Role of Smad Proteins and Transcription Factor Sp1 in p21Waf1/Cip1 Regulation by Transforming Growth Factor-β*

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Transforming growth factor-β (TGF-β) inhibits cell cycle progression, in part through up-regulation of gene expression of the p21Waf1/Cip1 (p21) cell cycle inhibitor. Previously we have reported that the intracellular effectors of TGF-β, Smad3 and Smad4, functionally cooperate with Sp1 to activate the human p21 promoter in hepatoma HepG2 cells. In this study we show that Smad3 and Smad4 when overexpressed in HaCaT keratinocytes lead to activation of the p21 promoter. Activation requires the binding sites for the ubiquitous transcription factor Sp1 on the proximal promoter. Induction of the endogenous HaCaT p21 gene by TGF-β1 is further enhanced after overexpression of Smad3 and Smad4, whereas dominant negative mutants of Smad3 and Smad4 and the inhibitory Smad7 all inhibit p21 induction by TGF-β1 in a dose-dependent manner. We show that Sp1 is a proximal promoter sequence that is required for efficient activation of the p21 promoter by wild type Smads. We also show that DNA-binding domain mutants of Smad3 and Smad4 are capable of transactivating the p21 promoter as efficiently as wild type Smads. Co-expression of Smad3 with Smad4 and Sp1 in SL-2 cells or co-incubation of phosphorylated Smad3, Smad4, and Sp1 in vitro results in enhanced binding of Sp1 to the proximal promoter sequences. We demonstrate that Sp1 physically and directly interacts with Smad2, Smad3, and weakly with Smad4 via their amino-terminal (Mad-Homology 1) domain. Finally, by using GAL4 fusion proteins we show that the glutamine-rich sequences in the transactivation domain of Sp1 contribute to the cooperativity with Smad proteins. In conclusion, Smad proteins play important roles in regulation of the p21 gene by TGF-β, and the functional cooperation of Smad proteins with Sp1 involves the physical interaction of these two types of transcription factors.

* This research was supported in part by a grant from the Human Frontier Science Program (to D. K. and A. M.), Institute of Molecular Biology and Biotechnology for internal funds (to D. K.), and a grant from the Dutch Cancer Society (to P. t. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a STINT post-doctoral fellowship from the Swedish Foundation for International Cooperation in Research and High Education.

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1 The abbreviations used are: TGF-β, transforming growth factor β; CKI, cyclin-dependent kinase inhibitor; Smad, Smad and Mad-related protein; MH, Mad-Homology; SBE, Smad-binding element; ALK, activin receptor-like kinase; CA, constitutively active; HaCaT, human keratinocyte cell line; HepG2, human hepatoma cell line; m.o.i., multiplicity of infection; GEMSA, gel electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; DDEEM, Dulbecco’s modified Eagle medium; PMSF, phenylmethylsulfonyl fluoride; CDKs, cyclin-dependent kinases; HA, hemagglutinin; DN, dominant negative; DTT, dithiothreitol.
sequences (17, 18). Sp1 is required for early embryogenesis and regulates the terminal differentiation state of cells by affecting the methylation of DNA CpG islands (19). The transcriptional activity of Sp1 is regulated by phosphorylation in a cell cycle-specific manner, acetylation by the co-activator p300, and glycosylation, which protects this factor from proteasome-dependent degradation (20–22). Sp1 exerts its transcriptional properties by interacting directly with factors of the basal transcription machinery and by cooperating with several transcriptional activators (23–29). Thus, although Sp1 traditionally appeared as a ubiquitous factor primarily serving the core activity of promoters, recent evidence increasingly implicates this protein in several instances of regulated gene transcription.

It is established that transcription factor Sp1 participates in the regulation of the p21 gene by TGF-β (16, 30). The TGF-β signaling pathway utilizes plasma membrane serine/threonine kinase receptors and their cytoplasmic effectors termed the Smad proteins (2, 31). Smad proteins are transcriptional activators that bind to DNA and cooperate with a fast growing list of transcription factors in regulating target gene expression (32–34). In a previous report (35) we have provided evidence for the role of Smad3 and Smad4 in mediating the induction of the p21 gene by TGF-β. The Smads were shown to cooperate functionally with Sp1 and depend on the Sp1-binding sites of the p21 promoter for their action. In addition, we have demonstrated that Jun family members, which are induced and activated by TGF-β, can also regulate the p21 promoter by physically interacting with Sp1 and utilizing the same G/C-rich motifs of the proximal promoter (12). In the present work we demonstrate that Smad3 and Smad4 enhance the level of endogenous HaCaT p21 gene induction by TGF-β, whereas dominant negative Smads and the inhibitory Smad7 block p21 induction by TGF-β in a dose-dependent manner. These results correlate very well with the p21 promoter transcription studies in the same cells. We show that Smads mediate enhancement of the Sp1 affinity for the p21 promoter, independent from a direct association of Smads to DNA, and Smad2, Smad3, and Smad4 physically interact with Sp1. We have mapped the domain of Smad3 and Smad4 required for this interaction and provide evidence for the involvement of specific Sp1 sequences in the functional cooperation between these two classes of transcription factors.

EXPERIMENTAL PROCEDURES

Materials—The purified baculoviral Smad3, TGF-β type I receptor-phosphorylated Smad3 and Smad4 proteins were a generous gift from F. M. Hoffman and A. Comer (36). Restriction enzymes and modifying enzymes (T4 DNA ligase, T4 polynucleotide kinase, Klenow fragment of DNA polymerase I, and calf intestinal alkaline phosphatase) were pur- 

phosphorylated Smad3 and Smad4 proteins were a generous gift from Dr. J. Massague (Memorial Sloan-Kettering Institute, New York). The expression vectors encoding the three deletion mutants of Smad3, Smad3(1–122), Smad3(1–248), and Smad3(122–424) were constructed by polymerase chain reaction amplification of the specified fragments using appropriate primers and subsequent subcloning of the amplified fragments into the expression vector pCDNA1/amp encoding the FLAG-tagged human Smad3 was described previously (12). The expression vector encoding the FLAG-tagged Smad4 protein was the generous gift of Dr. J. Massague (Memorial Sloan-Kettering Institute, New York). The expression vectors encoding the three deletion mutants of Smad3, Smad3(1–122), Smad3(1–248), and Smad3(122–424) were constructed by polymerase chain reaction amplifica-

tion of the specified fragments using appropriate primers and subsequent subcloning of the amplified fragments into the expression vector pCDNA1/amp encoding the FLAG-tagged human Smad4 was described previously (12). The expression vector encoding the FLAG-tagged Smad4 protein was the generous gift of Dr. L. Zhang, Harvard Medical School, Boston. The pGL3-CAT reporter containing five tandem GAL4-binding sites in front of the E1B minimal promoter and the CAT reporter gene was the generous gift of Dr. G. Gill, Harvard Medical School, Boston. The pGL3-CAT reporter containing five tandem GAL4-binding sites in front of the E1B minimal promoter and the CAT reporter gene was the generous gift of Dr. G. Mavrothalassitis, University of Crete Medical School, Heraklion, Greece. The bacterial expression vectors pGEX-Sp1 (GST-Sp1 83–778), pGEX-Sp1 516C (GST-Sp1 516–683), pGEX-Sp1 268C (GST-Sp1 268–435), pGEX-Sp1D (GST-Sp1 268–435), pGEX-Sp1A, pGEX-Sp1B, pGEX-Sp1C, pGEX-Sp1D, and pGEX-Sp1E were the generous gifts of Dr. S. Itoh of the Ludwig Institute, Uppsala, Sweden. The Drosophila expression vectors pPac-Sp1 and pPacO were the generous gifts of Dr. E. Flavey, Section of Molecular Genetics, Boston University Medical Center, Boston. The original Sp1 mutants were the generous gifts of Dr. R. Tjian, University of California, Berkeley. The bacterial expression vectors pGEX-Smad3, pGEX-Smad3MH1, pGEX-Smad3MH2, pGEX-Smad3MH3, pGEX-Smad3MH1, pGEX-Smad3MH1, pGEX-Smad3MH2, pGEX-Smad3MH2, pGEX-Smad3MH2, pGEX-Smad3MH2, and pGEX-Smad4MH2 were the generous gifts of Dr. S. Itoh of the Ludwig Institute, Uppsala, Sweden. The Drosophila expression vectors pPac-Sp1 and pPacO were the generous gifts of Dr. J. M. Horowitz, North Carolina State University, Raleigh, and J. Noti, Guthrie Research Institute, Sayre, PA, respectively. The Drosophila expression vectors pRactH-Smad3 and pRactH-Smad4 were constructed by transferring the corresponding Smad cDNA that includes only the protein-coding region from pCDNA1/amp-Smad3 and pCDNA-Smad4, respectively, into the BamHI and HindIII sites of the pGL3-CAT reporter gene. The pRactH and hsp-lacZ expression vector used for normalization of transfections in Drosophila SL2 cells were the generous gifts of Dr. O. Delidakis, University of Crete, and IMBB, Heraklion, Greece. The quality of all new DNA constructs was verified by DNA sequencing.

Cell Cultures, Transient Transfections, Adenoviral Infections, Reporter, and Western Blot Assays—Human HaCaT keratinocytes, human hepatoma HepG2 cells, and monkey kidney COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum, l-glutamine, and penicillin/streptomycin at 37 °C in a 5% CO₂ atmosphere. Drosophila Schneider’s SL2 cells were cultured in Schneider’s insect medium supplemented with 10% insect culture-tested fetal bovine serum and penicillin/streptomycin at 27 °C. Transient transfections of COS-7 cells for co-immunoprecipitation assays and of Schneider’s SL2 cells for nuclear extract isolation were performed using the liposome reagent Fugene-6 (Roche Molecular Biochemicals). Transient transfections using the calcium phosphate co-precipitation method, chloramphenicol acetyltransferase, luciferase, and β-galactosidase assays were performed as described previously (35). Nuclear extracts were isolated, and immunoblotting was performed as described previously (37). The dominant negative (DN) Smad3 and Smad4 encoding adenoviruses were the generous gifts of Dr. Theodora Fotakis, University of Ioannina, Ioannina, Greece. Under optimal conditions (C24420) antibodies were from Transduction Laboratories. The anti-phosphoserine rabbit polyclonal antibody (Poly-Z-PS1) was from Zymed Laboratories Inc. The anti-mouse horseradish peroxidase-conjugated secondary antibody was from Amersham Pharmacia Biotech. All other chemicals were obtained from the usual commercial sources at the purest grade available.

Plasmid Constructions—The p21 promoter plasmid −2,300/+8 p21 luc has been described previously (35). The p21 promoter deletion construct −143/+8 p21 luc was constructed by transfecting the XbaI to HindIII fragment of the promoter region from the −143/+8 p21 CAT plasmid (35) to pGL2-basic after digestion with XbaI and HindIII. The expression vectors encoding the pDNA−606pSmad2, pCDNA3−606pSmad3, pCDNA3−567pSmad3, pCDNA3−612/CAGA−MLP− luc were generously provided by Dr. S. Itoh of the Ludwig Institute, Uppsala, Sweden. The expression vector pCDNA1/amp encoding the FLAG-tagged human Smad3 was described previously (12). The expression vector encoding the FLAG-tagged Smad4 protein was the generous gift of Dr. J. Massague (Memorial Sloan-Kettering Institute, New York). The expression vectors encoding the three deletion mutants of Smad3, Smad3(1–122), Smad3(1–248), and Smad3(122–424) were constructed by polymerase chain reaction amplification of the specified fragments using appropriate primers and subsequent subcloning of the amplified fragments into the expression vector pCDNA1/amp encoding the FLAG-tagged human Smad4 was described in detail elsewhere. The GAL4/DDB-Sp1 fusion constructs pSG424/GAL4-Sp1A+B, pSG424/GAL4-Sp1B, pSG424/GAL4-Sp1Bn, pSG424/GAL4-Sp1Bc, and the pBXG1 plasmid containing the GAL4 DDB portion only were the generous gifts of Dr. G. Gill, Harvard Medical School, Boston. The pGL3-CAT reporter containing five tandem GAL4-binding sites in front of the E1B minimal promoter and the CAT reporter gene was the generous gift of Dr. G. Mavrothalassitis, University of Crete Medical School, Heraklion, Greece. The bacterial expression vectors pGEX-Sp1 (GST-Sp1 83–778), pGEX-Sp1 516C (GST-Sp1 ΔA), pGEX-Sp1 N619 (GST-Sp1 ΔD), and pGEX-Sp1 Δint 349 (GST-Sp1 ΔB+C) were the generous gifts of Dr. E. Flavey, Section of Molecular Genetics, Boston University Medical Center, Boston. The original Sp1 mutants were the generous gifts of Dr. R. Tjian, University of California, Berkeley. The bacterial expression vectors pGEX-Smad3, pGEX-Smad3MH1, pGEX-Smad3MH2, pGEX-Smad3MH3, pGEX-Smad3MH1, pGEX-Smad3MH1, pGEX-Smad3MH2, pGEX-Smad3MH2, pGEX-Smad3MH2, and pGEX-Smad4MH2 were the generous gifts of Dr. S. Itoh of the Ludwig Institute, Uppsala, Sweden. The Drosophila expression vectors pPac-Sp1 and pPacO were the generous gifts of Dr. J. M. Horowitz, North Carolina State University, Raleigh, and J. Noti, Guthrie Research Institute, Sayre, PA, respectively. The Drosophila expression vectors pRactH-Smad3 and pRactH-Smad4 were constructed by transferring the corresponding Smad cDNA that includes only the protein-coding region from pCDNA1/amp-Smad3 and pCDNA-Smad4, respectively, into the BamHI and HindIII sites of the pGL3-CAT reporter gene. The pRactH and hsp-lacZ expression vector used for normalization of transfections in Drosophila SL2 cells were the generous gifts of Dr. C. Delidakis, University of Crete, and IMBB, Heraklion, Greece. The quality of all new DNA constructs was verified by DNA sequencing.
conditions, more than 90% of the cells were infected as determined by the blue, β-galactosidase-positive staining using a lapZ control virus or the green fluorescent protein autofluorescence (for the DN-Smad3 and DN-Smad4 viruses). Routine infections were performed at a multiplicity of infection (m.o.i.) of 50 with single viruses. HaCaT cells were seeded at a density of 5 × 10^4 cells/well in 24-well tissue culture plates. The next day the cells were transfected with the reporter constructs using the Fugene-6 reagent. Twelve hours later the culture medium was changed to DMEM containing 5% fetal bovine serum and infected with different doses of each virus (see Fig. 2) 1 h later. Twenty four hours later the reporter assays were performed, which corresponds to 49 h post-transfection and 36 h post-infection. For p21 analysis, HaCaT cells were seeded at a density of 10^5 cells/well in 6-well tissue culture plates. Twelve hours later the culture medium was changed to DMEM containing 5% fetal bovine serum and infected with different doses of each virus (see Fig. 2) 1 h later. Twenty four hours later cells were treated with 10 ng/ml TGF-β1 for 20 h, and 20 μg of detergent-soluble cell extracts were analyzed by Western blotting with the p21-specific antibody, FLAG, HA, and Smad1/2/3-specific antibodies served as controls to measure expression of the co-infected proteins, and β-catenin antibody served as control to verify equal protein amount loading. Western analysis of transiently transfected SL2 cell extracts or purified baculoviral Smad3 proteins was performed in the same fashion using the antibodies described under legend.

Relative protein expression levels were quantified using the scanning densitometric software of the PhosphorImager Fujix BAS 2000. Ratios of band intensities of the tested protein (p21) over the control protein (β-catenin) were calculated, and the ground condition ratio was set to 1 or 100% relative to which all other conditions are expressed.

Co-immunoprecipitation Assays—Forty hours post-transfection of COS-7 cells, total detergent extracts were prepared by lysing the cells in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM aprotinin in 4 °C. The cell extracts were clarified from the insoluble material by a brief centrifugation at 10,000 rpm and were pre-cleared by incubation with protein A-Sepharose at 4 °C for 30 min. The pre-cleared extracts were incubated with the FLAG or GAL4-DDB antibody at 4 °C for 2 h, and the immunocomplexes were precipitated with protein-A Sepharose, washed with lysis buffer four times, and dissolved in Laemmli SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer. After 7% SDS-PAGE the resolved proteins were transferred to Hybond-C extra nitrocellulose (Amersham Pharmacia Biotech), and relevant proteins were detected after incubation with the anti-Myc (9E10) antibody followed by anti-mouse horseradish peroxidase-conjugated secondary antibody and homemade enhanced chemiluminescent substrate for autoradiography. Theobliteration of the expressed proteins was monitored by SDS-PAGE and Coomassie Brilliant Blue staining. All proteins were obtained at rather high levels and in relatively pure form as only the primary protein species were detectable without significant degradation products (Fig. 6, B, E, and H).

**GST Protein Interaction Assays—**Interaction assays of GST-Sp1 proteins with in vitro synthesized Smad proteins were performed as described previously (12). For the interaction assays of GST-Sp1 proteins with endogenous HaCaT Sp1, total cell extracts were prepared by lysis in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, 1% aprotinin, 50 mM NaF, 25 mM β-glycerophosphate, 1 mM NaOVO₃. Aliquots of the extracts corresponding to approximatively 10^5 cells were incubated with the glutathione-Sepharose beads carrying 10 μg of the GST-Smad fusions for 5 h at 4 °C. The bound proteins were washed with the lysis buffer, dissolved in Laemmli SDS loading buffer, resolved by 8% SDS-PAGE, and analyzed by Western blotting using the Sp1 antibody and the appropriate chemiluminescent substrate for autoradiography. Nuclear extracts from transiently transfected Schneider’s SL2 cells were prepared 40 h post-transfection by hypotonic lysis of the cells in 10% (v/v) glycerol, 20 mM Hepes, pH 7.9, 0.1 mM EDTA, 50 mM KCl, 2 mM DTT, 0.5 mM spermidine, 50 mM NaF, 1 mM NaOVO₃, 1 mM PMSF, 10 μM aprotinin by three sequential freeze (liquid nitrogen)-thaw (4 °C) cycles. The nuclear pellets obtained by centrifugation at 4,000 × g were solubilized by slow rotation for 45 min in hyperton buffer, 20% glycerol, 20 mM Hepes, pH 7.9, 0.1 mM EDTA, 600 mM KCl, 2 mM DTT, 50 mM NaF, 1 mM NaOVO₃, 1 mM PMSF, 10 mM aprotinin at 4 °C. The soluble nuclear extracts were collected after centrifugation at 12,000 × g, were aliquoted, flash-frozen, and stored at −80 °C. The immunoprecipitated Smad3 and Smad4-encoding cytoplasmic extracts were used for β-galactosidase assays to calibrate the extracts for transfection efficiency. The total protein in the nuclear extracts was measured by Bradford assay (Bio-Rad protein assay kit). The abundance of the transfected proteins in the nuclear extracts was estimated by Western blot analysis after 7% SDS-PAGE, using the relevant antibodies and enhanced chemiluminescence substrate for autoradiography.

Gel electrophoretic mobility shift assays (GEMSAs) were performed as described previously (12). The sequences of the oligonucleotides from the p21 promoter and the consensus Sp1 site used in the GEMSA experiments were described by Kardassis et al. (12). The sequence of the sense strand of the mutant double-stranded 5′-TGGTGCACCCTCTCCTAG-3′, where the underline indicates the mutated nucleotides. For comparison, the wild type oligonucleotide sequence is 5′-GGGTCGGCCTCCTAG-3′ (Fig. 1A). The quadruple Smad-binding element (SBE4) oligonucleotide was described by Jonk et al. (38). Oligonucleotides corresponding to the p21 promoter regions were synthesized, annealed, labeled with [α-32P]dCTP, and 10 fmol were incubated with 200 ng of each purified Smad protein or with aliquots of nuclear extracts from the transfected SL2 andigaved cells that were calibrated for total protein content, β-galactosidase activity, and specific protein abundance based on the Western blot signals. For the Smad-binding depletion experiment, purified Smad proteins were first incubated with excess (4 pmol) cold SBE4 oligonucleotide for 10 min at 4 °C, and then the Sp1 protein was added and incubated for another 10 min, and finally the labeled p21 promoter oligonucleotide was included in the reaction that proceeded for 30 min prior to 4% PAGE.

Relative bandshift intensities were quantified using the scanning densitometric software of the PhosphorImager Fujix BAS 2000. The ground condition (Sp1 alone) was set to 1 or 100% relative to which all other conditions are expressed.

**RESULTS**

**Smad3 and Smad4 Can Transactivate the p21 Promoter in Human HaCaT Keratinocytes—**The proximal (−124/−42) region of the human p21 promoter is G/C-rich and contains 5 sequence motifs that resemble or match exactly the recognition sequence of the ubiquitous transcription factor Sp1 (5′-GGGCGG-3′, Fig. 1A, double underline). The constitutive activity and induction of this promoter by extracellular signals in different cell systems depends on some of the Sp1-like motifs (16, 35, 39). Critical for TGF-β-mediated induction of the p21 promoter is one of these Sp1 sites (designated θp3RE in Fig. 1A) (16). Smads were found capable of transactivating the proximal p21 promoter in HepG2 cells (35). However, similar experiments performed in HaCaT cells failed to demonstrate Smad-mediated transactivation of the p21 promoter (data not shown and see Refs. 11 and 40). A reason for this could be the low Smad expression levels achieved after the inefficient transient transfection of HaCaT cells with various protocols (data not shown). Adenovirus-mediated gene transfer is highly efficient (80–95% infection rate), and relatively high levels of expression of the encoded protein can be obtained (37, 41). We thus combined transient transfections of HaCaT cells with three different p21 promoter-luciferase reporters (−2,300/+8 p21 Luc, −2,500/+8 S3p1 p21 Luc, and −143/+8 p21 Luc) with transient infections with Smad3- and Smad4-encoding viruses. To stimulate the TGF-β pathway we utilized another adenoviral vector encoding the constitutively active (CA) TGF-β type I receptor also termed activin receptor-like kinase 5, Akr1b1 (26). Akr1b1 was shown in Fig. 1B, CA-ALK-5 overexpression led to a moderate but distinct 2.2-fold activation of the −2,300/+8 p21 promoter. Overexpression of either Smad3 or Smad4 by means of the adenovirus system also resulted in the same level (2.2-fold) of transactivation in the absence of activated ALK-5 receptor (Fig. 1B). In the presence of the activated receptor the effect of...
the Smads was further augmented. Co-expression of Smad3 and Smad4 led to a higher level (3.7-fold) of transactivation, which reached levels as high as 5.5-fold after activated receptor co-expression. The transactivation potential of the adenovirally encoded Smads is specific as a control virus encoding for β-galactosidase resulted in transactivation levels similar to the uninfected condition. Thus, Smad proteins can transactivate the −2,300/+8 p21 promoter in HaCaT cells, and their potential is significantly increased by TGF-β receptor activation.

To pinpoint the importance of the Sp1 G/C-rich motifs of the proximal p21 promoter, we used a deletion mutant of the −2,300/+8 p21 promoter. As an independent control for the specificity of Smad-dependent transcriptional activation measured by the transient coupled transfection-adenovirus infection assay, we used the well-established reporter 12×(CAGA) Luc (43) whose transactivation by TGF-β depends solely on the Smads (16, 35). As expected, overexpressed Smad3 and Smad4 synergized with the CA-ALK-5 signal resulting in a robust 14-fold activation relative to the basal promoter activity. In conclusion, by using the adenovirus system we were able to demonstrate Smad-mediated transactivation of the human p21 promoter in HaCaT keratinocytes thus firmly establishing the importance of these factors in p21 gene regulation by TGF-β in this and other cell types.

Smad Proteins Contribute to the Regulation of the Endogenous p21 Gene by TGF-β in HaCaT Cells—To evaluate further the involvement of Smad proteins in the regulation of the endogenous p21 gene by TGF-β in HaCaT cells, we monitored p21 protein expression levels by Western blotting using a p21-specific antibody (Fig. 2). Treatment of HaCaT cells with...
10 ng/ml TGF-β1 for 20 h resulted in the robust accumulation of p21 protein (12-fold compared with untreated control, Fig. 2A, lanes 1 and 2) as previously reported (7). The TGF-β1 effect could be mimicked by the adenovirus-encoded constitutively active type I receptor ALK-5 in a dose-dependent manner. A selected m.o.i. of 50 of this virus resulted to a 3-fold activation (Fig. 2A, lane 3). Overexpression of adenovirus-encoded Smad3 and Smad4 also resulted in a dose-dependent increase of endogenous p21 protein levels. m.o.i. of 50 for each Smad-virus resulted in a low but reproducible 2-fold increase of p21 protein levels (Fig. 2A, lane 6). Combination of overexpressed Smad3 and Smad4 together with CA-ALK-5 or TGF-β1 treatment showed further increase of the p21 protein levels, which correspond to 5- and 14-fold, respectively, under the infection conditions shown in Fig. 2A (lanes 4 and 5).

To enhance the evidence that the Smad signaling pathway is involved in p21 protein accumulation in response to TGF-β1 in HaCaT cells, we also made use of dominant negative and inhibitory Smad proteins (Figs. 2, B–D). A Smad3 carboxy-terminal truncated mutant (DN-Smad3), the equivalent truncation of Smad4 (DN-Smad4), and the inhibitory Smad7 all resulted in a dose-dependent decrease of p21 accumulation in response to TGF-β1. The Smad3 and Smad4 mutants have been previously shown to interfere with TGF-β1 signaling in a dominant negative fashion with respect to specific target gene responses (44, 45), and we have shown that the DN-Smad4 mutant interferes with p21 promoter regulation in HepG2 cells (35). Similarly, the Smad7 inhibitor has also been previously reported to inhibit the induction of the p21 promoter-luciferase reporter by TGF-β1 in HaCaT cells (46). The combined p21 Western blotting results strongly support the involvement of the Smad signaling pathway in endogenous cell p21 regulation by TGF-β1.

Smad Proteins Do Not Associate with the Proximal p21 Promoter DNA—Smad3 contains DNA binding activity with low affinity toward 5'-TCTGAGAC-3' (termed the Smad-binding element (SBE)), whereas Smad4 toward both the SBE and G/C-rich motifs (42, 43, 47–49). The SBE is absent from the proximal p21 promoter (Fig. 1A) and exists in an upstream distal segment of the promoter (50) with no apparent functional significance (16, 35). On the other hand the p21 proximal promoter is G/C-rich. We thus tested the hypothesis of direct DNA binding of Smads to the proximal p21 promoter, which confers the inducibility of this gene to TGF-β and Smads (16, 35). Since mammalian cells express high levels of Sp1 protein, it has been rather difficult to identify TGF-β-specific nucleoprotein complexes by using GEMSA on the p21 or other Sp1-containing promoter sequences (16, 30). For this reason we used the well established Drosophila Schneider's SL2 cell line that lacks endogenous Sp1 activity (51). Fig. 3A shows representative GEMSA data produced from nuclear extracts of transiently transfected SL2 cells with the indicated combinations of Sp1 and Smad proteins and a radioactively labeled oligonucleotide corresponding to the TGF-β-responsive element (Tg(RE) (p21Pr(−86/−70)) of the proximal p21 promoter. Similar results were obtained when the upstream oligonucleotide containing two Sp1 motifs (p21Pr(−122/−84)) as shown in Fig. 1A was tested (data not shown). Our results showed the following.

(a) Under the conditions used, endogenous SL2 nuclear proteins do not recognize the p21 oligonucleotides (Fig. 3A, lane 1).

(b) Overexpression of Smad3 or Smad4 or both gives the same negative result as mock-transfected cells (Fig. 3A, lanes 2–4).

(c) Overexpression of Sp1 results in a specific nucleoprotein complex as expected (Fig. 3A, lane 8). (d) Overexpression of Smad3 together with Sp1 or Smad4 together with Sp1 results in a small 2- and 2.5-fold enhancement of the Sp1 nucleoprotein complex, respectively (Fig. 3A, lanes 5 and 6). (e) Overexpression of both Smad3 and Smad4 together with Sp1 results in a significant (5-fold) enhancement of the Sp1 nucleoprotein complex on both p21 probes (Fig. 3A, lane 7 and data not shown). In order to prove the specificity of the obtained Sp1 nucleoprotein complex in the SL2 nuclear extracts, we used competition experiments with excess amounts of cold oligonucleotides corresponding to the wild type p21 proximal promoter (Fig. 3B, lanes 3–5), the same oligonucleotides harboring point mutations in

![Fig. 2](image-url)
the p1-like elements (Fig. 3B, lanes 6–8) or a consensus Sp1 sequence (Fig. 3B, lane 9). These experiments confirmed that the nucleoprotein complexes formed with p21 promoter DNA probes are specific and contain only Sp1. In addition, unrelated, non-Sp1 sequence containing oligonucleotides including the SBE4 did not show any competition (data not shown). It must be noted that co-expression of Smads with Sp1 results only in relative enhancement of the nucleoprotein complex without any additional higher size complexes. Finally, the enhanced binding of Sp1 to the p21 proximal promoter after co-expression of Smad proteins in the SL2 cells could not result from nonspecific effects of differential protein expression in the transfected cells, as the levels of nuclear Sp1 and Smad proteins appeared rather comparable (Fig. 3C).

Thus the combined data demonstrate that Smad proteins do not associate with the proximal p21 sequences tested and co-expression of Smad3, Smad4, and Sp1 results in an enhanced nucleoprotein complex that retains the binding characteristics of Sp1.

GEMSA using nuclear extracts of transiently transfected Drosophila Schneider SL2 cells with the indicated Sp1 and Smad expression constructs and the radiolabeled −86/−70 p21 promoter oligonucleotide as a probe. The nucleoprotein complexes were resolved by 4% PAGE and detected by autoradiography. A representative autoradiogram is shown. The migrating positions of the free probe and the bandshift produced by Sp1 (lanes 5–8) are shown with arrows on the left side of the panel. Relative arbitrary densitometric values of the autoradiogram are boxed on top of the panel. B, GEMSA using the same nuclear extracts as above from SL2 cells transiently transfected with Sp1 alone (SL2/Sp1 NE) and radiolabeled p21Pr(−86/−70) (lanes 1–9) or the corresponding point mutant (mut) (lane 10) oligonucleotides as probes. A representative autoradiogram is shown. The migrating positions of the free probe and the bandshift produced by Sp1 are shown with arrows on the left side of the panel. In lane 1 half the amount of nuclear extract used in lanes 2–10 was used. Competitions with 50-fold excess (lanes 3, 6, and 9), 100-fold excess (lanes 4 and 7), and 200-fold excess (lanes 5 and 8) of the wild type (wt, lanes 3–5), the point mutant (mut, lanes 6–8) −86/−70 p21, or the consensus Sp1 (con, lane 9) oligonucleotide are shown on the top of the panel. C, expression levels of the indicated transiently transfected Smad and Sp1 proteins in the nuclear extracts of the Drosophila Schneider SL2 cells used in the GEMSA shown in A. Aliquots of the nuclear extracts were resolved by 7% SDS-PAGE, and Western blot analysis was performed using a Smad3-, FLAG- (for Smad4), and Sp1-specific antibody as indicated on the left side of each chemiluminogram. Only the relevant part of the panel. Relative arbitrary densitometric values of the autoradiogram are shown with arrows on the right side of the panel. Nonspecific protein species are indicated by asterisks. D, GEMSA using purified bacterially expressed Sp1 and baculovirally expressed Smad proteins and radiolabeled −86/−70 p21 promoter oligonucleotide. The nucleoprotein complexes were resolved by 4% PAGE and detected by autoradiography. A representative autoradiogram is shown. The migrating positions of the free probe and the two distinct bandshifts are shown with arrows on the left side of the panel. Competitions with 20-fold excess (lanes 6 and 10), 100-fold excess (lanes 7 and 11), and 200-fold excess (lanes 8 and 12) of the −86/−70 p21 oligonucleotide are shown on the left side of the panel. The exposure time for lanes 1–8 is 8 h and for lanes 9–16 is 16 h. E, GEMSA using the same reagents as in C, except that the two Smad proteins were first preincubated with excess (400-fold, lanes 2 and 3) cold SBE4 and then allowed to interact with (lane 2) or without (lane 3) Sp1 and finally with radiolabeled p21 promoter oligonucleotide (−86/−70). Only the relevant part of a representative autoradiogram is shown. The migrating positions of the bands are shown with arrows on the left side of the panel. Note the significant enhancement of the Sp1-specific bandshift when excess cold SBE4 oligonucleotide is provided in the reaction (lane 2). The exposure time of the autoradiogram was 3 h and must be compared with the 8-h exposure of lane 5 in D. F, quality and phosphorylation status of the two forms of baculovirally expressed Smad3 proteins used in the GEMSA shown in D and E. Aliquots of the purified proteins were resolved by 7% SDS-PAGE, and Western blot analysis was performed using a phosphoserine (P-Ser)- and histidine (His)-specific antibody as indicated on the left side of each chemiluminogram. Only the relevant part of the chemiluminogram is shown (WB, for Western blot). The migrating positions of the relevant proteins are shown with arrows on the right side of the panel.

**Fig. 3.** A–F, Smad proteins do not bind directly to the proximal p21 promoter sequences but enhance binding of Sp1 to these sequences. A,
Smad Proteins Induce an Increase in Sp1 Affinity for the DNA Sequences of the p21 Proximal Promoter—To examine thoroughly whether Smad proteins alter the Sp1 affinity for DNA, we relied on purified protein factors and in vitro GEMSA. By using purified unphosphorylated or phosphorylated (by CA-ALK-5 receptor) Smad3 and unphosphorylated Smad4 from a baculovirus system (36) and purified Sp1 from bacteria, we confirmed that Smad3, phospho-Smad3, Smad4 alone, or in combinations could not exhibit stable complexes with the p21 promoter DNA (Fig. 3D, lanes 2–4 and 13–15). The same proteins strongly and stably associated with the SBE DNA (data not shown and see Ref. 36). Purified Sp1 formed a strong complex with both p21 promoter probes (12); however, for the purpose of the experiments presented in this figure, a low concentration of Sp1 was used that reproducibly gave a rather weak binding (Fig. 3D, lane 9). Interestingly co-incubation of phospho-Smad3, Smad4, and Sp1 with the p21 probe resulted in a strong nucleoprotein complex of higher molecular mass (labeled Sp1') when compared with the complex obtained by Sp1 alone (Fig. 3D, lane 5). This effect was only obtained when phospho-Smad3 was used, as unphosphorylated Smad3 in combination with Smad4 and Sp1 resulted in the same weak bandshift as Sp1 alone (compare Fig. 3D, lanes 9 and 16). Western blot analysis of the two Smad3 preparations using anti-phosphoserine-specific antibodies confirmed that only phospho-Smad3 contained phosphorylated serines (Fig. 3D). Phospho-Smad3, Smad4, and Sp1 with the p21 probe resulted in a strong nucleoprotein complex of higher molecular mass (labeled Sp1') which could only be competed by excess SBE4 oligonucleotide (Fig. 3D, lanes 10). A 20-fold excess cold oligonucleotide could easily compete most of the Sp1 binding under the conditions used (lane 10), whereas complete competition of the higher mass complex was obtained when 200-fold excess of cold oligonucleotide (lane 8) was used. Similar in vitro GEMSA results to those shown in Fig. 3D for the p21Pr(−86/−70) probe were also obtained for the upstream p21Pr(−122/−84) probe (data not shown). Identical competition profiles were observed by the consensus Sp1 oligonucleotide, whereas the mutant p21Pr(−86/−70) oligonucleotide failed to compete like in the SL2 nuclear extract GEMSA results (Fig. 3B), and the unrelated SBE4 oligonucleotide not only failed to compete but exhibited enhancement of Sp1 binding to the p21 promoter (data not shown and see below). These experiments suggest that Smad proteins increase the affinity of Sp1 for its cognate G/C-rich-binding motif.

To understand further the nature of the induced enhancement of the Sp1 affinity for DNA by the Smads, we performed a preincubation-competition experiment using a quadruple concatamer of the consensus SBE (SBE₄) (Fig. 3E). In this experiment the phospho-Smad3 and Smad4 were preincubated with excess (400-fold) cold SBE₄ oligonucleotide to saturate the intrinsic binding of the Smad proteins for DNA, followed by addition of the Sp1 and the labeled p21 promoter probe (Fig. 3E, lane 2). The resulting complex was compared with that obtained when all proteins were mixed together in the absence of excess SBE₄ oligonucleotide (lane 1) or in the absence of Sp1 (lane 3). Surprisingly, this method of treatment from three independent experiments that included triplicate samples each. The analysis in B serves as control experiment for the results of A. The scale of the relative luciferase activity in B is broken to fit in the figure.
Smad Binding to DNA Is Not Essential for the Activation of the p21 Proximal Promoter—To examine whether Smad binding to DNA is required for the activation of the p21 promoter, we used point mutants in the conserved β-hairpin of the MH1 domain of Smad3 and Smad4 which is the DNA-binding domain of Smads (47). We used an R74K/K81R double mutant of Smad3 and the corresponding R81K/K88R double mutant of Smad4. These mutations in the β-hairpin of the MH1 domain, as predicted from the crystal structure, completely abolish binding of the Smads to the SBE. The prediction in our experiment was that these mutants should have wild type transactivation activity on the p21 promoter if DNA binding of Smads to the p21 proximal promoter is not important. Indeed, the experiments shown in Fig. 4A, performed in HepG2 cells, demonstrate that both Smad3 and Smad4 mutants can transactivate the proximal p21 promoter as efficiently as the wild type proteins. The constitutive effect of overexpression of the Smad proteins on the proximal p21 promoter was retained by the mutants, as was the inducible effect stimulated by the co-expression of CA-ALK-5.

As a control experiment we tested the transactivation of an artificial promoter that contains 12 copies of the SBE (CAGA) reporter stand in contrast to the p21 proximal promoter. The combined data presented in Fig. 5 demonstrate that Smad2 and Smad3 can co-precipitate with Sp1, whereas Smad4 is unable to do so under the conditions used.

Smad2, Smad3, and Smad4 Directly Associate with Sp1 via Their Conserved MH1 Domain—In order to map the domains of Smad and Sp1 proteins involved in their physical interaction, we used an in vitro interaction assay with GST-Sp1 and wild type or mutated Smad proteins synthesized in vitro. Fig. 6A shows schematically various Smad and GST-Sp1 constructs used in these experiments. The relative expression levels of affinity-purified Sp1, negative control GST, and in vitro synthesized Smad proteins are shown in Fig. 6B. When the in vitro synthesized Smad2, Smad3, and Smad4 were allowed to interact with GST-Sp1, all three Smad proteins were found capable for the direct interaction (Fig. 6C, lanes 4–6). However, among the three, Smad3 showed the strongest potential for interaction, whereas Smad2 and Smad4 interacted rather weakly (6 and 10% relative to Smad3 which is arbitrarily set to 100%, Fig. 6A). The interaction was specific as the GST moiety of the fusion protein when tested alone failed to support productive interaction (Fig. 6C, lanes 1–3). It must be noted here that these interactions are constitutive and do not depend on an activated TGF-β signaling pathway. In order to map the domain in Smad3 that is responsible for the interaction with Sp1, we used three different deletion mutants of Smad3 (Fig. 6A). Smad3-1(248) contains the MH1 and linker sequences and showed detectable, albeit weak, interaction with Sp1.

resulted in a further 3-fold enhancement of the Sp11 bandshift (compare lanes 1 and 2) which suggests that when the Smad proteins are provided with their DNA substrate they can still enhance the affinity of Sp1 for the p21 promoter DNA. These findings suggest that the Smad3-Smad4 complex bound or unbound to DNA leads to a significant increase in the affinity of Sp1 for its DNA-binding sequences.

Smad2 and Smad3 Co-immunoprecipitate with Sp1 in Transfected COS-7 Cells—Our previous work demonstrated a strong functional interaction between Smads and Sp1 (35). To test whether Smads and Sp1 physically interact, we applied several complementary experimental approaches. Fig. 5 shows the results of co-immunoprecipitation experiments performed with protein extracts from COS-7 cells transiently transfected with epitope-tagged Smad and GAL4-Sp1 fusion proteins. As a positive control we used the receptor activation-dependent interaction of Smad3 with Smad4 (Fig. 5A, lanes 2 and 3). Smad3 was found to co-precipitate with Sp1 at measurable levels even in the absence of stimulation of the signaling pathway (Fig. 5B, lane 2). However, co-expression of the CA-ALK-5 resulted in significant enhancement of co-precipitating Smad3 (Fig. 5B, lane 3). Essentially the same results were obtained for the co-immunoprecipitation of Smad2 with Sp1 (Fig. 5C, lanes 2 and 3). In contrast, we failed to detect co-precipitation of Smad4 with GAL4-Sp1, suggesting that the interaction of these two proteins might be either indirect or rather weak compared with the Smad3-Sp1 interaction (Fig. 5C, lanes 4 and 5).
FIG. 6. A–I, Smad2, Smad3, and Smad4 directly interact with transcription factor Sp1 via their MH1 domain. A, diagrammatic representation of the various Smad3, Smad2, and Smad4 proteins used for the in vitro interaction assays shown in B and C. The Smad amino-terminally conserved Mad homology (MH) 1 domain, the linker, and the conserved carboxyl-terminal MH2 domain are indicated with boxes of different shading. The β-hairpin loop of the MH1 domain, which is the DNA-binding domain of Smads, is shown as a small box with two pinheads representing the positions of the two point mutations described in Fig. 4. The amino- and carboxyl-terminal amino acids are numbered. The Sp1 functional domains A–D (23) are shown with brackets along with the conserved repeated Ser/Thr-rich and Gln-rich subdomains of the transactivation domains A and B, the zinc-finger DNA-binding motifs, and the transactivation modulatory region (−/+/). The relative sizes of the different proteins are not in scale. The column on the right-hand side summarizes the interaction results of C after densitometric analysis, and the values are presented as percentages relative to Smad3 that was set arbitrarily to 100. The actual arbitrary densitometric units (adu) of the reference sample (Smad3) are shown in parentheses. B, left, Coomassie Brilliant Blue staining of the input GST-Sp1 and control GST proteins coupled to glutathione beads (lanes 1 and 2) that were used in the interaction assay of C. Arrows indicate the two protein species. Molecular mass markers (expressed in kilodaltons) are shown to the left of the gel. Right, autoradiogram of the input 35S-labeled in vitro synthesized Smad proteins (lanes 3–8). Smad proteins were synthesized in vitro by a rabbit reticulocyte lysate. Each protein band represents 5% of the total amount of protein used in each interaction assay of C. Arrows indicate the positions of the relevant protein bands. Molecular mass markers (expressed in kilodaltons) are shown to the left of the autoradiogram. C, left, interaction of in vitro translated Smad proteins with GST-Sp1 immobilized on glutathione beads. The in vitro synthesized Smad2, Smad4, and Smad3 proteins (B, right) were allowed to interact with glutathione beads carrying GST alone (lanes 1–3) or GST-Sp1 (lanes 4–6), washed thoroughly, resolved by 12% SDS-PAGE, and detected by autoradiography. The positions of each relevant protein are marked with arrows. Molecular mass markers (expressed in kilodaltons) are shown to the left of the autoradiogram. Right, interaction of in vitro translated Smad3 deletion mutant proteins with GST-Sp1 immobilized to glutathione beads proves that the Smad3 MH1 domain is required for the interaction. Smad3 deletion mutants were synthesized in vitro by a rabbit reticulocyte lysate (input, B) and then allowed to interact with glutathione beads carrying GST alone (lanes 7–9) or GST-Sp1 (lanes 10–12), washed thoroughly, resolved by 12% SDS-PAGE, and detected by
compared with the wild type Smad3 (Fig. 6C, lane 10). Smad3- (1–122) contains the MH1 domain only, which is also truncated at its carboxyl terminus and exhibited similar weak interaction (23.9%) as the previous MH1-linker mutant (Fig. 6C, lane 11). Finally, Smad3-(122–424), which contains very few amino acids from the MH1 domain, the linker, and the MH2 domains, failed to support detectable interactions (<1.5%) with Sp1 (Fig. 6C, lane 12). The same Smad3 mutants failed to show specific retention to the GST affinity columns (Fig. 6C, lanes 7–9).

We conclude from the *in vitro* experiments that all three Smad proteins of the TGF-β signaling pathway, Smad2, Smad3, and Smad4, are capable of direct physical interaction with transcription factor Sp1, although Smad3 shows a more pronounced interaction (10–16-fold, based on densitometric analysis) in the *in vitro* pull-down assays. The MH1 domain of Smad3 is the primary determinant for this interaction, although the MH2 domain is necessary for fully productive interaction, as MH2 truncation significantly decreased (by 4–5-fold) the interaction potential of the residual Smad3 domains.

Although the previous experiments provide strong evidence for the physical association of Smad proteins and in particular Smad3 with Sp1, in these assays chimeric Sp1 proteins were always used (GAL4-Sp1 and GST-Sp1). To obtain evidence that the natural Sp1 molecule also interacts with Smad proteins, we used a series of GST-Smad fusion proteins that included full-length Smad3 and Smad4 as well as deletion mutants of these two proteins (Fig. 6D). Total detergent extracts from HaCaT cells were passed through the GST-Smad affinity columns (Fig. 6E) and washed, and the proteins bound to the columns were analyzed by Western blotting using an Sp1-specific polyclonal antibody (Fig. 6F). Endogenous Sp1 was readily detectable in the total HaCaT cell extract (Fig. 6F, lane 9). Sp1 was found to interact with GST fusions of full-length Smad4 (lane 1) and Smad3 (lane 3) but not GST alone (lane 8). The interaction with Smad3 was more efficient than with Smad4, which is in agreement with the *in vitro* experiments of Fig. 6A–C. Experiments with deletion mutants of the two Smad proteins also corroborated the previous results as they showed that the MH1 plus linker domains of Smad4 were capable of sustaining interaction with Sp1 but less efficiently than full-length protein (Fig. 6F, lane 2). In addition, the MH1 domain of Smad3 was the primary determinant for the interaction with Sp1 (Fig. 6F, lane 6); however, the presence of the linker (lane 5) and the MH2 domain (lane 3) enhances the interaction considerably. Furthermore, the isolated linker plus MH2 (ΔMH1, lane 4), MH2 (lane 7), and linker (not shown) domains did not support any detectable interaction, excluding the possibility that these domains could provide primary specificity to the intermolecular associations studied.

Therefore, these experiments are in full agreement with those shown in Figs. 5 and 6, A–C, and establish that the natural Sp1 molecule is capable of interacting with Smad proteins via their MH1 domains.

The Glutamine-rich Region of the Sp1 Transactivation Domain Can Sustain Functional Synergism with Smad Proteins—Since a first map of the interaction domain on Smad proteins was established, we were interested in defining the Sp1 sequences that were participating in the Smad-Sp1 interaction. For this reason we relied on the *in vitro* interaction assay by using a panel of GST-Sp1 deletion mutants (Fig. 6, G and H) and *in vitro* synthesized full-length Smad3 protein since this showed the best potential in the interaction assays (Fig. 6, C and F). The Sp1 mutants included deletions of the conserved and duplicated subdomain A of the major transactivation domain of this protein (23), the second subdomain B of the major transactivation domain together with domain C, which also confers transactivation potential to Sp1 and a carboxyl-terminus deletion of domain D (Fig. 6G). The three mutants and full-length Sp1 were produced as GST fusions (Fig. 6H). Smad3 exhibited direct interaction with full-length Sp1 (Fig. 6D, lane 3) as described above, which was not seen with GST alone (lane 2). Smad3 binding to Sp1 was decreased when subdomain A was deleted (lane 4). However, Smad3 binding was not affected at all by deletion of the subdomains B plus C (lane 5) and was mildly decreased by deletion of domain D (lane 6). Since none of the Sp1 mutants showed complete loss of interaction potential with Smad3, this suggested that the conserved and duplicated glutamine and serine/threonine-rich sequences of subdomains A and B and/or the DNA-binding domain of Sp1 (domain Zn2+ in Fig. 6G), which are included in all the Sp1 mutants, might be involved in the interaction. Alternatively, it is possible that Sp1 contains multiple sequence motifs that contribute to the interaction with Smad proteins.

To characterize in more detail sequences within the conserved domains A and B that might contribute to the functional synergism with Smads, we made use of another panel of deletion mutants of Sp1, which included shorter truncations of the major transactivation domain fused to GAL4 DNA-binding domain (GAL4-DBD, Fig. 7A). Fig. 7B shows that co-expression of Smad3 and Smad4 together with full-length Sp1 fused to GAL4 resulted in almost 20-fold activation of a reporter containing five concatameres GAL4-binding sites in front of a minimal thymidine kinase promoter, relative to the constitutive level of GAL4-Sp1 alone. Mutants Sp1 A + B, Sp1 B, and Sp1 Bc all exhibited significant transactivation (34–37-fold) by the Smad3-Smad4 complex, which was comparable with each other and higher than GAL4-Sp1. Mutant Sp1 Bc retains only the glutamine-rich region of domain B (amino acids 424–542).
with Smad proteins, and of course, since not all glutamines were mutated in the triple point mutant, the residual transactivation potential (7.5-fold) can be attributed to the remaining glutamine-rich protein surfaces.

Finally, we tested the functional interaction of the glutamine-rich domain of Sp1 with the three Smad proteins of the TGF-β signaling pathway, Smad2, Smad3, and Smad4 (Fig. 7C). The results showed that Smad3 exhibits the strongest cooperative activation (8-fold) together with Sp1-Bc, which can be enhanced further (13-fold) by co-expression of Smad4. Smad2 and Smad4 alone did not show any appreciable level of transactivation (1.1–1.4-fold) in agreement with the low or undetectable constitutive interactions described above. However, the Smad2 plus Smad4 combination resulted in measurable but small transactivation (2.9-fold), which is consistent also with the physical interaction data, since this combination partially mimics the activation of Smad2 by ligand.

In conclusion, these experiments suggest that the glutamine-rich subdomain of the transcription domain of Sp1 plays important roles in the functional synergism between Sp1 and Smad proteins and further strengthen the finding that Smad3 under all experimental conditions tested exhibits the best physical and functional cooperativity with transcription factor Sp1.

**DISCUSSION**

The experiments of Fig. 1 demonstrate the positive regulatory effect of Smad proteins on the activity of the p21 promoter in human keratinocytes HaCaT. This result is of particular significance as this cell line has been extensively analyzed for the mechanism of growth inhibition by TGF-β (4, 11, 52) and exhibits a rather dramatic induction of its endogenous p21 gene in response to TGF-β (7 and Fig. 2). The Smad effect also depends on the integrity of the G/C-rich, Sp1-occupied proximal promoter (Fig. 1C). It must be noted that the adenovirus-mediated expression of Smad3 and Smad4 proteins positively up-regulates the −2,300/+8 and the −143/+8 p21 promoters, to a lower extent than TGF-β (16). Thus, additional regulatory factors may be required for the maximal activation of the p21 promoter by TGF-β (see below). In addition, the promoter analysis of Fig. 1 supports our previous analysis in HepG2 cells where a distal inhibitory and a proximal stimulatory promoter segment were functionally defined (35). Removal of the distal sequences results in significant increase of the responsiveness of this promoter to the TGF-β signal and Smad proteins (35). For this reason the transcriptional activity of the proximal −143/+8 p21 promoter in response to TGF-β is relatively higher than the activity of the −2,300/+8 promoter (Fig. 1 and Ref. 35).

The p21 promoter studies in HaCaT cells (Fig. 1) and in HepG2 cells (35) are in strong agreement with the HaCaT experiments of Fig. 2 in which exogenous Smad3 and Smad4 were found to potentiate the response of the endogenous p21 gene to TGF-β. Overexpression of Smad3 and Smad4 by means of adeno-virus infection could significantly enhance endogenous p21 accumulation, an effect that could be further augmented by co-expression of the constitutively active type I receptor for TGF-β (CA-ALK5) or TGF-β1. This implies that activation of the Smads by receptors leads to more efficient p21 gene activation. These effects on endogenous p21 protein accumulation are dose-dependent for all tested activators, i.e., the ligand TGF-β1, the constitutively active type I receptor, and the Smads, and thus, conditions where the cell can tolerate excessive amounts of p21 accumulation can be obtained (Fig. 2A and data not shown). In addition, carboxyl-terminally truncated dominant negative mutants of Smad3 and Smad4 both inhibited p21 accumulation in response to
TGF-β1 (Fig. 2, B and C), in a dose-dependent manner. Since these mutants are known to interfere with Smad activation by receptors, oligomerization, nuclear translocation, and cooperation with transcription factors (34, 44, 45), the Smad signaling pathway must be required for endogenous p21 gene induction by TGF-β. The same results were obtained with increasing doses of the inhibitory Smad7. The combined data of Figs. 1 and 2 strongly confirm that activation of Smads by the type I receptor is a critical step in endogenous p21 responsiveness to TGF-β1.

Smad proteins are known to associate directly with DNA elements containing the TCGTAGAC or G/C-rich sequences, although with relatively low affinity (36, 42, 43, 47–49). The p21 proximal promoter lacks any obvious SBEs but contains a G/C-rich region between nucleotides −124 and −42 (Fig. 1A). GEMSA experiments (Fig. 3) showed that Smad3 or Smad4 does not bind to the G/C-rich region of the p21 promoter. The transactivation experiments using Smad3 and Smad4 proteins containing point mutations in their DNA-binding domains, which cannot recognize the SBE (Fig. 4), confirmed that p21 promoter regulation by Smads does not require the DNA-binding function of Smads. Control experiments using the multimerized SBE promoter confirmed the defective nature of the mutant Smads in HepG2 cells; however, their absolute negative effects could not be estimated since HepG2 cells contain endogenous Smad3 and Smad4. The mutant Smad3 and Smad4 proteins failed to transactivate the same SBE promoter in cells that lack the genes for Smad3 or Smad4.2 Finally, it is worth noting that despite the lack of SBE sequences in the proximal p21 promoter, such elements have been described in the distal segment of the promoter (42, 50). However, previous deletion analyses have shown that both TGF-β and Smad-dependent activation of the p21 promoter do not require this distal SBE (16, 35). The exact role of the distal SBE on the basal and inducible activity of the p21 promoter remains to be elucidated.

The GEMSA analyses illustrated in Fig. 3 showed that Smad3 and Smad4 proteins enhanced the formation of a nucleoprotein complex between Sp1 and p21 promoter oligonucleotides. This observation could support a mechanism of cooperativity between Smads and Sp1 in p21 promoter transactivation. On the other hand, binding of Smads to SBE sequences resulted in a stronger cooperativity with Sp1 (Fig. 3E). Such a mechanism would imply that TGF-β-responsive promoters that contain both SBE sequences and G/C-rich Sp1-binding motifs would provide more optimal substrates for a cooperative function between Smads and Sp1 in transcriptional regulation. Such examples might be the p15ink4b, the Smad7, the TGF-β1, and the TGF-β type I and type II receptor promoters (55–57).

In contrast to previously published examples of nucleoprotein complex formation between Smads and other transcription factors (53, 58), we observed the presence of a distinct nucleoprotein complex with only slightly slower mobility than the Sp1-DNA complex (Fig. 3D). This implies that the Sp1-Smad nucleoprotein complex cannot withstand GEMSA conditions or that alternatively high affinity Sp1-DNA complex formation induced by Smad proteins may rapidly lead to Smad dissociation from the complex. A qualitatively similar result has been observed in studies of interaction and cooperation between the co-activator p300 and Sp1 (21). Thus, Smads could induce the following: (a) oligomerization of Sp1, which results in higher affinity binding to the p21 promoter; (b) recruitment of additional cooperating factors (such as p300 or c-Jun) that could possibly stabilize or enhance the transcriptional activity of Sp1; and (c) modulation of the phosphorylation or acetylation status of Sp1 with concomitant effects in DNA binding and transcriptional potencies. Recent reports indicate that the mitogen-activated protein kinase (MAPK) pathway is also activated by TGF-β and contributes positively to the regulation of the p21 gene (59–61). Thus, it is of interest to examine whether MAPKs directly modulate the transcriptional activity of Sp1, which is phosphorylated in a cell cycle-specific manner (20). Alternatively, transcription factor targets which themselves are activated by the MAPK cascade and which are known to bind to the p21 promoter sequences, such as the Ets-like factor E1AF (62) might also cooperate with the Sp1-promoter complex.

The obvious corollary of the above results has been that Smads may physically interact with Sp1. This hypothesis was tested by co-immunoprecipitation and GST pull-down analyses (Figs. 5 and 6), which led to the conclusion that Smad2, Smad3, and Smad4 proteins all can directly interact with Sp1. Smad3 showed the strongest constitutive association with Sp1 (Fig. 6, C and F). This result, combined with the strong transactivation potential of this protein on various TGF-β-inducible promoters, can explain a series of previous data attesting to a dominant and ligand-independent function of Smad3 in activating Sp1-dependent transcriptional events (35). However, activation of the TGF-β signaling pathway by means of a constitutively active type I receptor (ALK-5) increased the levels of Smad3 species that co-immunoprecipitated with Sp1 in transiently transfected COS-7 cells (Fig. 5B). In contrast, the constitutive association of Smad2 and Smad4 with Sp1 is much weaker (Fig. 5B, 6, and 6F). Type I receptor activation leads to much stronger enhancement of Smad2 association with Sp1 when compared with the enhancement seen for Smad3 (Fig. 5, B versus C). Although the constitutive association of Smad4 with Sp1 was readily detectable with two independent techniques (Fig. 6), the co-immunoprecipitation assay failed to measure constitutive or ligand-dependent association (Fig. 5C). Since the constitutive Smad-Sp1 interactions were detected using in vitro synthesized Smad proteins and bacterially purified Sp1 (Fig. 6C), these interactions must be direct and do not require any additional intermediates. However, this result does not exclude the possibility that additional factors participate in the Smad-Sp1 nuclear complex in vivo.

The same set of experiments resulted in mapping the Smad interaction domain with Sp1 as the amino-terminal conserved MH1 domain (Fig. 6, C and F). Interestingly, although the MH1 domain is the primary determinant for interactions with Sp1, the linker and MH2 domains contribute positively to the interaction in a progressive manner, making the full-length protein more capable of associating with Sp1 (Fig. 6, B and C). The MH1 domain of Smad proteins is known to associate specifically with several other transcription factors, such as Jun family members, ATF-2, and vitamin D receptor (34). In addition, the MH1 domain contains the DNA-binding domain of the Smad proteins (47). In our efforts to finely map the interaction domain in Smad3, we have collected preliminary data suggesting that sequences proximal to the Smad3 DNA-binding β-hairpin domain may be responsible for the specific interaction with Sp1 (data not shown). Thus, the structural model proposed by Shi et al. (47) for the cooperation between Smad3 and Jun family members (leucine zipper proteins) might also apply for transcription factor Sp1 (a prototype for zinc finger proteins).

On the other hand, one possible domain of Sp1 that could confer specificity to the Sp1-Smad cooperativity maps to the conserved and duplicated glutamine-rich region of the Sp1 transactivation domains A and B (Fig. 7). This hypothesis is in agreement with previous results showing the importance of the glutamine-rich sequences in TGF-β1-mediated activation of GAL4-Sp1 fusion proteins (30). However, additional sequences
in Smad proteins with transcription factor Sp1. Our study suggests that the glutamine-rich segment exhibits functional cooperativity with Sp1. On the other hand, Sp1 and Smad proteins interact directly via the MH1 domain of the latter (53, 54). Thus, one can envision complex intermolecular interactions between these three classes of transcription factors, whereby complexes of all three factors might simultaneously occur as discussed in our previous work (12). The importance of such higher order complexes in the regulation of the p21 promoter by TGF-β requires future investigation.

In conclusion, the present data provide strong evidence for the involvement of the Smad signaling pathway in both p21 promoter and endogenous p21 gene regulation by TGF-β in HaCaT cells. In addition, we demonstrate the physical association of Smad proteins with transcription factor Sp1. One of the consequences of such interactions is the apparent enhancement of Smad proteins with transcription factor Sp1. One of the HaCaT cells. In addition, we demonstrate the physical association of Smad proteins, bacterial and mammalian expression vectors, and antibodies used in this study. We also thank S. Itoh for technical advice; K. J. Noti, C. Delidakis, C. Rorsman, and S. Itoh for various reporter plasmids, bacterial and mammalian expression vectors, and antibodies used in this study. We also thank S. Itoh for technical advice; K. Shiraishi and G. Koutsodendis for excellent technical assistance; S. Grimbs for automated sequencing of plasmids; and C.-H. Heldin and J. Ericsson for discussions and a critical reading of this manuscript.

Acknowledgments—We are grateful to M. Fujii, K. Miyazono, and T. Fotsis for the adenoviruses; F. M. Hoffman and A. Comer for the baculoviral Smad proteins; Santa Cruz Biotechnology for the anti-Sp1 and anti-Smad1/2/3 (H2) antibodies; B. Vogelstein, R. Derynck, J. Massagué, X.-F. Wang, G. Gill, G. Mavrothalassitis, E. Flavey, R. Tjian, J. Noti, C. Delidakis, C. Rorsman, and S. Itoh for various reporter plasmids, bacterial and mammalian expression vectors, and antibodies used in this study. We also thank S. Itoh for technical advice; K. Shiraishi and G. Koutsodendis for excellent technical assistance; S. Grimbs for automated sequencing of plasmids; and C.-H. Heldin and J. Ericsson for discussions and a critical reading of this manuscript.

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