The EMBO Journal Vol.16 No.17 pp.5353–5362, 1997

TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad4

Atsuhito Nakao[1], Takeshi Imamura[2], Serhiy Souchelnytskyi, Masahiro Kawabata[2], Akira Ishisaki, Eiichi Oeda[2], Kiyoshi Tamaki, Jun-ichi Hanai[2], Carl-Henrik Heldin, Kohei Miyazono[2] and Peter ten Dijke

Ludwig Institute for Cancer Research, Box 595, S-751 24 Uppsala, Sweden, [2]Department of Biochemistry, The Cancer Institute, Tokyo, Japanese Foundation for Cancer Research, and Research for the Future Program, Japan Society for the Promotion of Science, 1-37-1 Kami-ikebukuro, Toshima-ku, Tokyo 170, Japan

[1]Corresponding author
email: Ludwig@LICR.uu.se

A.Nakao and T.Imamura contributed equally to this work

Smad family members are newly identified essential intracellular signalling components of the transforming growth factor-β (TGF-β) superfamily. Smad2 and Smad3 are structurally highly similar and mediate TGF-β signals. Smad4 is distantly related to Smads 2 and 3, and forms a heteromeric complex with Smad2 after TGF-β or activin stimulation. Here we show that Smad2 and Smad3 interacted with the kinase-deficient TGF-β type I receptor (TβR)-I after it was phosphorylated by TβR-II kinase. TGF-β1 induced phosphorylation of Smad2 and Smad3 in Mv1Lu mink lung epithelial cells. Smad4 was found to be constitutively phosphorylated in Mv1Lu cells, the phosphorylation level remaining unchanged upon TGF-β1 stimulation. Similar results were obtained using HSC4 cells, which are also growth-inhibited by TGF-β. Smads 2 and 3 interacted with Smad4 after TβR activation in transfected COS cells. In addition, we observed TβR-activation-dependent interaction between Smad2 and Smad3. Smads 2, 3 and 4 accumulated in the nucleus upon TGF-β1 treatment in Mv1Lu cells, and showed a synergistic effect in a transcriptional reporter assay using the TGF-β-inducible plasminogen activator inhibitor-1 promoter. Dominant-negative Smad3 inhibited the transcriptional synergistic response by Smad2 and Smad4. These data suggest that TGF-β induces heteromeric complexes of Smads 2, 3 and 4, and their concomitant translocation to the nucleus, which is required for efficient TGF-β signal transduction.

Keywords: nuclear translocation/phosphorylation/signal transduction/transforming growth factor-β

Introduction

Transforming growth factor-β1 (TGF-β1) is the prototype of a family of structurally related cytokines, which also includes activins and bone morphogenetic proteins (BMPs). TGF-β superfamily members induce a multitude of effects and have been shown to control proliferation, differentiation, migration and apoptosis of many different cell types (Roberts and Sporn, 1993). They act via ligand-induced hetero-oligomerization of type I and type II serine/threonine kinase receptors (Lin and Lodish, 1993; Derynck, 1994; Massagué and Weis-Garcia, 1996; ten Dijke *et al*., 1996). The molecular mechanism for TGF-β receptor activation, in which a constitutively active type II receptor phosphorylates and activates a type I receptor, is fairly well understood (Wrana *et al*., 1994). However, little is known about the intracellular events, which occur following receptor activation.

Using a genetic modifier screen of decapentaplegic, a TGF-β-like protein in *Drosophila*, *mothers against dpp* (*Mad*) was identified (Sekelsky *et al*., 1995). Recent studies indicate that Mad is the prototype for a novel family of 50–60 kDa proteins that perform essential roles in the intracellular signal transduction pathways of TGF-β superfamily members (Derynck and Zhang, 1996; Massagué, 1996). In *Caenorhabditis elegans*, *Mad* homologues were identified and termed *Sma-2*, *Sma-3* and *Sma-4* genes (Savage *et al*., 1996). In vertebrates, five *Mad* and *Sma* homologues have thus far been identified and termed '*Smad*' genes (Derynck *et al*., 1996). Alterations in *Smad2* and *Smad4* genes have been found in specific tumour subsets, and thus Smads may have a tumour suppressor function (Eppert *et al*., 1996; Hahn *et al*., 1996; Riggins *et al*., 1996). Smad proteins have conserved N-terminal (MH1) and C-terminal (MH2) domains, which are linked by diverse sequences rich in proline residues. The Smad C-terminal domain, when fused to a heterologous DNA binding domain, has transcriptional activity (Liu *et al*., 1996; Meersseman *et al*., 1997). In addition, Smad2 was found to interact with the winged-helix transcription factor FAST-1 in a multiprotein complex that regulates the activin early response gene *Mix-2* (Chen,X. *et al*., 1996). Thus, Smads may act as transcriptional activators.

Functional studies in *Xenopus* revealed that different Smads can specify different responses (Baker and Harland, 1996; Graff *et al*., 1996; Thomsen, 1996); Smad1 induced ventral mesoderm, a BMP-like response, whereas Smad2 induced dorsal mesoderm, a TGF-β/activin-like response. Biochemical studies in vertebrates indicated that Smads become rapidly phosphorylated and translocate to the nucleus upon ligand stimulation (Eppert *et al*., 1996; Hoodless *et al*., 1996; Lechleider *et al*., 1996; Liu *et al*., 1996; Macías-Silva *et al*., 1996; Yingling *et al*., 1996; Nakao *et al*., 1997), which also occurs in a ligand-specific manner; Smad1 is activated after BMP stimulation, while Smad2 was found to be a direct substrate for the activated TGF-β type I receptor (TβR-I). In addition, Smad2

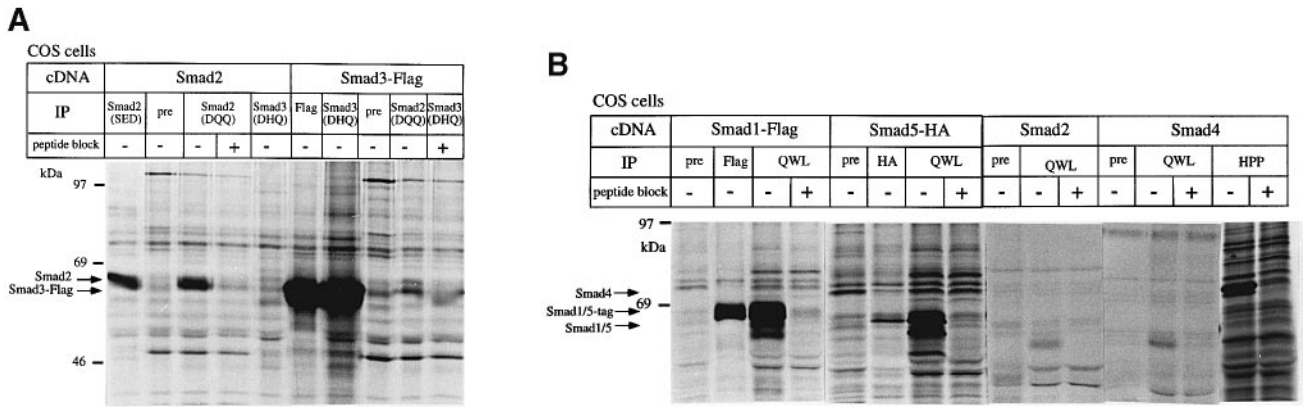

Fig. 1. Specificity of antisera against Smads. (**A**) DQQ antiserum against Smad2 and DHQ antiserum against Smad3 are specific and do not cross-react with each other. COS cells transfected with expression plasmids containing Smad2 or Smad3-Flag were metabolically labelled and immunoprecipitated (IP) with indicated antisera. To test for Smad2 and Smad3 expression immunoprecipitation was performed with SED antiserum or anti-Flag antibody, respectively. pre, preimmune serum. (**B**) HPP antiserum recognizes Smad4, and QWL antiserum recognizes Smad1 or Smad5, but not Smad2. Specificity of antiserum is shown by the absence of cross-reactivity, and/or lack of precipitation with preimmune serum and by blocking of immunoprecipitation by addition of 10 μg of cognate peptide. Smads are indicated by arrows. Smad2 and Smad1/5 co-migrate on SDS-gels. Immunoprecipitates were analysed by SDS–PAGE and fluorography.

phosphorylation at C-terminal serine residues was shown to be required for its nuclear translocation (Macías-Silva *et al*., 1996). Smad3, which is closely structurally related to Smad2, was also found to become phosphorylated after TGF-β stimulation and to interact with TβR-I (Zhang *et al*., 1996).

Smad2 or Smad3 has been shown to synergize with Smad4 in TGF-β signalling (Lagna *et al*., 1996; Zhang *et al*., 1996), and Smad4 restored the sensitivity to TGF-β when transfected into the breast cancer line MDA-MB468 which lacks Smad4 (Lagna *et al*., 1996; de Winter *et al*., 1997). In addition, Lagna *et al*. showed that Smad4 is present as a homomeric complex in the absence of ligand, and forms heteromeric complexes with Smad1 or Smad2 in response to BMP or TGF-β/activin, respectively (Lagna *et al*., 1996). Therefore, Smad2 and/or Smad3 together with Smad4 appear to be part of the intracellular signal transduction pathway of TGF-β. However, it is not known whether Smad2 and Smad3 are redundant in mediating the TGF-β/activin-specific responses.

Here, we show that Smad2, Smad3 and Smad4 physically and functionally interact, suggesting that TGF-β signalling occurs via complex(es) of three Smad proteins.

Results

Specificity of antisera against Smad proteins

Many studies on Smad activation make use of transfected cells and epitope-tagged Smads. In order to study expression and activation of members of the Smad family under more physiological conditions in non-transfected cells, we raised specific antisera against them. Peptide sequences, corresponding to highly diverged proline-rich linker regions in different Smads, were selected based upon their prediction to be highly immunogenic (Jameson and Wolf, 1988) and to have a high surface probability (Emini *et al*., 1995).

In order to characterize the affinities and specificities of the antisera, individual Smads were transfected into COS cells; after metabolic labelling with [^{35}S]methionine and [^{35}S]cysteine, cell extracts were prepared and subjected to immunoprecipitation with the different antisera. The previously described antiserum against Smad2, termed SED (Nakao *et al*., 1997), cross-reacted weakly with the related Smad3. Therefore, we made another Smad2 antiserum termed DQQ, raised against a Smad2-derived peptide from a more divergent region; the corresponding Smad3 peptide was used to raise the DHQ antiserum against Smad3. DQQ and DHQ antisera recognized Smad2– and Smad3–Flag, respectively, with similar efficiencies to SED antiserum and Flag antibody, respectively (Figure 1A). The 62 kDa Smad2– and 58 kDa Smad3–Flag were not precipitated when preimmune sera were used or when the antisera were blocked by addition of excess cognate peptides. The DHQ antiserum did not cross-react with Smad2, and the DQQ antiserum did not cross-react with Smad3 (Figure 1A). Neither did the DHQ and DQQ antisera immunoprecipitate Smad1, Smad4 or Smad5 from transfected COS cells (data not shown).

The antiserum raised against Smad4, termed HPP, specifically immunoprecipitated a component of 70 kDa from COS cells expressing Smad4 (Figure 1B), but did not cross-react with other Smads (data not shown). We made an antiserum against a peptide derived from the conserved MH2 domain of Smad1, termed QWL. In COS cells transfected with a Smad1–Flag construct, we found that both QWL and Flag antibodies immunoprecipitated a 62 kDa component. The QWL antiserum recognized Smad5 equally as efficiently as Smad1, but not Smad2 or Smad4, in transfected COS cells (Figure 1B). In addition, QWL antiserum recognized a component of 58 kDa in COS cells, which was not recognized by the Flag antibody (Figure 1B). This component probably represents endogenous Smad1/Smad5, because it was not precipitated when preimmune serum or the antiserum blocked with cognate peptide, were used.

Endogenous Smad expression in Mv1Lu cells

We used the specific antisera against Smad2, 3 and 4 and the Smad1/Smad5 cross-reactive antiserum, to investigate which Smads were endogenously expressed in Mv1Lu cells. The specific antisera to Smad2 and Smad3 recognized 58 kDa and 54 kDa components, respectively, which were not seen when preimmune sera were used or when

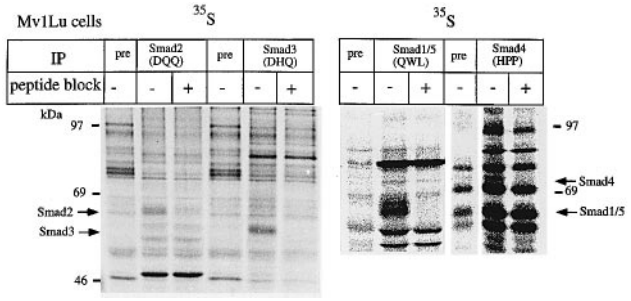

Fig. 2. Endogenous Smad expression in Mv1Lu cells. Cell lysates from metabolically labelled Mv1Lu cells were subjected to precipitation (IP) using preimmune sera (pre) or DQQ, DHQ, HPP or QWL antisera, which recognize Smad2, Smad3, Smad4 or Smad1/5, respectively. Smads are indicated by arrows. Immunoprecipitates were analysed by SDS–PAGE and fluorography or Fuji X Bio-Imager.

excess blocking peptides were added together with these antisera (Figure 2). Using metabolically labelled Mv1Lu cell extracts, the HPP antiserum against Smad4, and the QWL antiserum against Smad1/Smad5 precipitated 70 kDa and 58–62 kDa components, respectively (Figure 2). The two bands observed with QWL antiserum may possibly represent Smad1 and Smad5 or alternative spliced gene products thereof. The experimentally observed molecular masses of Smad1/Smad5, Smad2, Smad3 and Smad4 in Mv1Lu cells are somewhat higher than the masses predicted from the human cDNA sequences, which are 52.2 kDa, 52.3 kDa, 47.8 kDa and 60 kDa, respectively. Thus, Mv1Lu cells contain Smad2, Smad3, Smad4 and Smad1/Smad5.

TGF-β1-induced phosphorylation of Smad2 and Smad3 and constitutive phosphorylation of Smad4 in non-transfected cell lines

The effect of TGF-β1 on the phosphorylation of different members of the Smad family was analysed using [^{32}P]orthophosphate-labelled Mv1Lu cells. In the absence of ligand, Smad2 and Smad3 were not or very weakly phosphorylated. This is in contrast to Smad1/Smad5 and Smad4 which were both phosphorylated in unstimulated cells (Figure 3A and C). The phosphorylation of Smad2 and Smad3, but not of Smad1/Smad5 and Smad4, were strongly induced upon addition of TGF-β1 in Mv1Lu cells (Figure 3A). Using TGF-β-sensitive HSC4 cells (Ichijo *et al.*, 1990), we also observed TGF-β1-induced phosphorylation of Smad2 and Smad3, and constitutive phosphorylation of Smad4 that remained unchanged after TGF-β stimulation (Figure 3B and C). We were unable to detect expression of Smad1/5 in HSC4 cells, and did not observe any constitutive or TGF-β-induced phosphorylation of Smad1/Smad5 in these cells (data not shown).

Association of Smad2 and Smad3 with TβR-I

COS cells transfected with Smad2 or Smad3 constructs alone or in combination, together with TβR-II and wild-type TβR-I or a kinase-deficient form of TβR-I (TβR-I/K232R) were affinity labelled with [^{125}I]TGF-β1, and cell lysates were subjected to immunoprecipitation with antiserum against epitope tag present in Smads. In accordance with previous results (Macías-Silva *et al.*, 1996), we found that Smad2 formed a complex with TβR-I which was kinase-inactive and phosphorylated by TβR-II kinase,

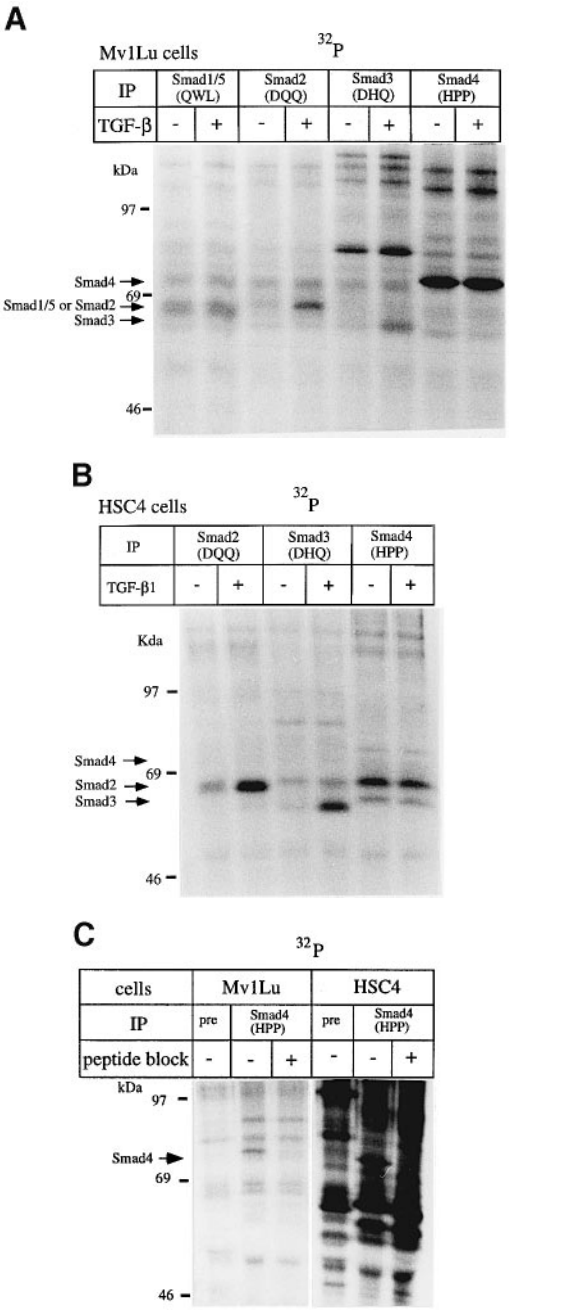

Fig. 3. TGF-β-mediated phosphorylation of Smad2 and Smad3 and constitutive phosphorylation of Smad4 in non-transfected cells. (**A**) Mv1Lu cells were labelled with [^{32}P]orthophosphate in the absence or presence of TGF-β. Cell lysates were subjected to immunoprecipitation (IP) with DQQ, DHQ, HPP or QWL antisera that recognize Smad2, Smad3, Smad4 or Smad1/5, respectively, and analysed by SDS–PAGE and autoradiography. (**B**) HSC4 cells were labelled with [^{32}P]orthophosphate in the absence or presence of TGF-β1. Cell lysates were subjected to immunoprecipitation with DQQ, DHQ and HPP antisera, and analysed by SDS–PAGE and autoradiography. (**C**) Mv1Lu cells and HSC4 cells were labelled with [^{32}P]orthophosphate in the absence of TGF-β1, and cell lysates were subjected to immunoprecipitation with HPP antiserum or preimmune serum (pre) with and without excess HPP peptide, and analysed by SDS–PAGE and autoradiography.

but not with wild-type TβR-I (data not shown). N-terminally Flag-tagged Smad3 (F-Smad3) formed a complex with the kinase-inactive form of TβR-I (Figure 4). However, we noticed that the C-terminally Flag-tagged

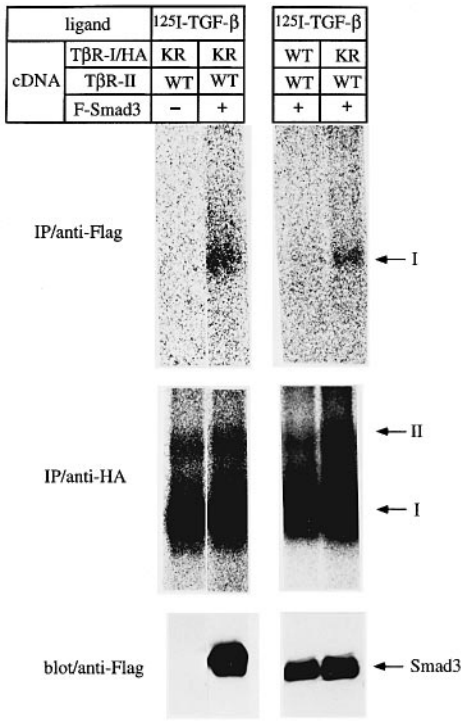

Fig. 4. Association of Smad3 with TβR-I. COS cells were transfected with N-terminally Flag-tagged Smad3 (F-Smad3) in combination with TβR-II and wild-type (WT) or kinase-inactive (KR) forms of TβR-I. TβR-I constructs tagged at the C-terminus with a HA epitope were used. The receptors were affinity-labelled with [^{125}I]TGF-β1. Cell lysates were subjected to immunoprecipitation and co-immunoprecipitation of receptors was analysed by SDS–PAGE and autoradiography. Expression of Smad3 and receptors was determined by immunoblotting by Flag antibody and immunoprecipitation by HA antibody using aliquots of cell lysates, respectively.

Smad3 (Smad3-F), in contrast to F-Smad3, associated also with wild-type TβR-I (data not shown), consistent with observations made by Zhang *et al*. (1996). Thus, both Smad2 and Smad3 form stable complexes with kinase inactive TβR-I; complexes with wild-type TβR-I may be too transient to be able to be detected with this method unless Smads are tagged in the C-terminus where phosphorylation occurs (Macías-Silva *et al*., 1996).

TGF-β1-dependent interaction of Smad2 and Smad3 with Smad4

TGF-β-dependent heteromeric complex formation of Smad2 and Smad4 has been shown before (Lagna *et al*., 1996). We examined the interaction of Smad2 and Smad3 with Smad4 using transfected COS cells. COS cells were transfected with indicated Smad constructs alone or together with TβR-I and TβR-II and the interactions between Smad3 and Smad4, and Smad2 and Smad4 were investigated using immunoprecipitation followed by Western blotting. COS cells were transfected with F-Smad3 or Smad3-F and N-terminally Myc-tagged Smad4 (M-Smad4), and analysed by immunoprecipitation with anti-Flag antibody followed by immunoblotting with anti-Myc antibody (Figure 5A). Upon co-transfection with TβR-II and TβR-I, an interaction between F-Smad3 and M-Smad4 could be demonstrated which strongly increased after stimulation by ligand. The interaction between F-Smad3 and M-Smad4 in the absence of ligand is likely caused by TβR-I activation through ligand-independent complex formation of TβR-I and TβR-II upon their overexpression in COS cells. Smad3-F did not interact with M-Smad4 (Figure 5A), suggesting that tagging at the C-terminus is functionally disruptive.

To examine the interaction between Smad2 and Smad4, COS cells were transfected with N-terminally Flag-tagged Smad2 (F-Smad2) and M-Smad4 in the absence or presence of constitutively active variants of the individual type I receptors (Figure 5B). Immunoprecipitation with anti-Flag antibody followed by anti-Myc immunoblotting showed that heteromeric complexes were formed between Smad2 and Smad4 only in the presence of the constitutively active forms of TβR-I (TβR-I/T204D) or the structurally and functionally related activin type IB receptor (ActR-IB) (ActR-IB/T206D). This response was specific as constitutively active variants of activin receptor like-kinase (ALK)-1 (ALK-1/Q201D), activin type I receptor (ActR-I) (ActR-I/Q207D), BMP receptor (BMPR)-IA (BMPR-IA/Q233D) and BMPR-IB (BMPR-IB/Q204D), with intracellular domains distinct from TβR-I and ActR-IB, were unable to induce an interaction between F-Smad2 and M-Smad4 (Figure 5B).

The formation of heteromeric Smad complexes *in vivo* was further studied using the sequential immunoprecipitation technique. Cell lysates from metabolically labelled COS cells transfected with Myc-tagged Smad3 (M-Smad3) and Flag-tagged Smad4 (F-Smad4) were subjected to immunoprecipitation with a Myc antibody (against Smad3); bound components were then dissociated and reprecipitated by a Flag antibody (against Smad4). F-Smad4 was detected in the second precipitation, but only upon co-transfection with TβR-II and TβR-I and addition of TGF-β1 (Figure 5C). Using the sequential immunoprecipitation technique we also observed the ligand-dependent interaction between Smad2 and F-Smad4 (Figure 5D).

TβR-1 activation induces interaction between Smad2 and Smad3

In addition to the interactions between Smad2 or Smad3 and Smad4, the interaction between Smad2 and Smad3 was studied upon TβR-I activation. COS cells were transfected with F-Smad2 and M-Smad3 in the absence or presence of constitutively active TβR-I. We studied the co-immunoprecipitation of Smad3 with Smad2 after immunoprecipitation with Flag antibody directed towards Smad2 followed by immunoblotting with anti-Myc antibody against Smad3. Only upon co-transfection of constitutively active TβR-I did we observe co-immunoprecipitation of the Smad3 protein (Figure 6A).

Interaction between Smad2 and Smad3 was also investigated by the sequential immunoprecipitation technique. We observed interaction between F-Smad2 and M-Smad3 (Figure 6B). Although we observed no ligand-dependency in this case, the complex formation was increased after co-expression of Smads with TβR-I and TβR-II. This increase can be explained by ligand-independent complex formation of TβR-I and TβR-II (with concomitant TβR-I activation) (Chen and Weinberg, 1995), sufficient for induction of Smad2 and Smad3 complex formation.

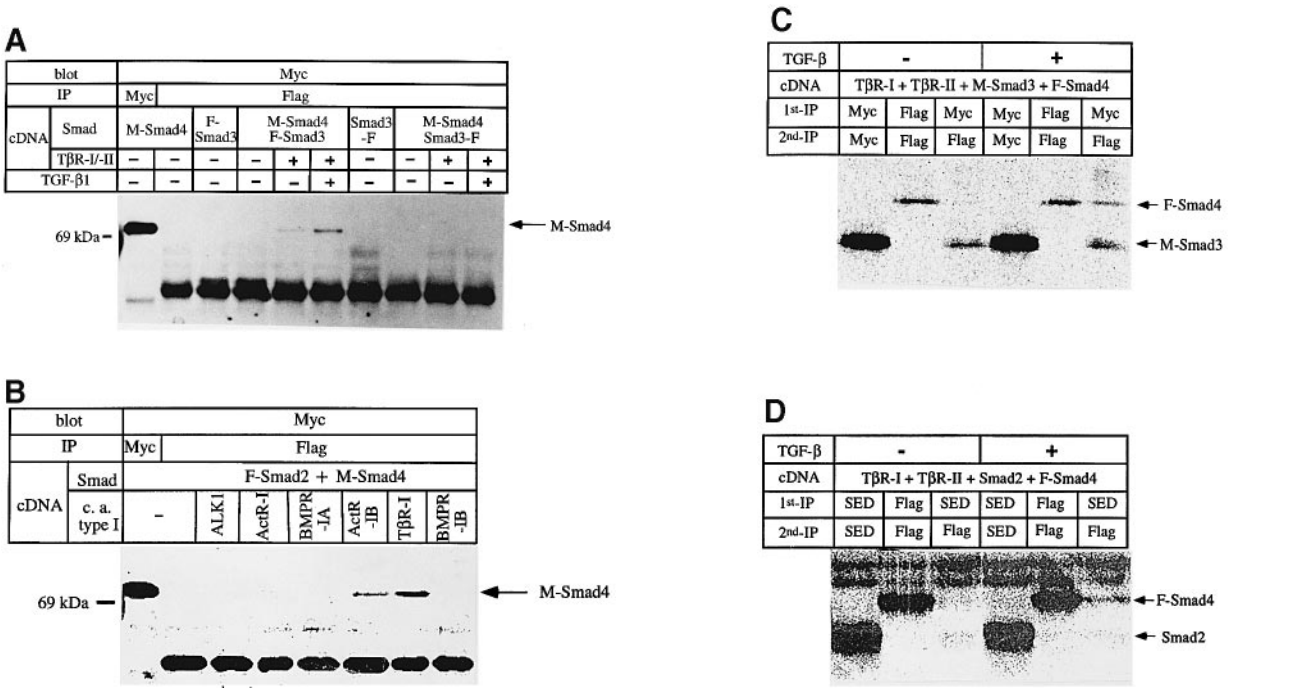

Fig. 5. TGF-β-dependent interaction of Smad2 with Smad4, and of Smad3 with Smad4. (**A**) The *in vivo* heteromeric complex formation of Smad3 and Smad4 was studied using COS cells transfected with combinations of Smads, in the absence or presence of TGF-β receptor-mediated activation. Cell lysates were subjected to immunoprecipitation (IP) followed by Western blotting (blot) to show the TGF-β-dependent interaction of N-terminally Myc-tagged Smad4 (M-Smad4) and N-terminally Flag-tagged Smad3 (F-Smad3), but not C-terminally Flag-tagged Smad3 (Smad3-F). The band detected in both lanes is immunoglobulin. (**B**) Interaction between Flag-tagged Smad2 (F-Smad2) and M-Smad4 in the presence of constitutively active type I receptors (c.a. type I). Interaction was determined using immunoprecipitation of F-Smad2 followed by Western blotting of M-Smad4 with the indicated antibodies. Myc antibody was used for immunoprecipitation to show the expression of M-Smad4. (**C** and **D**) Cell lysates of metabolically labelled COS cells transfected with different combinations of Smads were subjected to sequential immunoprecipitation. The immunoprecipitated proteins were analysed by SDS–PAGE and Fuji X Bio-Imager. For detection of the interaction between M-Smad3 and F-Smad4, Myc and Flag antibodies were used, respectively. and for detection of Smad2 and F-Smad4, SED antiserum and Flag antibody, respectively.

Translocation of Smad2, Smad3 and Smad4 in Mv1Lu cells after stimulation with TGF-β1

The subcellular localization of different Smads in Mv1Lu cells before or after stimulation with TGF-β was analysed by immunofluorescence using specific antisera. In the absence of ligand, staining for Smad2 and Smad3 as well as Smad4 was seen predominantly in the cytoplasm of the cells, whereas after stimulation with TGF-β1 for 1 h, the staining in each case significantly accumulated in the nucleus (Figure 7A–F). In contrast, using the Smad1/Smad5 antiserum we observed a cytoplasmic staining in the absence of ligand that remained unaltered upon TGF-β1 stimulation (Figure 7G and H). However, when Mv1Lu cells were stimulated with BMP-7, we observed a nuclear accumulation of Smad1/Smad5 (S.Souchelnytskyi, unpublished results). These data suggest a nuclear function for Smad2, Smad3 and Smad4, but not of Smad1/Smad5 after TGF-β1 stimulation in Mv1Lu cells.

Synergistic effect of Smad2, Smad3 and Smad4 in transcriptional response using p3TPLux reporter

Previously, synergistic effects of combinations of Smad3 and Smad4 (Zhang *et al*., 1996) and of Smad2 and Smad4 (Lagna *et al*., 1996) were shown using a transcriptional response assay with the p3TPLux reporter construct. We confirmed the synergistic effects of Smad2 and Smad4, and of Smad3 and Smad4, by transfection of the p3TPLux reporter with Smads in Mv1Lu cells deficient in TβR-I (R mutant cells). R mutant cells were used as they can be transfected more efficiently than wild-type Mv1Lu cells. Smad2 co-transfected with Smad4, or Smad3 with Smad4, induced significantly higher levels of luciferase activity than Smad2, Smad3 or Smad4 alone (Figure 8A). In addition, Smad2 and Smad3 synergized, albeit to a lesser extent than combinations of Smad2 and Smad4, or Smad3 and Smad4. Notably, the highest luciferase response was found when Smad2, Smad3 and Smad4 were co-transfected all together. It should be noted that the total amount of Smad DNA transfected in each transfection was constant. Substitution of Smad2 or Smad3 with Smad1, which acts in the BMP pathway, decreased the response to levels similar to those found with combinations of Smad3 and Smad4, or Smad2 and Smad4, respectively. Furthermore, the synergistic effect of Smad2 and Smad4 in the p3TPLux luciferase assay was significantly inhibited by co-transfection of a dominant-negative-acting Smad3 (Smad3 with truncation at the C-terminus) (Figure 8B). These data indicate that Smad2, Smad3 and Smad4 functionally synergize in a TGF-β-inducible transcriptional response assay.

Discussion

After ligand-mediated activation of serine/threonine kinase receptors, particular Smad family members become phosphorylated, form heteromeric complexes and translocate to the nucleus, where they may direct transcriptional responses (Derynck and Zhang, 1996; Massagué, 1996).

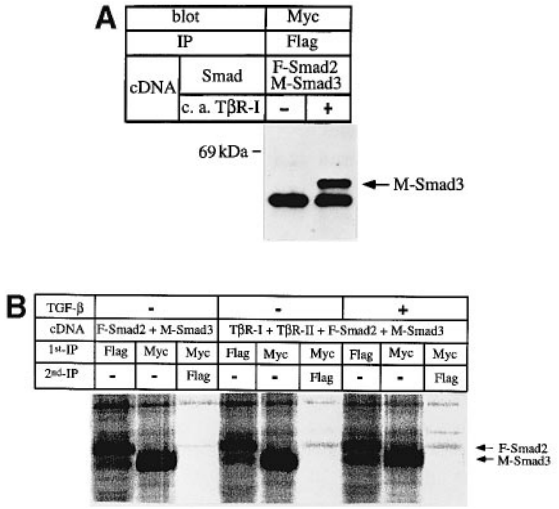

Fig. 6. TβR-I activation induces interaction between Smad2 and Smad3. (**A**) The complex formation of Smad2 and Smad3 was studied using COS cells transfected with combinations of N-terminally Flag-tagged Smad2 (F-Smad2) and N-terminally Myc-tagged Smad3 (M-Smad3) in the absence or presence of the constitutively active form (c.a.) of TβR-I. Cell lysates were subjected to immunoprecipitation (IP) followed by Western blotting (blot) to show the TβR-I activation-dependent interaction between F-Smad2 and M-Smad3. (**B**) COS cells were transfected with combinations of F-Smad2 and M-Smad3 in the presence or absence of TβR-I and TβR-II, and stimulated with TGF-β1. Cells were metabolically labelled and cell lysates subjected to sequential immunoprecipitation with Myc and Flag antibodies. The immunoprecipitated proteins were analysed by SDS–PAGE and Fuji X Bio-Imager.

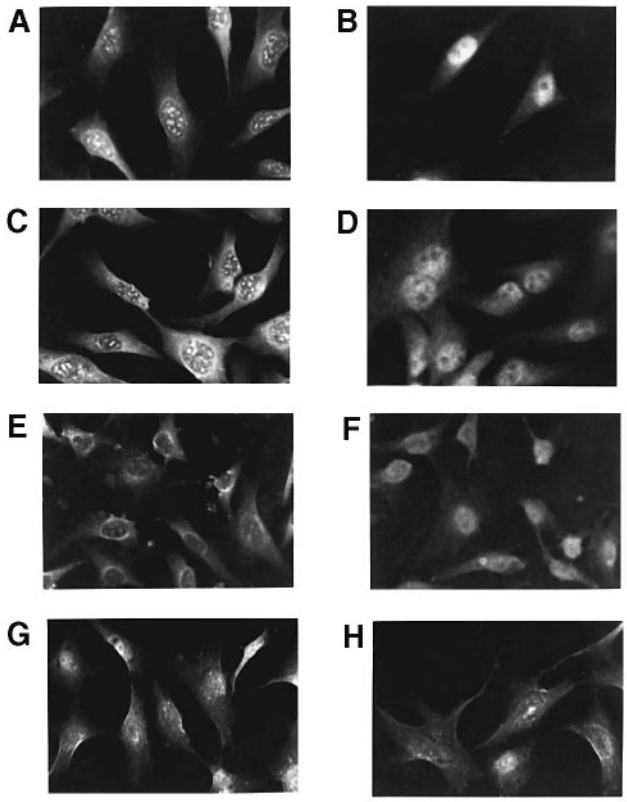

Fig. 7. Nuclear translocation of Smad2, Smad3 and Smad4 after TGF-β stimulation. Mv1Lu cells were incubated in the absence (**A**, **C**, **E** and **G**) or presence (**B**, **D**, **F** and **H**) of TGF-β for 1 h. Smad2 (A and B), Smad3 (C and D), Smad4 (E and F) and Smad1/Smad5 (G and H) were localized in the cells by immunofluorescence using specific antisera. Smad2, Smad3, Smad4 and Smad5 staining was predominant in the cytoplasm in the absence of TGF-β, whereas nuclear staining for all Smads, except Smad1/Smad5, was observed after TGF-β stimulation.

In the present report, we show that TGF-β induces the activation of Smad2, Smad3 and Smad4 via TβR-II and TβR-I. In addition, TGF-β stimulates the formation of physical heteromeric complexes between Smad2, Smad3 and Smad4. Furthermore, the three Smads were shown to functionally synergize in a TGF-β-inducible transcriptional response assay. Taken together, these results suggest that complex formation between Smad2, Smad3 and Smad4 is required for efficient TGF-β signal transduction (Figure 9).

Specific antisera to Smad2, Smad3 and Smad4 were raised against peptide sequences corresponding to highly divergent sequences in the proline-rich linker regions (Figure 1). These reagents enabled us to study endogenous Smad activation. TGF-β was found to induce the phosphorylation and nuclear translocation of Smad2 and Smad3 in non-transfected cells (Figures 3 and 7A–D). These findings extend and strengthen the physiological significance of previous studies using transfected cells and epitope-tagged Smads (Chen,Y. *et al*., 1996; Eppert *et al*., 1996; Zhang *et al*., 1996). As shown in this paper, the latter experimental conditions may alter the signalling properties of Smads (Figures 4 and 5A), and provide a possible explanation why, in contrast to our results on endogenous Smad proteins, Myc-tagged Smad3 was constitutively present in the nucleus upon its overexpression in COS cells (Chen,Y. *et al*., 1996).

Association between TβR-I and N-terminally tagged Smad3 (and Smad2) could be detected when TβR-II was co-transfected with kinase-deficient TβR-I, but not wild-type TβR-I (Figure 4). These findings are in agreement with a previous report by Macías-Silva *et al*. (1996) in which Smad2 was shown to be a direct substrate of activated TβR-complex. In contrast to N-terminally tagged Smad3, C-terminally tagged Smad3 was shown to interact with wild-type TβR-I, and thus to act similarly to the Smad2 dominant-negative mutant, in which the three C-terminal serine residues of Smad2 were mutated to alanine residues (Macías-Silva *et al*., 1996). These results may explain the difference in TβR-I interaction with Smad2 versus Smad3 that was previously reported (Macías-Silva *et al*., 1996; Zhang *et al*., 1996).

Phosphorylation of Smad4 has thus far been reported only after stimulation by activin using transfected cells (Lagna *et al*., 1996). In the present study, we showed that Smad4 was constitutively phosphorylated in non-transfected cells. This is in contrast to Smad4 over-expressed in 293 cells, which was not phosphorylated (Zhang *et al*., 1996). The Smad4 kinases, of which the identity is unknown, may exert a regulatory role, which may or may not be regulated by TGF-β. Upon TGF-β stimulation, Smad4 translocated to the nucleus, whereas the phosphorylation level of Smad4 remained unaltered (Figures 3C and 7E and F). However, it is possible that due to a high basal level of phosphorylation, a change in phosphorylation at a particular site(s) occurred, but could not be detected. In particular, as Smad4—unlike Smad2 and Smad3—appears not to be a direct substrate for TβR-I (Macías-Silva *et al*., 1996). A possible candidate for an

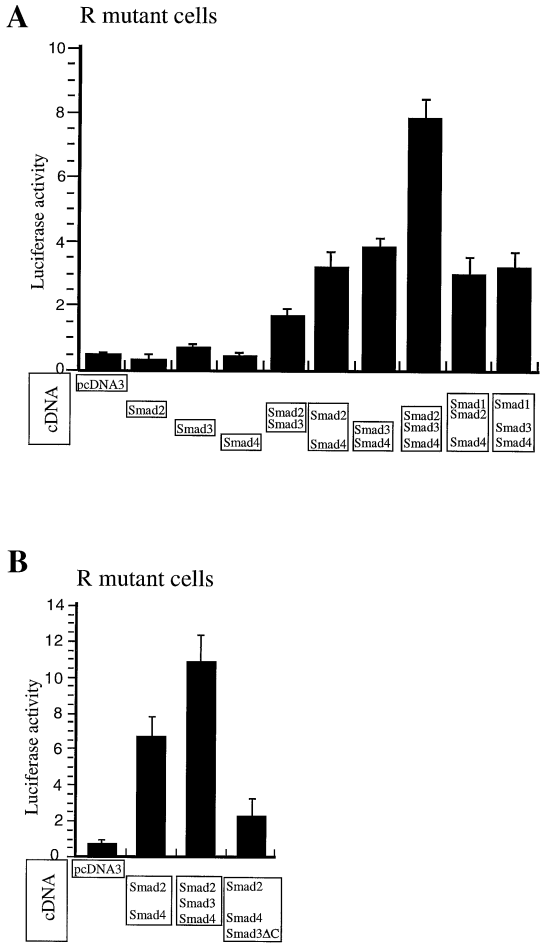

Fig. 8. Synergism of Smad2, Smad3 and Smad4 on TGF-β-inducible p3TPLux reporter. (**A**) The effect of Smad2, Smad3 or Smad4, transfected alone or in various combinations, on p3TPLux transcriptional response was measured in R-mutant cells. In each case (except for pcDNA3) an identical amount of Smad was transfected; in single transfection 6 μg of the indicated Smad was used, in double transfection 3 μg of each Smad was used, in triple transfection 2 μg of each Smad was used. (**B**) Dominant-negative Smad3 (Smad3 C) inhibits the synergistic effect of Smad2 and Smad4. For double transfection 3 μg of each Smad was used, for triple transfection 2 μg of each Smad was used. The values were normalized for transfection efficiency using the β-gal reporter gene under transcriptional control of the cytomegalovirus promoter. Results shown are representative of at least three independent experiments, and data points are the average of triplicate measurements.

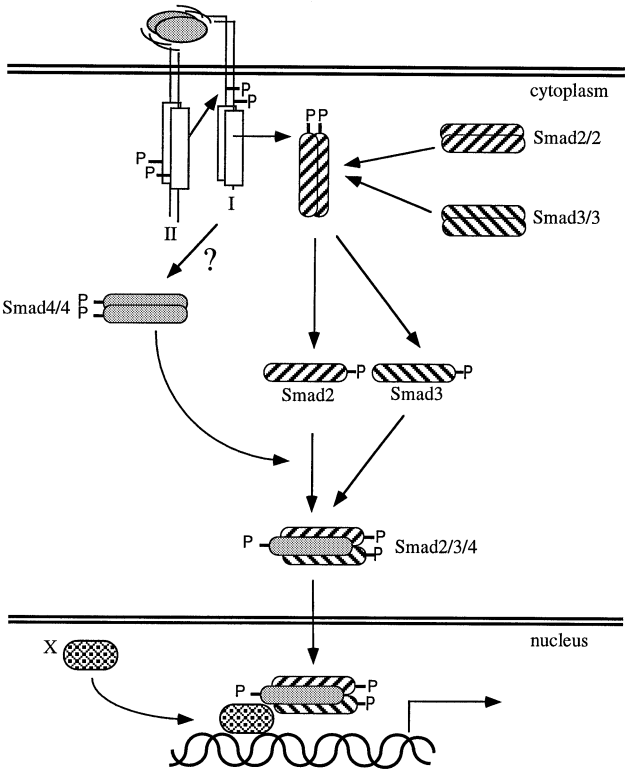

Fig. 9. Schematic model of TGF-β-mediated Smad activation and heteromeric complex formation. TGF-β binding induces heteromeric complex formation of TβR-II and TβR-I, in which the constitutively active TβR-II kinase phosphorylates and activates TβR-I. Smad2, Smad3 and Smad4 exist as homomeric complexes in the absence of ligand (homodimers are shown, but higher order complexes cannot be excluded). Smad2 and Smad3 become directly phosphorylated by transient interaction with activated TβR-I, whereas Smad4 is indirectly activated. Upon activation, Smad2, Smad3 and Smad4 assemble in a common complex, or possibly several types of heteromeric complexes, of which the stoichiometry between the components is unknown. Thereafter, the Smad complex(es) translocate(s) to the nucleus, where it (or they) may interact with specific DNA binding proteins and direct transcription of target genes.

activating kinase of Smad4 is the MAP kinase kinase kinase homologue, TGF-β-activated kinase (TAK)-1 (Yamaguchi *et al*., 1995). Interestingly, both TAK-1 and Smad4 were shown to act in both TGF-β and BMP signalling pathways (Yamaguchi *et al*., 1995; Lagna *et al*., 1996).

Previously, Lagna *et al*. (1996) reported that in the absence of ligand homomeric complexes of Smad 2 or Smad4 occur in transfected Mv1Lu cells, and that TGF-β induces a heteromeric complex of Smad2 and Smad4 in these cells. Here, we confirm and extend their findings by showing TGF-β heteromeric complex formation of Smad3 and Smad4, as well as Smad2 and Smad3 *in vivo* (Figure 5). The interaction of Smad2 or Smad3 with TβR-I is very transient and it is thus unlikely that the interaction is mediated by simultaneous binding of Smad2 and Smad3 to TβR-I. Further experiments are needed to determine whether Smad2–Smad3 interaction requires Smad4, e.g. using cells deficient in Smad4. COS cells express endogenous Smad4 which may circumvent the need for co-transfection of Smad4 for the interaction between Smad2 and Smad3. Thus far, attempts to show the formation of a heteromeric complex in non-transfected cells using the specific Smad antisera on lysates from cells labelled with [^{35}S]methionine/cysteine or [^{32}P]orthophosphate have failed. A possible reason could be that the antisera made towards the linker regions in the Smads may interfere with heteromeric complex formation. Specificity of downstream signalling responses was shown by the association between Smad2 and Smad4 upon activation of TβR-I and the structurally related ActR-IB, but not upon activation of the more divergent ActR-I, BMPR-IA and BMPR-IB.

A cooperative effect of Smad2, Smad3 and Smad4 on p3TPLux reporter was observed (Figure 8). Combinations of two Smads showed an enhanced response, but importantly, highest synergism was observed with all three Smads. The specificity of this response was shown by the inability of Smad1, which acts in the BMP signalling pathway, to substitute functionally for Smad2 or Smad3.

In addition, we found that a dominant-negative Smad3 inhibited the synergistic effect between Smad2 and Smad4. Thus, efficient TGF-β signalling as measured with p3TPLux reporter requires all three Smads. The finding of a response by transfection of two Smads only, cannot be taken as evidence that two Smads are enough for signalling, since all three Smads were found to be endogenously expressed in Mv1Lu cells (Figure 2). The synergistic effect on the p3TPLux reporter is observed in the absence of ligand, and receptor activation did not significantly increase this effect (data not shown). The mere overexpression of Smads in Mv1Lu cells may lead to a small increase in heteromeric complex formation and nuclear translocation, which is sufficient for p3TPLux response, but which is below the detection limit of biochemical assay.

Importantly, our data on ligand-dependent activation, complex formation and functional synergism suggest a non-redundant role for Smad2 and Smad3 in TGF-β signalling with respect to p3TPLux, and may provide a reason why in one cell Smad2 and Smad3 are both phosphorylated and translocated to the nucleus upon TGF-β challenge. A functional requirement of complex formation of three Smads in TβR-mediated signalling implies co-expression of all three Smads in the same cell, and a broad distribution between different tissues similar to what is found for TβR-I and TβR-II. Indeed, our results on Smad protein and mRNA expression in various cell lines and tissues (Nakao *et al*., 1997; A.Nakao *et al*., unpublished results), and analysis of tissues where Smad2, Smad3 and Smad4 expression sequence tags have been identified (Washington University–Merck EST project), support the notion that Smad2, Smad3 and Smad4 are simultaneously expressed in many different cell types.

Involvement of multiple Smads appears not to be limited to TGF-β-mediated signalling. Three Smads, i.e. Smad1, Smad4 and Smad5 have been implicated in BMP signalling (Lagna *et al*., 1996; S.Souchelnytskyi *et al*., unpublished results). In addition, genetic findings in *C.elegans* point to the formation of a heteromeric signalling complex of Sma-2, Sma-3 and Sma-4, as mutant phenotypes for each of the three *sma* genes mimic the mutant phenotype of *daf-4* (Savage *et al*., 1996). Our future studies will be aimed at determining the assembly mechanism of heteromeric complex(es) and the stoichiometry between the components, and at the elucidation of the specific functional roles of Smad2, Smad3 and Smad4 in the heteromeric complex(es).

Materials and methods

Constructs and reagents

Expression constructs for TβR-I, TβR-II, Smad2, Smad1 and Smad4 have been described previously (ten Dijke *et al*., 1994; de Winter *et al*., 1997; Nakao *et al*., 1997). Constitutively active forms of TβR-I, ALK1, ActR-I, ActR-IB, BMPR-IA, BMPR-IB and the kinase-inactive form of TβR-I were made by a polymerase chain reaction (PCR)-based approach and subcloning into pcDNA3 vector. F-Smad2, F-Smad4 and M-Smad4 were made using the PCR technique and by subcloning into pCMV5Flag and pCMV5BFlag, respectively (the latter vectors were provided by Dr J.Wrana). Smad3-F cDNA and cDNA encoding Smad3 with C-terminal truncation, acting in a dominant-negative manner (Zhang *et al*., 1996) were provided by Dr R.Derynck (University of California, San Francisco). Smad5-HA was a gift from Dr J.M.Yingling (Duke University, Durham, NC). M-Smad3 and F-Smad3 were made by a PCR-mediated approach and subcloned into appropriate epitope-tagged pcDNA3 vectors. Anti-Flag, anti-Myc and anti-haemagglutinin (HA; 12CA5) antibodies were purchased from Kodak (New Haven, CT), Santa Cruz Biotechnology (Santa Cruz, CA) and Boehringer-Mannheim (Mannheim), respectively.

Cell lines

COS cells and Mv1Lu mink lung epithelial cells were obtained from the American Type Culture Collection. HSC4 human oral squamous cell carcinoma cells were obtained from Dr F.Momose (Tokyo Medical and Dental University, Tokyo). R mutant Mv1Lu cells were obtained from Dr J.Massagué. Cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% fetal bovine serum, 100 units/ml penicillin and 50 μg/ml streptomycin.

Preparation of polyclonal antisera

Antisera raised against a Smad2 peptide that weakly cross-reacts with Smad3 (denoted SED; peptide SEDGETSDQQLNQSMDTG), and against Smad4 (denoted HPP; HPPSNRASTETYSTPALLA), have been described previously (de Winter *et al*., 1997; Nakao *et al*., 1997). Specific antisera were raised against synthetic peptides corresponding to amino acid sequences of the variable proline-rich linker regions of Smad2 (termed DQQ; peptide DQQLNQSMDTGSPAELSPTTL) and Smad3 (termed DHQ; peptide DHQMNHSMDAGSPNPM). Smad1/Smad5 cross-reactive antiserum was obtained using a peptide derived from the MH2 domain of Smad1 (termed QWL; peptide QWLDKLTQMGSPHNPISSVS). The peptides were coupled to keyhole limpet haemocyanin (Calbiochem-Behring) with glutaraldehyde, mixed with Freund's adjuvant, and used to immunize rabbits.

Transient transfection, metabolic labelling, immunoprecipitation, [^{32}P]orthophosphate labelling of cells and SDS–PAGE

Transient transfection, metabolic labelling, immunoprecipitation, [^{32}P]orthophosphate labelling of cells and SDS–PAGE were performed as described previously (Nakao *et al*., 1996). Proteins were electrotransferred to nitrocellulose membrane and immunoblotted with the indicated antibodies and developed using an enhanced chemiluminescence detection system.

Iodination of TGF-β1 and affinity crosslinking

TGF-β1 was iodinated using the chloramine T method according to Frolik *et al*. (1984). Crosslinking was performed as previously described (Franzén *et al*., 1993). Aliquots of cell lysates were subjected to immunoprecipitation using indicated antibodies as previously described (Franzén *et al*., 1993), but protein A–Sepharose beads were washed once with washing buffer. To determine the expression of Smad proteins, cell lysates were electrotransferred to nitrocellulose membrane and immunoblotted with the Flag antibody and developed using an enhanced chemiluminescence detection system (Amersham).

Immunoprecipitation followed by Western blotting

COS cells were transfected with expression constructs for Smads alone, or in combination with receptor cDNAs using DMRIE-C transfection reagent (Gibco-BRL). At 48 h after transfection, cells were washed, scraped and solubilized in a buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1% Trasylol, 1 mM PMSF. After 20 min on ice, the cell lysates were pelleted by centrifugation and incubated with the Flag or Myc antibodies for 2 h, followed by incubation with protein G–Sepharose beads for 30 min at 4°C. The beads were washed four times with the buffer used for cell solubilization. Thereafter, the immunocomplexes were eluted by boiling for 3 min in SDS sample buffer (100 mM Tris–HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS) containing 10 mM DTT and subjected to SDS–PAGE. Proteins were then electrotransferred to nitrocellulose membrane and immunoblotted with the Myc antibody and developed using an enhanced chemiluminescence detection system.

Sequential immunoprecipitation

COS cells were transfected with expression constructs for Smads and TβRs using the DEAE–dextran method. At 48 h after transfection, cells were labelled with [^{35}S]methionine and [^{35}S]cysteine for 4 h. The cells were treated with 10 ng/ml TGF-β1 for the last 1 h of labelling. Thereafter, the cells were washed in TBS (20 mM Tris–HCl pH 7.4, 150 mM NaCl) and solubilized in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Trasylol, 1 mM PMSF). After 20 min on ice, the cell lysates were pelleted by centrifugation and precleared once with protein A–Sepharose (Pharmacia-LKB). Sub-

sequently, samples were incubated with the first antisera for 2 h, followed by an incubation with protein A–Sepharose beads for 30 min at 4°C. The beads were spun down and washed three times with lysis buffer. Thereafter, 50 μl of TBS containing 1% SDS and 10 mM DTT was added to the beads and samples were boiled for 2 min. After cooling to room temperature, 50 mM iodoacetamide was added to the samples, which after incubation at room temperature for 15 min were centrifuged. Supernatants were saved and diluted 10-fold with lysis buffer, followed by incubation with the second antibody for 2 h. Thereafter, protein A–Sepharose beads were added for 30 min and subsequently the beads were washed three times with lysis buffer. The immunocomplexes were eluted by boiling for 3 min in SDS sample buffer in the presence of 10 mM DTT and analysed by SDS–PAGE.

Transcriptional response assay

R mutant cells were transiently transfected with p3TPLux promoter reporter construct in the presence of various combinations of the indicated Smad expression plasmids. In each experiment equal amounts of DNA were transfected. Luciferase activity was measured as previously described (Yamashita *et al*., 1995). The β-gal reporter gene in pCMV5 vector (Stratagene) was co-transfected in each transfection for normalization of the transfection efficiency. β-gal activity was analysed using the β-gal reporter kit (Promega).

Immunofluorescence study

Subcellular localization of Smad2, Smad3 and Smad4 in Mv1Lu cells was determined as previously reported (Nakao *et al*., 1996). Cells grown in LAB TEK chambers (Nunc, Naperville, IL) were washed with PBS, fixed with acetone and incubated with 5% normal goat serum. After that cells were incubated with Smad antiserum for 60 min. The DHQ antiserum against Smad2 or DQQ antiserum against Smad3 were used at a 1000- to 2000-fold dilution. The HPP antiserum against Smad4 was affinity purified and used at 2.6 μg/ml. After washing in PBS, the cells were incubated with biotinylated antibodies against rabbit-Ig for 60 min, then incubated further with FITC-labelled strepavidin. After a final wash, the cells were covered with glycerine and observed by fluorescence microscopy. Mv1Lu cells were incubated in the absence or presence of 10 ng/ml TGF-β1 for 1 h.

Acknowledgements

We thank our colleagues for stimulating discussions, Nils-Erik Heldin for the help with microscopic examination, and Irie Nakao for encouragement. We are also grateful to Johan de Winter for Smad4 cDNA, Rik Derynck for Smad3 cDNA, Jonathan Yingling for Smad5 cDNA, Hideya Ohashi for TGF-β1, Kuber Sampath for BMP-7, Joan Massagué for p3TP-Lux reporter construct and R mutant cells, and Fumio Momose for the HSC4 cell line.

References

Baker,J.C. and Harland,R.M. (1996) A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. *Genes Dev.*, **10**, 1880–1889.

Chen,F. and Weinberg,R.A. (1995) Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor β receptor kinase. *Proc. Natl Acad. Sci. USA*, **92**, 1565–1569.

Chen,X., Rubock,M.J. and Whitman,M. (1996) A transcriptional partner for MAD proteins in TGF-β signalling. *Nature*, **383**, 691–696.

Chen,Y., Lebrun,J.-J. and Vale,W. (1996) Regulation of transforming growth factor β- and activin-induced transcription by mammalian Mad proteins. *Proc. Natl Acad. Sci. USA*, **93**, 12992–12997.

Derynck,R. and Zhang,Y. (1996) Intracellular signalling: the Mad way to do it. *Curr. Biol.*, **6**, 1226–1229.

Derynck,R. (1994) TGF-β-receptor-mediated signaling. *Trends Biochem. Sci.*, **19**, 548–553.

Derynck,R. *et al*. (1996) Nomenclature: vertebrate mediators of TGFβ family signals. *Cell*, **87**, 173.

de Winter,J.P., Roelen,B.A.J., ten Dijke,P., van der Burg,B. and van den Eijnden-van Raaij,A.J.M. (1997) DPC4 (SMAD4) mediates transforming growth factor-β1 (TGF-β1) induced growth inhibition and transcriptional response in breast tumour cells. *Oncogene*, **14**, 1891–1900.

Emini,E.A., Hughes,J.V., Perlow,D.S. and Boger,J. (1985) Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *J. Virol.*, **55**, 836–839.

Eppert,K. *et al*. (1996) MADR2 maps to 18q21 and encodes a TGFβ-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell*, **86**, 543–552.

Franzén,P., ten Dijke,P., Ichijo,H., Yamashita,H., Schulz,P., Heldin,C.-H. and Miyazono,K. (1993) Cloning of a TGFβ type I receptor that forms a heteromeric complex with the TGFβ type II receptor. *Cell*, **75**, 681–692.

Frolik,C.A., Wakefield,L.M., Smith,D.M. and Sporn,M.B. (1984) Characterization of a membrane receptor for transforming growth factor-β in normal rat kidney fibroblasts. *J. Biol. Chem.*, **259**, 10995–11000.

Graff,J.M., Bansal,A. and Melton,D.A. (1996) *Xenopus* Mad proteins transduce distinct subsets of signals for the TGFβ superfamily. *Cell*, **85**, 479–487.

Ichijo,H., Momose,F. and Miyazono,K. (1990) Biological effects and binding properties of transforming growth factor-β on human oral squamous cell carcinoma cells. *Exp. Cell Res.*, **187**, 263–269.

Hahn,S.A. *et al*. (1996) *DPC4*, a candidate tumor suppressor gene at human chromosome 18q21.1 *Science*, **271**, 350–353.

Hoodless,P.A., Haerry,T., Abdollah,S., Stapleton,M., O'Connor,M.B., Attisano,L. and Wrana,J.L. (1996) MADR1, a MAD-related protein that functions in BMP2 signaling pathways. *Cell*, **85**, 489–500.

Jameson,B.A. and Wolf,H. (1988) The antigenic index: a novel algorithm for predicting antigenic determinants. *Comput. Appl. Biosci.*, **4**, 181–186.

Lagna,G., Hata,A., Hemmati-Brivanlou,A. and Massagué,J. (1996) Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. *Nature*, **383**, 832–836.

Lechleider,R.J., de Caestecker,M.P., Dehejia,A., Polymeropoulo,M.H. and Roberts,A.B. (1996) Serine phosphorylation, chromosomal localization, and transforming growth factor-β signal transduction by human *bsp-1*. *J. Biol. Chem.*, **271**, 17617–17620.

Lin,H.Y. and Lodish,H.F. (1993) Receptors for the TGF-β superfamily. *Trends Cell Biol.*, **11**, 972–978.

Liu,F., Hata,A., Baker,J.C., Doody,J., Cárcamo,J., Harland,R.M. and Massagué,J. (1996) A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature*, **381**, 620–623.

Macías-Silva,M., Abdollah,S., Hoodless,P.A., Pirone,R., Attisano,L. and Wrana,J.L. (1996) MADR2 is a substrate of the TGFβ receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell*, **87**, 1215–1224.

Massagué,J. (1996) TGFβ signaling: receptors, transducers, and mad proteins. *Cell*, **85**, 947–950.

Massagué,J. and Weis-Garcia,F. (1996) Serine/threonine kinase receptors: mediators of transforming growth factor β signals. *Cancer Surv.*, **27**, 41–64.

Meersseman,G. *et al*. (1997) The C-terminal domain of Mad-like signal transducers is sufficient for biological activity in the *Xenopus* embryo and transcriptional activation. *Mech. Dev.*, **61**, 127–140.

Nakao,A., Röijer,E., Imamura,T., Souchelnytskyi,S., Stenman,G., Heldin,C.-H. and ten Dijke,P. (1997) Identification of Smad2, a human MAD-related protein in the TGF-β signaling pathway. *J. Biol. Chem.*, **272**, 2896–2900.

Riggins,G.J. *et al*. (1996) *Mad*-related genes in the human. *Nature Genet.*, **13**, 347–349.

Roberts,A.B. and Sporn,M.B. (1993) Physiological actions and clinical applications of transforming growth factor-β (TGF-β). *Growth Factors*, **8**, 1–9.

Savage,C., Das,P., Finelli,A.L., Townsend,S.R., Sun,C.-Y., Baird,S.E. and Padgett,R.W. (1996) *Caenorhabditis elegans* genes *sma-2*, *sma-3*, and *sma-4* define a conserved family of transforming growth factor β pathway components. *Proc. Natl Acad. Sci. USA*, **93**, 790–794.

Sekelsky,J.J., Newfeld,S.J., Raftery,L.A., Chartoff,E.H. and Gelbart, W.M. (1995) Genetic characterization and cloning of *Mothers against dpp*, a gene required for *decapentaplegic* function in *Drosophila melanogaster*. *Genetics*, **139**, 1347–1358.

ten Dijke,P., Yamashita,H., Ichijo,H., Franzén,P., Laiho,M., Miyazono,K. and Heldin,C.-H. (1994) Characterization of type I receptors for transforming growth factor-β and activin. *Science*, **264**, 101–104.

ten Dijke,P., Miyazono,K. and Heldin,C.-H. (1996) Signaling via hetero-oligomeric complex of type I and type II serine/threonine kinase receptors. *Curr. Opin. Cell Biol.*, **8**, 139–145.

Thomsen,G.H. (1996) *Xenopus mothers against decapentaplegic* is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. *Development*, **122**, 2359–2366.

Wrana,J.L., Attisano,L., Wieser,R., Ventura,F. and Massagué,J. (1994) Mechanism of activation of the TGF-β receptor. *Nature*, **370**, 341–347.
Yamaguchi,K., Shirakabe,K., Shibuya,H., Irie,K., Oishi,I., Ueno,N., Taniguchi,T., Nishida,E. and Matsumoto,K. (1995) Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. *Science*, **270**, 2008–2011.
Yamashita,H., ten Dijke,P., Huylebroeck,D., Sampath,T.K., Andries,M., Smith,J.C., Heldin,C.-H. and Miyazono,K. (1995) Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J. Cell Biol.*, **130**, 217–226.
Yingling,J.M., Das,P., Savage,C., Zhang,M., Padgett,R.W. and Wang, X.-F. (1996) Mammalian dwarfins are phosphorylated in response to transforming growth factor β and are implicated in control of cell growth. *Proc. Natl Acad. Sci. USA*, **93**, 8940–8944.
Zhang,Y., Feng,X.-H., Wu,R.-Y. and Derynck,R. (1996) Receptor-associated Mad homologues synergize as effectors of the TGF-β response. *Nature*, **383**, 168–172.

Received on April 16, 1997; revised on June 5, 1997