Smad7 Differentially Regulates Transforming Growth Factor β-mediated Signaling Pathways*

(Received for publication, June 24, 1999, and in revised form, August 17, 1999)

Hong-Jian Zhu‡, Josephine Iaria, and Andrew M. Sizeland

From the Ludwig Institute for Cancer Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia

Smad7 has been identified as a negative regulator of transforming growth factor β (TGF-β) signaling by interfering with the phosphorylation of other Smad proteins by TGF-β receptor type 1 (TβRI). We established a mink lung epithelial (Mv1Lu) cell line where ectopic expression of Smad7 is tightly controlled by doxycycline using an improved Tet-on system. Once induced by doxycycline, the recombinant Smad7 was localized predominantly in the perinuclear region and in the cytoplasm. However, the type of culture surface alters the subcellular localization of Smad7: on plastic or on fibronectin-coated glass, Smad7 was localized in the cytoplasm; but when the cells were cultured on glass, nuclear localization was observed. TGF-β stimulation did not alter substantially the cellular distribution of Smad7. Importantly, the expression of recombinant Smad7 differentially inhibited TGF-β signaling pathways. Consistent with previous studies, Smad7 inhibited TGF-β-stimulated induction of type 1 plasminogen activator inhibitor as measured by p3TP-Lux reporter. However, expression of Smad7 had little effect on TGF-β-induced growth inhibition.

Members of the transforming growth factor β (TGF-β) family mediate a diverse range of cellular responses including cell proliferation, differentiation, migration, organization, and death (1). TGF-β signals through a heteromeric receptor complex of two distinct type I and type II serine/threonine kinase receptors, TβRI and TβRII (2). In the absence of TGF-β, TβRI and TβRII can form a latent receptor complex (3–5), and ligand binding is required for the activation of the receptor complex (6, 7). Upon TGF-β binding, the receptors rotate relatively within the complex (8), resulting in phosphorylation of TβRI by the constitutively active and autoprophosphorylated TβRII and thereby activation of TβRI (2). The activated TβRI then directly signals to downstream intracellular substrates, Smads (9–12). The first member of Smad protein family, Mad (mothers against dpp (decapentaplegic)) was identified in a genetic screen in Drosophila (13, 14), followed by cloning of sma-2, sma-3, and sma-4 in Caenorhabditis elegans (15). Subsequently, eight vertebrate Smad proteins in three different functional classes have been identified (16). Smads 1, 2, 3, 5, and 8 make up the receptor-regulated Smad subfamily with a conserved carboxyl-terminal SSXS motif; Smad4, also called DPC4 (deleted in pancreatic carcinoma locus 4), is a collaborating Smad (or common Smad); and Smads 6 and 7 form an inhibitory Smad subfamily (16). All Smad proteins share two regions of sequence similarity: Mad homology (MH) 1 at the NH2 terminus and MH2 at the COOH terminus. The receptor-regulated Smads contain a conserved SSXS motif at their COOH terminus, whereas the common Smad4 and inhibitory Smads 6 and 7 lack this motif. The receptor-regulated Smads 1, 5, and 8 appeared to mediate specifically signaling downstream of bone morphogenetic protein and its receptors, whereas Smads 2 and 3 function in TGF-β and activin signaling pathways (10, 11, 16). TGF-β-activated TβRII transiently and directly interacts with Smad2 (17, 18) and Smad3 (19), resulting in phosphorylation of the SSXS motif (20–22). Once phosphorylated, Smad2 and Smad3 associate with Smad4 and translocate to the nucleus (23). In the nucleus, this Smad complex associates with the forkhead DNA-binding protein FAST2 and binds to DNA, forming a transcriptionally active DNA complex (24, 25).

The inhibitory subfamily of Smads was first identified as genes induced by shear stress in vascular endothelial cells (26, 27). Expression of recombinant Smad6 is able to inhibit bone morphogenetic protein signaling and in part TGF-β signaling (28, 29). Smad7 inhibits both TGF-β signaling (30, 31) and bone morphogenetic protein signaling (31). Endogenous expression of Smad7 is induced rapidly by TGF-β, suggesting that the inhibitory Smads participate in a negative feedback loop that may control the intensity and/or duration of the response to TGF-β (31). Although Smad6 and Smad7 have both MH1 and MH2 domains, they lack the SSXS phosphorylation motif, and their MH1 domains are short (27, 28, 30, 31). When recombinant Smad6 and Smad7 are expressed, they exert their inhibitory effects by binding to TGF-β family receptors, thereby blocking the receptor-regulated Smads from interacting with the receptor (28, 30, 31). This mechanism would result in nonspecific negative regulation of TGF-β signaling pathways by inhibitory Smads (16). It is not clear whether physiologic levels of inhibitory Smads can also interfere with receptor-mediated phosphorylation of receptor-regulated Smads. However, it has been demonstrated that at low levels, Smad6 does not block receptor-mediated phosphorylation of Smad1 (29). Smad6 does compete with Smad4 for binding to phosphorylated Smad1, forming an inactive Smad1-Smad6 complex (29). This mechanism may provide selective inhibition by inhibitory Smads (16). TGF-β mediates multiple cellular responses (1), and it is not yet clear whether the inhibitory Smads selectively or nonselectively inhibit TGF-β signaling pathways.

Unlike receptor-regulated Smads, a recent report showed that in the absence of TGF-β, recombinant Smad7 localizes in the nucleus but exports from the nucleus to the cytoplasm after TGF-β stimulation (32). In addition, using a zinc-inducible system, this report (32) concluded that Smad7 inhibits TGF-β-mediated growth inhibition. However, one clone, 10–3, in the report by Itoh et al. (32), showed little inhibition by Smad7.

* 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.

‡ To whom correspondence should be addressed. Tel.: 61-3-9341-3150; Fax: 61-3-9341-3104; E-mail: Hong-Jian.Zhu@ludwig.edu.au.

The abbreviations used are: TGF-β, transforming growth factor-β; TβRI, TGF-β receptor; MH, mad homology; PAl, plasminogen activator inhibitor; CDTA, trans-1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid.
Furthermore, Smad7 expression only partially inhibited TGF-β-mediated growth inhibition in two other clones, 7-5s and 7-10. In the present work, we have investigated the negative regulatory role of Smad7 on two TGF-β-mediated biological responses using an improved tetracycline (Tet)-inducible system, where the expression of Smad7 is under the tight control of doxycycline. Once induced by doxycycline, Smad7 was expressed predominantly in the perinuclear region and in the cytoplasm. TGF-β stimulation did not alter substantially the cellular distribution of Smad7. Recombinant Smad7 inhibited TGF-β-stimulated induction of type 1 plasminogen activator inhibitor (PAI-1) as measured by the p3TP-Lux reporter. However, expression of Smad7 had little effect on TGF-β-induced growth inhibition. Thus, Smad7 inhibits differentially at least two TGF-β-mediated signaling pathways.

**EXPERIMENTAL PROCEDURES**

Establishing a Doxycycline-regulated Smad7-expressing Mv1Lu Cell Line—Mouse Smad7 cDNA with a flag tag at its NH₂ terminus in pcDNA3 (31) was a generous gift from P. ten Dijke (Ludwig Institute for Cancer Research,Upsala, Sweden). To subclone the flag-tagged Smad7 cDNA into a tetracycline-inducible vector, pTRE (CLONTECH), to obtain pTRE-Smad7, a BamHI-XbaI fragment encoding full-length Smad7 and flag tag at its NH₂ terminus and an XhoI-BamHI fragment from pTRE were ligated into pTRE at its XhoI and XbaI sites. An improved pTet-on vector (CLONTECH), pEPFpurp-Tet-on, was generously provided by G. Varo (AMRAD, Melbourne, Australia). Briefly, the gene encoding the “reverse” tetracycline repressor (33, 34) was subcloned into a pEP-BOS (35) vector, pEP-FPGpurpAv18, which confers puromycin resistance. Thus, the EF-1α promoter drives the expression of the reverse tetracycline repressor, and stable cell lines can be selected by puromycin. The improved system resulted in robust induction and a minimum level of leakage (see Fig. 1). To obtain doxycycline (a tetracycline derivative)-induced Smad7 expression Mv1Lu cell lines, pTRE-Smad7 and pEPFpurp-Tet-on were cotransfected into Mv1Lu cells by electroporation, and then the cells were selected in puromycin. Positive clones were screened first by luciferase assay according to CLONTECH protocol and then selected for their ability to express Smad7 in the presence of doxycycline by Western analysis using M2 antibody (IBI, Eastman Kodak Co.). Four clones were obtained, clones C, G, N, and Q.

Cell Culture and Transient Transfection—Mink lung epithelial (Mv1Lu) cells were a gift from A. B. Roberts (National Institutes of Health). The cells were grown in a 5% CO₂ atmosphere at 37 °C in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum (CSL, Australia), 60 μg/ml penicillin, and 100 μg/ml streptomycin. Puromycin (2 μg/ml) was added to the medium for Smad7 expression clones. Transient transfections were performed using FuGENE™ 6 (Boehringer Mannheim) protocol, and transfected cells were assayed 48 or 72 h later.

Western Blotting—Cells were seeded at about 40% confluence in six-well plates and treated with doxycycline at a designated concentration for 24 h, then treated with or without TGF-β1 (10 ng/ml) for 1 h. The cells were then washed with phosphate-buffered saline and lysed for 1 h at 4 °C in 150 μl of lysis buffer consisting of 25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, 1% Triton X-100, and 1.5% Trasylol (Bayer). The total cell lysates were subjected to SDS-gel electrophoresis using 12% polyacrylamide and Western blotting using M2 antibody (IBI). To prepare soluble and insoluble fractions of cell lysates, the lysis buffer contained 5 mM EDTA, 30 mM Hepes, 150 mM NaCl, 1% Triton X-100, 1 μM pepstatin, 1 μM phenylmethylsulfonyl fluoride, 0.2 mg/ml 1–10 phenanthroline, 1% Trasylol, and 0.2 mg/ml leupeptin. Cells were lysed in this lysis buffer for 1 h at 4 °C, and the cell lysates were then microcentrifuged to obtain a clear supernatant as the soluble fraction. The precipitates were washed twice with phosphate-buffered saline, suspended in SDS sample buffer containing 0.125 M Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, and 20% glycerol, and this constitutes the insoluble fraction. Both the soluble and insoluble fractions were then subjected to Western analysis similar to that of total cell lysates using M2 antibody (IBI) or cyclin A antibody (Santa Cruz Biotechnology, Inc.).

Immunofluorescence—Cells were grown in 24-well plates (Nunc, Denmark) or in LAB TEC chambers (Nunc, Naperville, IL) and were treated the same way as cells for Western analysis. To coat the glass surface of a LAB TEC chamber with fibronectin, 40 μg/ml human plasma fibronectin (Life Technologies, Inc.) in phosphate-buffered saline was added to the chamber and incubated at 37 °C for 3 h. The cells were washed twice with phosphate-buffered saline, fixed in methanol for 15 min, washed three times with phosphate-buffered saline, then incubated with M2 antibody (20 μg/ml in phosphate-buffered saline with 1% bovine serum albumin) for 30 min. Subsequently, these cells were washed again three times with phosphate-buffered saline and incubated with Cy3-conjugated goat anti-mouse IgG antibody (Zymed
nitrocellulose membranes were stripped of antibodies and Western blotted with cyclin A antibody (bottom panels) (soluble and insoluble fractions as described under “Experimental Procedures.” Both the soluble fraction (top panels) and the insoluble fraction (middle and bottom panels) were subjected to SDS-gel electrophoresis and Western blotting using M2 antibody (top and middle panels). Then the nitrocellulose membranes were stripped of antibodies and Western blotted with cyclin A antibody (bottom panels). B, immunofluorescence. Clone C cells were plated in a 24-well plate and incubated with doxycycline (2 mg/ml) for 24 h and with or without TGF-β1 (10 ng/ml) for 1 h. Subsequently, the cells were immunofluorescence stained, and confocal images were taken as in Fig. 1.

Laboratories Inc.) for 30 min and washed three times with phosphate-buffered saline and once with H2O. Finally, immunofluorescence images were obtained using a Bio-Rad MRC-1000 confocal microscope.

Luciferase Assay—The p3TP-Lux (36) TGF-β-inducible luciferase reporter construct, containing a region of the human PAI-1 gene promoter and three repeats of 12-O-tetradecanoylphorhbol-13-acetate-responsive elements upstream of luciferase gene (36), was obtained from A. B. Roberts. p3TP-Lux (0.25 μg/well) was transfected into inducible Smad7 expression Mv1Lu cells grown in 24-well plates (0.125 μg/well for cells in 8-well LAB TEK chambers). At 6 h post-transfection, doxycycline was added to the medium to achieve the designated concentration. At 28 h post-transfection, the medium was changed to Dulbecco’s modified Eagle’s medium and 0.2% bovine serum albumin with the designated doxycycline concentration and stimulated with TGF-β1 for 20 h. Thereafter, cells were lysed in 50 μl/well lysis buffer consisting of 25 mM Tris, pH 7.8, 2 mM diethiothreitol, 2 mM CDAT, 10% glycerol, 1% Triton X-100 and assayed for luciferase activity using the luciferase assay system (Promega). Cell lysates (20 μl/well) were used to measure the total light emission in 10 s using a ML 3000 Microtiter Plate Luminometer (Dynatech Laboratories, Inc., Chantilly, VA).

Growth Inhibition Assay—Cells were seeded at 2 × 10⁴ cells/well in 96-well plates (or 16-well LAB TEK chambers) and treated in a way similar to that in Western blotting analysis, but the treatment of TGF-β1 was for 20 h, and a quadruplicate of each concentration of treatment was used. Cells were pulsed with 0.2 μCi of [3H]thymidine for 4 h at 37°C. Subsequently, 20 μl of 0.5 M NaOH was added to each well, and the cells were incubated for 30 min at room temperature. Then cells were harvested using Filtermate Harvester (Packard Instrument Co., Meriden, CT), and the [3H]radioactivity incorporated into DNA was counted by Microplate Scintillation Counter (Packard Instrument Co.).

RESULTS

Induced Expression and Localization of Smad7—To investigate how Smad7 regulates TGF-β-mediated signaling pathways, flag-tagged Smad7 was stably transfected into Mv1Lu cells. The transcription of the tagged Smad7 was under the control of doxycycline using an improved tetracycline-inducible system in which the expression of the reverse tetracycline repressor (33, 34) was driven by the EF-1α promoter (35). In the absence of doxycycline, no Smad7 was detected in total cell lysate by Western analysis (lane 1, Fig. 1A). The level of Smad7 expression correlated with the amount of doxycycline present in the cell growth medium (Fig. 1A). These results shown in Fig. 1A demonstrated that the expression of Smad7 in Mv1Lu clone C cells is tightly regulated by doxycycline. Using flag antibody M2-mediated immunofluorescence (Fig. 2B), it was confirmed that Smad7 expression was only detectable after exposure to doxycycline. Furthermore, the immunofluorescence experiments demonstrated that Smad7 is expressed predominantly in the perinuclear region (seen as a ring around the nucleus) and the cytoplasm (Fig. 1B, c–e), with less than 5% of the cells exhibiting nuclear localization of Smad7 (data not shown). To analyze further the doxycycline-induced expression and subcellular localization of Smad7, we used Western blots to analyze both the soluble (containing membrane-binding and cytoplasmic proteins) and insoluble (containing nuclear and cytoskeleton proteins) fractions of Triton X-100 cell lysates. As shown in Fig. 2A, doxycycline induced Smad7 expression in the soluble fraction (top panels, Fig. 2A) where the nuclear expressing protein cyclin A was not detected (data not shown). In the insoluble fraction, Smad7 was absent (middle panels, Fig. 2A), whereas the expression of cyclin A was easily detected (bottom panels, Fig. 2A). A similar expression pattern of Smad7 was observed after TGF-β stimulation (right panels, Fig. 2A), indicating that TGF-β treatment does not alter Smad7 subcellular localization substantially. Analysis by immunofluorescence showed that the intense Smad7 perinuclear ring appeared to be diffused, probably to the cytoplasm, after TGF-β treatment (Fig. 2B).

To investigate whether the above results are caused by clonal selection, three more clones expressing recombinant Smad7 (G, N, and Q) were examined. In all three clones, Smad7 expression was induced by doxycycline, and the expressed Smad7 was detectable only in the cytoplasmic fraction (top panel, Fig. 3A) and not in the nuclear fraction (middle and bottom panels, Fig. 3A). As in clone C, cytoplasmic expression of Smad7 was observed for clones G, N, and Q by immunofluorescence (Fig. 3B). TGF-β did not mediate substantial change in the subcellular localization of Smad7 in clones G, N, and Q (Fig. 3B).
Smad7 Inhibits TGF-β-induced Transcriptional Activation of p3TP-Lux but Has Little Effect on TGF-β-induced Growth Inhibition—Smad7 was first identified as a negative regulator of TGF-β signaling because of its ability to inhibit TGF-β-induced transcriptional activation of the p3TP-Lux reporter gene (27, 30, 31), in which the PAI-1 promoter drives expression of luciferase (36). Cells with inducible Smad7 expression are an appropriate system to analyze this result further because the expression of Smad7 is induced by stimulation to cells (31, 32, 37). As shown in Fig. 4, A and C, in clone C, the TGF-β-induced transcriptional activation of the p3TP-Lux reporter measured as luciferase activity was inhibited by the treatment of doxycycline, which corresponded to expression of Smad7 (Figs. 1 and 2). The extent of the inhibition correlated with the amount of doxycycline and therefore correlated with the level of Smad7 expression. TGF-β-induced cell growth arrest is the other biological function of TGF-β, which has been used often as readout of TGF-β signaling activity. Contrary to the inhibition of p3TP-Lux, in clone C, the doxycycline-induced expression of Smad7 had little effect on TGF-β-induced growth arrest, as measured by [3H]thymidine incorporation (Fig. 4, B and C). Clearly, Smad7 acts differentially: it inhibits TGF-β-induced p3TP-Lux activation (PAI-1 expression) but plays little role in the TGF-β-induced cell growth arrest pathways.

We subsequently examined the effect of induced expression of Smad7 on TGF-β-controlled signaling pathways in several independent clones G, N, and Q. For all of the clones, the treatment with doxycycline, which corresponds to induced expression of Smad7, resulted uniformly in the block of the majority of TGF-β-induced p3TP-Lux activation (Fig. 5A). However, in no case did the expression of Smad7 reverse the TGF-β-mediated cell growth arrest (Fig. 5B). Mv1Lu cells die when they are cultured with TGF-β for a long period, such as 3–4 days (36). In the presence of TGF-β (5 ng/ml), almost all of the cells died in 3 days with or without the treatment of doxycycline (data not shown), further supporting that Smad7 expression does not block the TGF-β-induced growth inhibition pathway. Overall, our results on several cell lines clearly demonstrate that Smad7 inhibits TGF-β-mediated p3TP-Lux activation but has little effect on the growth arrest pathway.

The Cell Culture Surface Determines the Subcellular Localization of Smad7—Smad7 has been reported previously to be localized in the nucleus (32). Our results of cytoplasmic localization of Smad7 in the Mv1Lu cells are inconsistent with that

**Fig. 3.** Cytoplasmic expression of Smad7 in clones G, N, and Q. A, Western blotting. Cells from clones G, N, and Q were incubated with or without doxycycline (2 μg/ml) for 24 h and then with or without TGF-β1 (10 ng/ml) for an additional 20 h. Subsequently, the cells were lysed, and the lysates were separated into soluble (top panel) and insoluble fractions (middle and bottom panels) and subjected to SDS-gel electrophoresis and Western blotting using M2 antibody (top and middle panels). The nitrocellulose membranes were stripped of antibodies and Western blotted with cyclin A antibody (bottom panel). B, immunofluorescence. In 24-well plate, cells from clones G (panels a–d), N (panels e–h), and Q (panels i–l) were incubated with or without doxycycline (Dox; 2 μg/ml) for 24 h and then with or without TGF-β1 (10 ng/ml) for 1 h. Subsequently, the cells were immunofluorescence stained using M2 antibody and Cy3-conjugated secondary antibody as described under “Experimental Procedures.” The images were obtained using a confocal microscope. Panels a, e, and i are transmission images of cells immunofluorescence stained in panels b, f, and j.
In our immunofluorescence experiments, the Mv1Lu cells were grown on plastic; however, the cells were grown on glass in the report from Itoy et al. (32). Interestingly, when we grew cells on glass, Smad7 was localized in the nucleus (panel a, Fig. 6A). Fewer than 10% of the cells exhibited cytoplasmic localization and nuclear exclusion of Smad7 (data not shown). Treatment of Mv1Lu cells, grown on glass, with TGF-β resulted in a redistribution of some Smad7 in the cytoplasm, but it was not excluded from the nucleus (panel b, Fig. 6A). Our results demonstrated that the nature of the surface on which cells grow affects the subcellular localization of Smad7. To investigate this notion further, we subsequently examined cellular localization of Smad7 in cells grown on fibronectin-coated glass. The fibronectin-coated glass resulted in nuclear exclusion and cytoplasmic localization of Smad7 (panels e and f, Fig. 6A) for more than 70% of the cells, as on plastic (panels c and d, Fig. 6A). Interestingly, 2 h after seeding, the cells spread and attached well on either plastic or fibronectin-coated glass but not directly on glass. However, overnight the cells attached well to all three surfaces (data not shown). These results demonstrate that the nature of surface on which cells grow may determine the subcellular localization of Smad7.

To investigate whether the culture surface and therefore the cellular localization of Smad7 affect the differential regulation of TGF-β signaling pathways by Smad7, we cultured cells on plastic and glass surfaces. On glass, as on plastic, Smad7 expression inhibited TGF-β-mediated p3TP-Lux activation (Fig. 6B) but had little effect on growth inhibition (Fig. 6C). These results suggest that the culture surface is without effect at least on two of the TGF-β signaling pathways, although the surface determines the subcellular localization of Smad7.

**DISCUSSION**

Since the identification of the Smad protein family our understanding of TGF-β signaling pathways has advanced quickly (13–16). It is now clear that Smad proteins are substrates of TGF-β-activated receptors, and they transduce TGF-β signals from the cell surface to the nucleus (9–12). It is also well established that TGF-β mediates a diverse range of cellular responses (1); however, it is not clear how TGF-β acti-
Differential Inhibition by Smad

Fig. 6. Surface effect on subcellular localization of Smad7. A, clone C cells were grown in a LAB TEK chamber (glass surface, panels a and b), a 24-well plate (plastic surface, panels c and d), or a human plasma fibronectin-coated LAB TEK chamber (fibronectin-coated glass surface, panels e and f) and incubated with doxycycline (2 μg/ml), then stimulated with or without TGF-β1 (10 ng/ml). Subsequently, the cells were immunofluorescence stained using a monoclonal M2 antibody and a Cy3-conjugated goat anti-mouse IgG secondary antibody. The images were obtained using a confocal microscope. B, cells were transfected with p3TP-Lux and treated with or without doxycycline (2 μg/ml) and/or with or without TGF-β1 (5 ng/ml), then pulsed with [3H]thymidine as described under “Experimental Procedures.” The results of [3H]thymidine incorporation are of quadruplicate experiments and are representative of two separate experiments.

Selective targeting of receptor-regulated Smad2 and Smad3 has been reported recently (24, 38, 39). In particular, in contrast to Smad2, Smad3 can bind to regions of PAI-1 promoter (38–42), suggesting that Smad3, not Smad2, is responsible for TGF-β-induced PAI-1 expression and the activation of the p3TP-Lux pathway. Given our result that Smad7 selectively inhibits the p3TP-Lux pathway, it is possible that Smad7 selectively blocks Smad3 activation. In supporting this possibility, a recent report (37) demonstrated that interferon-γ-induced expression of Smad7 blocked the phosphorylation and association with Smad4 and nuclear translocalization of Smad3. It has been reported previously that another inhibitory Smad, Smad6, specifically competes with Smad4 for binding to activated Smad1, thereby selectively inhibiting bone morphogenetic protein signaling (29, 16). To understand the molecular mechanism of specificity and selectivity of Smad proteins in TGF-β signaling, we are currently establishing cell lines in which TGF-β signaling pathways are selectively activated.

Subcellular localization of a signaling molecule plays an important role in its function during signal transduction. It is essential for a signaling molecule to be in the right place at the right time to function. The receptor-regulated Smad2 and Smad3 and the common Smad4 are usually localized in the cytoplasm (10, 16). It is in the cytoplasm where Smad2 and Smad3 are phosphorylated, activated, and form complexes with Smad4 (17–23). However, when Smad complexes are translocated into the nucleus where they bind to target genes (16, 24, 25). Smad7 is a negative regulator of TGF-β signaling by antagonizing the activation of receptor-regulated Smads (27–32), therefore it is in the cytoplasm where its antagonizing function is executed. Endogenous Smad7 is not constitutively expressed, and its expression appears to be stimulated to in-
hhibit TGF-β signaling (31, 32, 37). The cytoplasmic localization of Smad7 we observed is consistent with its function. Furthermore, fibronectin (a component of extracellular matrix proteins) coating of glass resulted in cytoplasmic localization of Smad7 instead of nuclear localization on untreated glass (32, Fig. 6A). Thus the cytoplasmic localization of Smad7 may be physiologically relevant, but the nuclear localization may not. Nevertheless, Smad7 differentially regulates TGF-β signaling pathways regardless of its prior cellular localization either in the cytoplasmic or in the nucleus. However, further investigation is still required to understand the effect of the extracellular environment on the cellular localization of signaling proteins, and more importantly, the effect on cellular biological responses.

Acknowledgments—We thank A. W. Burgess for critical reading of the manuscript and for continuing support and encouragement. We also thank P. ten Dijke for Smad7 cDNA, A. B. Roberts for p3TP-Lux reporter and MvI1L cells, G. Varo for eFPepuor-Tet-on vector, R. Whitehead for advice on immunofluorescence experiments, and S. Cody for help in obtaining confocal images.

REFERENCES

1. Roberts, A. B., and Sporn, M. B. (1990) in Peptide Growth Factors and Their Receptors: Part I (Sporn, M. B., and Robert, A. B., eds) pp. 419–472, Springer-Verlag, Berlin.
2. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) Nature 370, 341–347
3. Yamashita, H., ten Dijke, P., Franzien, P., Miyazono, K., and Heldin, C.-H. (1994) J. Biol. Chem. 269, 20172–20178
4. Chen, R.-H., Moses, H. L., Maruoka, E. M., Derynck, R., and Kawabata, M. (1995) J. Biol. Chem. 270, 12235–12241
5. Wells, R. G., Gilboa, L., Sun, Y., Liu, X., Henis, Y. I., and Lodish, H. F. (1999) J. Biol. Chem. 274, 5716–5722
6. ten Dijke, P., Miyazono, K., and Heldin, C.-H. (1996) Curr. Opin. Cell Biol. 8, 139–145
7. Attisano, L., and Wrana, J. L. (1996) Cytokine Growth Factor Rev. 7, 327–339
8. Zhu, H.-J., and Sizeland, A. M. (1999) J. Biol. Chem. 274, 11773–11781
9. Zhang, Y., and Derynck, R. (1996) Curr. Biol. 6, 1226–1229
10. Heldin, C.-H., Miyazono, K., and ten Dijke, P. (1997) Nature 390, 465–471
11. Attisano, L., and Wrana, J. L. (1998) Curr. Opin. Cell Biol. 10, 188–194
12. Kretzschmar, M., and Massague, J. (1998) Curr. Opin. Genet. Dev. 8, 103–111
13. Seckel, S. J., Newfeld, S. J., Rafferty, L. A., Chartoff, E. H., and Gelbart, W. M. (1995) Genetics 139, 1347–1358
14. Rafferty, L. A., Twombly, V., Wharton, K., and Gelbart, W. M. (1995) Genetics 139, 241–245
15. Savage, C., Das, P., Finelli, A., Townsend, S. R., Sun, C.-Y., Baird, S. E., and Padgett, R. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 790–794
16. Massague, J. (1998) Annu. Rev. Biochem. 67, 783–791
17. Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. (1996) Cell 87, 1215–1224
18. Nakao, A., Roinier, E., Imamura, T., Soucekulnytskii, S., Stennan, G., Heldin, C.-H., and ten Dijke, P. (1997) J. Biol. Chem. 272, 2896–2900
19. Zhang, Y., Peng, X., We, R., and Derynck, R. (1996) Nature 383, 168–172
20. Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, J. L. (1997) J. Biol. Chem. 272, 27678–27685
21. Soucekulnytskii, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P., and Heldin C.-H. (1997) J. Biol. Chem. 272, 28107–28115
22. Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10669–10674
23. Nakao, A., Imamura, T., Soucekulnytskii, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C.-H., Miyazono, K., and ten Dijke, P. (1997) EMBO J. 16, 5353–5362
24. Labbe, E., Silvestri, C., Hoodless, P. A., Wrana, J. L., and Attisano, L. (1998) Mol. Cell 2, 109–120
25. Zhou, S., Zavbel, L., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998) Mol. Cell 2, 121–127
26. Topper, J. N., Cai, J., Falb, D., and Gimbrone, M. A., Jr. (1998) Proc. Natl. Acad. Sci. U. S. A. 93, 10417–10422
27. Topper, J. N., Cai, J., Qiu, Y., Anderson, K. R., Xu, Y. Y., Deeds, J. D., Feeley, R., Gimeno, C. J., Wood, E. A., Taylor, O., Mays, G. G., Sampson, B. A., Schoen, F. J., Gimbrone, M. A., Jr., and Falb, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9314–9319
28. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., and Miyazono, K. (1997) Nature 389, 622–626
29. Hata, A., Lagna, G., Massague, J., and Hemmatti-Brivanlou, A. (1998) Genes Dev. 12, 186–197
30. Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Jr., Wrana, J. L., and Falb, D. (1997) Cell 89, 1165–1173
31. Nakao, A., Afarkhate, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C.-H., and ten Dijke, P. (1997) Nature 389, 631–635
32. Itoh, S., Landstrom, M., Hermansson, A., Itoh, F., Heldin, C.-H., Heldin, N., and ten Dijke, P. (1998) J. Biol. Chem. 273, 29195–29201
33. Hillen, W., and Berene, C. (1994) Annu. Rev. Microbiol. 48, 345–369
34. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) Science 268, 1766–1769
35. Mizushima, S., and Nagata, S. (1996) Nucleic Acids Res. 18, 5322
36. Carcamo, J., Zentella, A., and Massague, J. (1995) Mol. Cell. Biol. 15, 1573–1581
37. Uleio, L., Doody, J., and Massague, J. (1999) Nature 397, 710–713
38. Denuiller, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) EMBO J. 17, 3091–3100
39. Denuiller, S., Huet, S., and Gauthier, J. M. (1999) Oncogene 18, 1643–1648
40. Stoockstein, S. L., Wang, W., and Luo, K. (1999) J. Biol. Chem. 274, 9431–9441
41. Yingling, J. M., Datto, M. B., Wong, C., Frederick, J. P., Liberati, N. T., and Wang, X.-F. (1997) Mol. Cell. Biol. 17, 7019–7028
42. Wong, C., Rougier-Chapman, E. M., Frederick, J. P., Datto, M. B., Liberati, N. T., Li, J. M., and Wang, X.-F. (1999) Mol. Cell. Biol. 19, 1821–1830