lux. Similar to the effect observed on $(CAGA)_9$-lux, PGE_2 antagonized TGF-β-induced *PAI-1* promoter activity, an effect that was abolished by H-89

(Fig. 6C). Taken together, these results indicate that a PKA-dependent mechanism mediates PGE_2 action against TGF-β-driven SMAD3/4 signaling and resulting *PAI-1* transactivation.

DISCUSSION

Our results provide definitive evidence that cAMP-elevating agents interfere with TGF-β-specific SMAD3/4-dependent gene expression in a PKA-dependent manner. Such interference with SMAD-dependent transcription occurs in the nucleus via reduction of SMAD3-CBP/p300 interactions and possible sequestration of the transcriptional co-activators CBP and p300 by phosphorylated CREB, whereas SMAD translocation in response to TGF-β is not affected. Likewise, the ability of SMAD proteins to bind DNA *in vitro* is not affected by cAMP-inducing agents. Such a mechanistic model applies to the regulation of PAI-1, a natural TGF-β/SMAD target. It also provides a molecular explanation to the inhibitory effect of cAMP-elevating agents, membrane-permeable 8-Br-cAMP and the AC activator cholera toxin, against TGF-β-induced connective tissue growth factor (CTGF) gene expression and collagen deposition (Duncan et al., 1999), since the CTGF, PAI-1 and fibrillar collagen genes are direct SMAD targets (Dennler et al., 1998; Leask et al., 2001; Verrecchia et al., 2001a).

Escape of epithelial cells from TGF-β growth control is a hallmark of many cancers. The presence of inactivating mutations in genes encoding TGF-β receptors and SMADs in human carcinomas and various studies of tumor development in mouse models underscore the role of TGF-β signaling as a tumor suppressor pathway controlling epithelial cell growth and maintaining genetic stability in early carcinogenesis (de Caestecker et al., 2000; Gold, 1999; Reiss, 1999). Inversely, TGF-β may become pro-oncogenic at later stages of carcinogenesis, and high levels of TGF-β expression correlate with the advanced clinical stage of a tumor. Tumor-derived TGF-β exacerbates the malignant phenotype of transformed and tumor-derived cells, and may contribute to tumor growth indirectly by suppressing immunosurveillance, stimulating the production of pro-angiogenic factors, or facilitating invasion and metastasis (de

Caestecker et al., 2000; Gold, 1999; Reiss, 1999). It is expected that deciphering the complex cross-talks between the SMAD cascade and other signaling pathways will lead to a better understanding of the bifunctional tumor suppressor/oncogenic role of TGF-β that depends on the state of tumorigenesis. It may be interpreted that chemoprevention is likely to require mechanisms that potentiate TGF-β/SMAD signaling, whereas, on the other hand, late-stage carcinogenesis will require approaches that either inhibit TGF-β signals to allow immune rejection of tumors, or restore TGF-β growth control in tumor cells.

A growing body of evidence indicates that cyclooxygenase, hence prostaglandin synthesis, is involved in carcinogenesis, inducing the production of angiogenic factors and enabling resistance to apoptosis of cancer cells (Sheng et al., 1998; Tsujii et al., 1998). PGE_2, which stimulates the growth of both normal colon and colon cancer cells (Sheng et al., 2001), exhibits elevated levels in colorectal cancer (Levy, 1997). Conversely, cyclooxygenase inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to exert anti-neoplastic effects, and to decrease the risk of colorectal, breast or oesophagal carcinomas (Corley et al., 2003; Johnson et al., 2002; Smalley et al., 1999). Similarly, PKA inhibitors may also be considered as therapeutic agents for cancer therapy, as it has been shown that combined blocade of PKA and EGF receptor activities exerts a synergistic antitumor effect in a variety of experimental models both *in vitro* and *in vivo* (Tortora & Ciardiello, 2002). Moreover, PKA inhibitors cooperate with cytotoxic drugs and ionizing radiations to inhibit tumor growth and angiogenesis (Bianco et al., 2000; Tortora et al., 1997).

In the present report, we have shown that agents that activate PKA, e.g. PGE_2 and cAMP analogs, are able to antagonize SMAD signaling. These results provide a mechanistic explanation for some of the observed effects of NSAIDs and PKA inhibitors in the context of chemoprevention and cancer treatment, as these drugs, through the modulation of PKA

activity, may directly interfere with SMAD signaling initiated by TGF-β, a complex gatekeeper in carcinogenesis (de Caestecker et al., 2000; Gold, 1999; Reiss, 1999).

MATERIALS AND METHODS

Cell culture and biochemical reagents. The human epidermal HaCaT keratinocyte and human lung WI-26 fibroblast cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 mg/ml streptomycin-G and 0.25 mg/ml Fungizone™) in a humidified atmosphere of 5% CO_2. The human melanoma cell line 1205-Lu, kind gift from Dr. M. Herlin (Wistar Institute, Philadelphia, PA) shown previously to have functional, even exacerbated, SMAD signaling (Rodeck et al., 1999), was grown in a composite medium (W489) consisting of three parts MCDB153 and one part L15 supplemented with 4% FCS. Human recombinant TGF-β1, as well as recombinant human TNF-α, was purchased from R&D Systems Inc. (Minneapolis, MN). It is referred to as TGF-β throughout the text. PGE_2, forskolin, 3-isobutyl-1-methylxanthine (IBMX), dibutyryl-cAMP (db-cAMP), and the PKA inhibitor H-89 were purchased from Sigma Chemical Co, Saint-Louis, MO.

Immunofluorescence. Cells seeded into 8-well tissue chambers (Lab-Tek, Nalge Nunc Int, Naperville, IL) were fixed and permeated with 3% paraformaldehyde for 15 min., followed by Tween 0,5% for additional 30 min. Non specific binding was blocked with BSA 0,1%. Endogenous SMAD2/3 content was visualized by incubation for 1 h at room temperature with a monoclonal anti-SMAD2/3 antibody (1:200, Transduction Laboratories, Lexington, Kentucky). After washing, bound antibodies were visualized by secondary sheep anti-mouse IgG antibodies conjugated with Cy3 (1:250, Sigma). After 3 final washes, slides were counterstained with DAPI to visualize cell nuclei, mounted in Vectashield™ (Vecta Laboratories, Burlingame, CA) and stored at +4°C protected from light until use. For negative

controls, whole mouse serum was used instead of the primary antibody or, the primary antibody was omitted. Cells were imaged with a confocal laser scanning microscope (BioRad Cell Science Division, Hemel Hempstead, U.K.).

Plasmid Constructs. $(CAGA)_9$-Lux, a gift from Drs. S. Dennler and J.-M. Gauthier (Glaxo-Welcome, Les Ulis, France), a SMAD3/4-specific reporter has been described previously (Dennler et al., 1998). p800-Lux, which contains 800 bp of human PAI-1 promoter linked to the luciferase gene, was a kind gift of Dr. D.J. Loskutoff (Research Institute of Scripps Clinic, La Jolla, CA)(Keeton et al., 1991). pCRE -lux (Mercury™ pathway luciferase system, Clontech, Palo Alto, CA) was used to determine CRE-driven transcription. The GAL4-luciferase plasmid G5E1b-Luc (Gal4-lux) containing five GAL4 binding sites driving the expression of luciferase and the Gal4-SMAD3 fusion protein expression vector have been described previously (Atfi et al., 1997). Additional expression vectors used in this study were: Gal4-p300 (a kind gift from Dr. A. Giordano, Thomas Jefferson University, Philadelphia, PA) (Yuan et al., 1996), Gal4-CREB, VP-CBP, the catalytic subunit of PKA (Molina et al., 1993), and pCMV-CREB (BD Biosciences-Clontech, Palo Alto, CA), pCMV-CREB133 (Gonzalez & Montminy, 1989) encoding the S133-CREB mutant that cannot be phosphorylated by PKA (a gift from Dr. D.D. Ginty, Johns Hopkins University Medical School, Baltimore, MD).

Transient cell transfections, reporter- and mammalian two-hybrid assays. For reporter assays, cells were seeded in 35 mm diameter dishes and transfected at approximately 60-70% confluency with a liposome-based protocol (Fugene™, Roche Diagnostics, Indianapolis, IN) in fresh medium containing 1% FCS. db-CAMP or PGE_2 was added as needed, 3 to 18 hours after transfection (see *figure legends*). Following incubation, cells were rinsed twice with phosphate-buffered saline, harvested by scraping and lysed in 100 µl of reporter lysis buffer

(Promega Corp., Madison, WI). Aliquots corresponding to identical protein amounts were used for each reporter assay. Luciferase activity was determined with a commercial assay kit according to the manufacturer's protocol (Promega).

Electrophoresis mobility shift assays. A 39-base pair SMAD3/4-specific probe, 3xCAGA (Dennler et al., 1998), was used to determine the effect of forskolin and db-cAMP on TGF-β-induced SMAD3/4-DNA complex formation. Nuclear extracts were isolated using a small-scale preparation (Andrews & Faller, 1991), aliquoted into small fractions to avoid repetitive freeze-thawing, and stored at -80 °C until use. The protein concentration in the extracts was determined using a commercial assay kit (Bio-Rad Laboratories, Hercules, CA). Binding mixtures were separated electrophoretically on 4% acrylamide gels in 0.5% Tris-Borate-EDTA as described previously (Verrecchia et al., 2001b).

Western blotting. Subconfluent HaCaT cells were placed in fresh medium containing 1% FCS for 24 hours before stimulation with various cAMP-elevating agents. The cells were washed with cold PBS and lysed by addition of SDS lysis buffer (100 mM Tris-HCl (pH 6.8), 1% SDS, 0.1 M dithiothreitol, 0,1 mM orthovanadate) followed by boiling for 5 min. Proteins (50 μg/lane) were separated on 10% or 12% SDS-polyacrylamide gels and transferred to nitrocellulose Hybond ECL$^+$ (Amersham Pharmacia Biotech, Uppsala, Sweden). After blocking with 5% nonfat dry milk in 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature, filters were incubated with either anti-CREB (1:2000 dilution), anti-phospho-CREB (1:1000 dilution) (both from New England Biolabs, Beverly, MA), monoclonal C9 anti PAI-1 (1:200 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA), or anti-actin (Sigma Co., 1:5000 dilution) antibodies in blocking solution overnight at 4°C. After three washes in 20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween 20, membranes were

incubated with species-specific (anti-rabbit or anti-mouse) IgG-horseradish peroxidase-conjugated antibodies (1:5000 dilution) (Santa Cruz Biotechnology Inc.) in blocking solution for 1 h at room temperature. Membranes were then washed three times with wash buffer (see above), and detection of immunoreactive proteins was performed with the ECL^{+}Plus chemiluminescent reagent according to the manufacturer's instructions (Amersham Pharmacia Biotech) using a phsophorimager (Storm 840, Amersham Pharmacia Biotech).

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FIGURES LEGENDS

FIG. 1. The cAMP-PKA-CREB cascade inhibits TGF-β-specific SMAD-dependent transcription. A, Subconfluent HaCaT keratinocytes were transfected with $(CAGA)_9$-lux in medium supplemented with 1 % fetal calf serum. Three hours after transfection, cAMP-elevating agents, db-cAMP, IBMX and forskolin, were added at the indicated concentrations, without (-) or with (+) TGF-β□ □10 ng/ml). Incubations were continued for 20 hours after which the reporter gene activity was determined. B, Subconfluent HaCaT keratinocytes were transfected with $(CAGA)_9$-lux, together with expression vectors for either the catalytic subunit of PKA or CREB. Six hours later, TGF-β (10 ng/ml) was added (+) and incubations continued for another 20 hours before reporter gene activity was determined. C, HaCaT keratinocytes were transfected with pCRE-lux in the absence or presence of pPKA expression vector without (-) or with (+) S133-CREB expression vector. Results of a representative experiment performed on duplicate samples is shown as relative luciferase activity. Note that S133-CREB overexpression prevents PKA-induced CRE transactivation. D, Subconfluent HaCaT cultures were transfected with $(CAGA)_9$-lux, in the absence or presence of pPKA expression vector, with or without S133-CREB expression vector. Six hours later, TGF-β (10 ng/ml) was added (+) and incubations continued for another 20 h before reporter gene activity was determined. Results in panels A, B and D are presented as a percentage of construct activation in cells receiving TGF-β alone, and represent the mean $\pm$ SD of three independent experiments performed each on duplicate samples.

FIG. 2. CRE-dependent response in HaCaT keratinocytes. A, Subconfluent HaCaT cells were transfected with pCRE-lux in medium supplemented with 1 % fetal calf serum. Three hours after transfection, A, db-cAMP (1 mM) or forskolin (20 μM) was added in the absence (-

) or presence (+) of the PKA inhibitor H-89 (20 μM). Incubations were continued for 20 hours after which reporter gene activity was determined. B, Subconfluent HaCaT keratinocyte cultures were treated (+) or not (-) with db-cAMP (1 mM), in the absence or presence of H-89 (20 μM). Thirty min. later, cells were lysed and samples processed for Western blotting with specific antibodies for phospho-CREB and CREB. C, Subconfluent HaCaT cells were transfected with $(CAGA)_9$-lux in medium supplemented with 1 % fetal calf serum. Three hours after transfection, TNF-α (10 ng/ml) or db-cAMP (1 mM) was added in the absence (-) or presence (+) of H-89, 30 min. before addition of TGF-β (10 ng/ml). Promoter activity was determined 20 h later. Results, presented as relative promoter activity, are the mean ± SD of three experiments performed each on duplicate samples.

FIG. 3. cAMP does not alter SMAD nuclear translocation and SMAD/DNA binding in response to TGF-β. A, Subconfluent HaCaT cultures in 8-well tissue chambers were incubated for 30 min. with db-cAMP (1mM) before additon of TGF-β (10 ng/ml). Thirty min. later, cells were fixed and processed for visualization of endogenous SMAD2/3 (panel A, left column) or with DAPI to visualize cell nuclei (panel A, right column) with a confocal laser scanning microscope. Similar gain settings were used to compare control and TGF-β-treated samples. B, EMSAs were performed using the SMAD3/4-specific 3xCAGA oligonucleotide (9) as a probe, together with nuclear extracts from HaCaT keratinocyte cultures treated for 30 min. with TGF-β (10 ng/ml) after pre-incubation with db-cAMP (1 mM) for various time periods, as indicated. The identity of the protein/DNA complexes induced by TGF-β was verified with an anti SMAD3 antibody (lower panel), as described (43).

FIG. 4. db-cAMP prevents SMAD3-CBP and SMAD3-p300 interactions in a mammalian two-hybrid assay. A, Subconfluent HaCaT keratinocyte cultures were transfected with Gal4-

lux and Gal4-SMAD3, without (-) or with (+) VP-CBP. Six hours later, TGF-β (10 ng/ml) was added where indicated, following (+) or not (-) a 30 min. pre-incubation with db-cAMP (1 mM). Gal4-lux activity was determined 20 h later. B, Subconfluent HaCaT keratinocyte cultures were transfected with Gal4-lux and Gal4-p300, without (-) or with (+) VP-SMAD3. Six hours later, TGF-β (10 ng/ml) was added where indicated, following (+) or not (-) a 30 min. pre-incubation with db-cAMP (1 mM). Gal4-lux activity was determined 20 h later. C, Subconfluent HaCaT keratinocytes were transfected with $(CAGA)_9$-lux, together with either empty pRSVe or p300 expression vector. Six hours later, db-cAMP (1 mM) was added and incubations continued for another 20 hours in the absence (-) or presence (+) of TGF-β (10 ng/ml) before reporter gene activity was determined. Results from representative experiments performed with duplicate samples are shown.

FIG. 5. The cAMP-PKA-CREB cascade inhibits TGF-β-induced PAI-1 gene expression. A, Subconfluent HaCaT keratinocytes were transfected with the PAI-1 promoter/reporter gene construct p800-lux in medium supplemented with 1 % fetal calf serum. Three hours after transfection, cAMP-elevating agents, db-cAMP, IBMX and forskolin, were added at the indicated concentrations, without (-) or with (+) TGF-β□ □10 ng/ml). Incubations were continued for 20 hours after which the reporter gene activity was determined. B, Subconfluent HaCaT keratinocytes were transfected with p800-lux. Three hours after transfection, cells were treated (+) or not (-) with H-89. One hour later, db-cAMP was added, without (-) or with (+) TGF-β□ □10 ng/ml) C, Subconfluent HaCaT keratinocytes were transfected with p800-lux, together with empty RSVe or PKA expression vector. Six hours later, TGF-β (10 ng/ml) was added (+) and incubations continued for another 20 hours before reporter gene activity was determined. D, subconfluent HaCaT keratinocyte cultures were treated (+) or not (-) with either db-cAMP (1 mM) or forskolin (20 μM) for 30 min. before TGF-β (10 ng/ml) was added.

Twenty-four hours later, cells were lysed and samples processed for Western blotting with specific antibodies for PAI-1 and actin. Results from a representative experiment are shown.

FIG. 6. PGE_2 inhibits TGF-β-induced SMAD-dependent transcription and PAI-1 promoter transactivation. Subconfluent HaCaT keratinocyte cultures were transfected with pCRE-lux (panel A), $(CAGA)_9$-lux (panel B) or p800-lux (panel C) in medium supplemented with 1 % fetal calf serum. Three hours after transfections, PGE_2 (1 μM) was added, in the absence or presence of H-89. TGF-β□ □10 ng/ml) was added 30 min. later, where indicated. Incubations were continued for 20 hours after which the reporter gene activity was determined. Results, expressed as relative promoter activity, are mean ± SD of three separate experiments performed with each construct.