Phosphorylation of Smad7 at Ser-249 Does Not Interfere with Its Inhibitory Role in Transforming Growth Factor-β-dependent Signaling but Affects Smad7-dependent Transcriptional Activation*

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Smad proteins are major components in the intracellular signaling pathway of transforming growth factor-β (TGF-β), and phosphorylation is an important mechanism in regulation of their functions. Smad7 was identified as a potent inhibitor of TGF-β-dependent signaling. We have identified serine 249 in Smad7 as a major phosphorylation site, the phosphorylation of which was not affected by TGF-β/β1. Abrogation of the phosphorylation by substitution of Ser-249 with alanine or aspartic acid residues did not affect the ability of Smad7 to inhibit TGF-β1 and BMP7 signaling. No differences were found in the stability or in the intracellular distribution of Smad7 mutants compared with the wild-type molecule. However, Smad7 fused to the DNA-binding domain of GAL4 induced transcription from a reporter with mutated TATA minimal promoter in a Ser-249-dependent manner. Moreover, a reporter with the SV40 minimal promoter was inhibited by GAL4-Smad7, and this effect was also dependent on Ser-249 phosphorylation. The amplitude of effects on transcriptional regulation was dependent on cell type. Our results suggest that phosphorylation of Smad7, unlike phosphorylation of the receptor-regulated Smads, does not regulate TGF-β signaling but rather affects TGF-β-independent effects of Smad7 on transcriptional regulation.

Discovery of Smad proteins provided insights into the intracellular signaling by TGF-β family members. Three groups of Smads have been described. Receptor-regulated Smads (R-Smads) are direct targets of activated receptors and provide the signaling specificity. Common mediator (Co-Smad) Smad4 forms complexes with R-Smads and is involved in signal propagation. Finally, Smad7 and Smad6 were identified as inhibitors (I-Smads) of signaling by members of the TGF-β family (1–3). Smad7 has been suggested to inhibit TGF-β signaling by inhibiting the phosphorylation of R-Smads by type I receptors (1–4). Interestingly, Smad7 was found to occur abundantly in the nuclei of certain cells and to be exported from the nucleus upon TGF-β stimulation or a change in cell substrate (5, 6). This suggests that Smad7 may also have a function in the nucleus, which may be independent of the inhibition of ligand-induced signaling at the receptor level.

Since expression of Smad7 is up-regulated after TGF-β stimulation, it has been suggested that Smad7 is involved in negative feedback of TGF-β signaling. Induction of Smad7 has also been described as the pivotal mechanism whereby tumor necrosis factor-α and interferon-γ inhibit TGF-β signaling (7, 8). Smad7 has been found to be up-regulated in human tumors, but no mutation in human cancers has yet been described (9).

Phosphorylation has been found to be a potent regulatory mechanism of Smad functions (10–13). Sequential phosphorylation of R-Smads at two C-terminal serine residues by activated receptors is the triggering event in ligand-dependent intracellular signaling (13). Phosphorylation in the linker region, presumably by mitogen-activated protein kinase, may affect nuclear translocation of R-Smads (12). Moreover, phosphorylation of R-Smads by other kinases has also been shown to influence intracellular signaling (14, 15). Phosphorylation of mammalian Smad4 has not been reported, but Xenopus Smad4β has been identified as a phosphoprotein (16). Among inhibitory Smads, Smad6 is a phosphoprotein (17), whereas phosphorylation of Smad7 has not been characterized.

We show here that Smad7 is phosphorylated, and we identify Ser-249 as a major phosphorylation site. Mutation of Ser-249 did not affect the inhibitory effect of Smad7 on TGF-β or BMP7 signaling and did not interfere with nuclear localization of Smad7. However, the TGF-β-independent transcriptional activity of Smad7 was affected by mutation of Ser-249, suggesting that phosphorylation of Smad7 at Ser-249 is important for its ligand-independent ability to regulate transcription.

EXPERIMENTAL PROCEDURES

Constructs, Cells, and Reagents—pDNA3-based expression vectors for full-length mouse Smad7, as well as for its N-terminally truncated (Smad7C) and C-terminally truncated (Smad7N) mutants, were described previously (5). Expression vectors for Smad7 point mutants were generated by a polymerase chain reaction-based approach (Quick-Change, Stratagene, La Jolla, CA) and sequenced to confirm absence of undesired mutations. The luciferase reporter constructs CAGA(12)-luc and SBE(4)-luc were described earlier (18, 19), GCCG(12)-luc was obtained from Kohei Miyazono, p800-luc from Kunihiro Matsumoto, pH6-2, and GAL4-TK-luc, or BMP7stim-

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The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; MH domain, mad homology domain; SAD, Smad activation domain; PAGE, polyacrylamide gel electrophoresis; TK, thymidine kinase.
were obtained from Johan Ericsson, and GAL4-SV40-luc, containing an SV40 early promoter, was from Cory Abate. COS-1, Mv1Lu, and NIH-3T3 cell lines were obtained from ATCC, Manassas, VA. DR-26 clone of Mv1Lu cells, lacking TGF-β type II receptor, was obtained from Joan Massague. For stable transfection, wild-type FLAG-Smad7 was subcloned in pMEP4 vector. The construct and empty vector were transfected in DR-26 and NIH-3T3 cells, followed by selection in presence of hygromycin B.

All data in figures are presented as average ± S.E. Standard tests were used to evaluate statistical significance of the obtained results.

**Identification of Phosphorylation Sites in Smad7**—[32P]Orthophosphate labeling and mapping of tryptic phosphopeptides, as well as phosphoamino acid analysis, were performed as described (13). FLAG-Smad7 protein was immunoprecipitated with M2 anti-FLAG antibody (Sigma). Visualization of phosphopeptides was carried out using the Fujix BAS2000 imaging system.

**RESULTS**

**Smad7 Is a Phosphoprotein**—To investigate whether Smad7 is a phosphoprotein, full-length and N-terminal or C-terminal deletion mutants of FLAG-tagged Smad7 were transiently expressed in Mv1Lu and COS-1 cells. The cells were incubated with [32P]orthophosphate, treated or not with 10 ng/ml TGF-β1 for 2 h, and FLAG-Smad7 proteins were immunoprecipitated with anti-FLAG antibodies; immunoprecipitates were analyzed by SDS-PAGE and quantitated using the Fujix BAS2000 imaging system.

**Smad7 Is Phosphorylated at Serine 249**—To identify phosphopeptide sites, Smad7 was immunoprecipitated from [32P]orthophosphate-labeled cells and subjected to tryptic digestion; the generated peptides were separated by two-dimensional mapping (data not shown). Therefore, the activity of kinase(s) and/or phosphatase(s) regulating Smad7 phosphorylation may be dependent on the serum starvation of cells but is not directly regulated by TGF-β1.

**Smad7 Phosphorylation**

The fact that the phosphorylation of Smad7 was TGF-β-independent prompted us to attempt to evaluate how this phosphorylation is regulated. We observed that the level of Smad7 phosphorylation was higher if cells were serum-starved and that the phosphorylation was decreased if cells were cultured in the presence of serum. The effect of serum starvation was found to be especially strong for FLAG-Smad7C. No changes in the level of protein expression were detected, suggesting that the differences in phosphorylation were not related to the quantity of Smad7. The phosphopeptide patterns of Smad7 from serum-starved and proliferating cells were also similar, as evaluated by two-dimensional mapping (data not shown).

**Smad7 Phosphorylation**

The composite spot 3, consisting of three differently migrating phosphopeptides unique to FLAG-Smad7C and not seen in full-length FLAG-Smad7, was very prominent. The phosphopeptide maps of Smad7 from serum-starved and proliferating cells were also similar, as evaluated by two-dimensional mapping (data not shown). Therefore, the activity of kinase(s) and/or phosphatase(s) regulating Smad7 phosphorylation may be dependent on the serum starvation of cells but is not directly regulated by TGF-β1.

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rylated residue in this group of phosphopeptides was found to be Ser-206, since upon mutation of Ser-206 to an alanine residue, all three phosphopeptides of spot 3 disappeared (Fig. 2E). Interestingly, Ser-206 is neighbored by a PPPPY sequence, which may be involved in regulation of ubiquitin-dependent degradation (20). Therefore, phosphorylation of Ser-206 in the truncated Smad7 may interfere with its degradation, which may explain the higher level of FLAG-Smad7C protein compared with full-length FLAG-Smad7. However, because phosphorylation at Ser-206 was found only in the truncated mutant and not in the full-length protein, we considered it an artifact caused by the truncation. Thus, we have identified Ser-249 as a major phosphorylation site in Smad7.

Smad7-dependent Inhibition of TGF-β and BMP Signaling Is Not Dependent on Smad7 Phosphorylation—To investigate whether phosphorylation of Smad7 affects its inhibitory action on TGF-β and BMP signaling, we used luciferase reporter assays, which are dependent on the respective intact R-Smad-dependent pathways. CAGA(12)-luc is activated upon treatment of cells with TGF-β but not BMP, and its activation requires a receptor-dependent phosphorylation of Smad3 (18). Wild-type Smad7 inhibits the receptor-dependent phosphorylation of R-Smads and blocks TGF-β-induced activation of the CAGA(12)-luc reporter (18). We found that S249A and S249D mutants of Smad7 were as potent inhibitors as wild-type Smad7 (Fig. 3A). Neither was any significant effect of the mutation of Ser-249
found on inhibition of BMP7-dependent activation of the GCCG(12)-luc reporter (Fig. 3B). Stimulation of this reporter is dependent on receptor-induced activation of Smad1 and Smad5, which are specific for the BMP signaling pathway (21). In addition, the S249A and S249D mutants of Smad7 were equally efficient as wild-type Smad7 in inhibition of ligand-induced activation of another reporter responsive to TGF-β and BMP, SBE(4)-luc (data not shown).

Because CAGA(12)-luc and GCCG(12)-luc reporters have artifically designed promoters, we also tested how reporters with natural promoters, responsive to the ligand, were affected by interference with Smad7 phosphorylation. When reporters containing 800 base pairs of the PAI-1 promoter (p800-luc), 296 base pairs of collagen α2(I) promoter (pH6-luc), promoters of the cyclin-dependent kinase inhibitors p21 and p15 (p21-luc, p15-luc), or an E2F1-luc reporter were analyzed, the inhibition of the TGF-β-induced activation by Smad7 was not found to be affected by mutations of Ser-249 (Fig. 3, C and D, data not shown). The diversity of molecular mechanisms, by which Smads regulate different promoters used in this study, supported the conclusion that phosphorylation at Ser-249 does not affect the ability of Smad7 to interfere with Smad-mediated TGF-β and BMP signaling pathways.

Phosphorylation of Smad7 Phosphorylation Does Not Affect Its Stability and Nuclear Localization—Since regulation of Smad biosynthesis and degradation has been shown to be an important mechanism of modulation of TGF-β signaling (20), we investigated whether phosphorylation of Smad7 affects its stability. Transfection of similar quantities of cDNAs of the wild-type and the S249A and S249D mutants of Smad7 resulted in comparable protein expression levels (data not shown). Moreover, pulse-chase experiments showed that interference with phosphorylation at Ser-249 did not affect the half-life of Smad7 (Fig. 4). In the presence of the protein synthesis inhibitor cycloheximide, the half-life of FLAG-Smad7 was ~1 h, and no significant differences were found for the mutants. In absence of cycloheximide the apparent half-life was estimated at ~4 h both for wild-type Smad7 and for Smad7 mutants (data not shown). Thus, the phosphorylation of Smad7 at Ser-249 appears not to affect its stability and half-life.

In two recent reports, the nuclear localization of Smad7 has
containing 30 m pulse-labeled with [35S]methionine for 1 h and chased with medium porter was used in COS-1 (Fig. 6 obtained for the wild-type and S249A mutant when this re-
duction of a negative charge (Fig. 6 due resulted in an effect similar to the wild-type Smad7,
tation (\textsuperscript{T})). Similar results were obtained for the wild-type and S249A mutant when this re-
porter was used in COS-1 (Fig. 6C) and Mv1Lu cells (data not shown). For Mv1Lu cells, however, the effect was weaker than for COS-1 cells. Interestingly, using the reporter containing an SV40 minimal promoter instead of TGA, wild-type Smad7 was found to repress luciferase expression; in this case the S249D mutant of Smad7 was mimicking the wild-type Smad7 effect on transcription, and the S249A mutant even induced it (Fig. 6D). Results obtained with the GAL4-TK-luc reporter were similar to the data for the GAL4-SV40-luc reporter (data not shown). Thus, Smad7 can act as a transcriptional activator or as a repressor depending on the type of promoter. Our data suggest that the phosphorylation at Ser-249 regulates the transcriptional activity of Smad7, probably through regulation of

been described (5, 6). These observations also suggested that the nuclear localization is a regulated process. Therefore, we tested whether phosphorylation at Ser-249 affects the intracellular distribution of Smad7. Fig. 5 shows that mutation of Ser-249 did not lead to significant differences in intracellular localization, since S249A and S249D mutants and wild-type of FLAG-Smad7 all localized to the cell nucleus to a similar extent. Thus, the intracellular distribution of Smad7 is not affected by its phosphorylation at Ser-249.

Transcriptional Activity of Smad7 Is Dependent on Phosphorylation at Ser-249—The findings that Smad7 is a nuclear protein, together with the observation that a GAL4-Smad7 fusion protein showed transcriptional activity in PC3U cells,\textsuperscript{2} prompted us to investigate whether the phosphorylation of Smad7 affects its transcription-regulating activity. We tested the effect of full-length Smad7, fused to the DNA-binding domain of GAL4, on the response of various reporters containing different minimal promoters downstream of the GAL4-binding elements (GAL4-TGTA-luc, GAL4-SV40-luc, and GAL4-TK-luc). We found that, in NIH-3T3 cells, wild-type Smad7 up-regulated the GAL4-TGTA-luc reporter containing a point-mutated TATA box to decrease the background activity (Fig. 6A). Abrogation of Smad7 phosphorylation at Ser-249 by its substitution to an alanine residue significantly inhibited this stimulation ($p < 0.01$). Substitution of Ser-249 to aspartic acid residue resulted in an effect similar to the wild-type Smad7, probably due to partial mimicking of phosphorylation by introduction of a negative charge (Fig. 6A). Similar results were obtained for the wild-type and S249A mutant when this reporter was used in COS-1 (Fig. 6C) and Mv1Lu cells (data not shown). For Mv1Lu cells, however, the effect was weaker than for COS-1 cells. Interestingly, using the reporter containing an SV40 minimal promoter instead of TGA, wild-type Smad7 was found to repress luciferase expression; in this case the S249D mutant of Smad7 was mimicking the wild-type Smad7 effect on transcription, and the S249A mutant even induced it (Fig. 6D). Results obtained with the GAL4-TK-luc reporter were similar to the data for the GAL4-SV40-luc reporter (data not shown). Thus, Smad7 can act as a transcriptional activator or as a repressor depending on the type of promoter. Our data suggest that the phosphorylation at Ser-249 regulates the transcriptional activity of Smad7, probably through regulation of its interaction with other components in transcriptional complexes.

FIG. 4. Phosphorylation status does not influence the stability of Smad7 \textit{in vivo}. COS-1 cells were transiently transfected with FLAG-Smad7 (wild-type, diamonds; S249A, squares; S249D, triangles), pulse-labeled with \textsuperscript{[35S]}methionine for 1 h and chased with medium containing 30 \mu g/ml cycloheximide to inhibit further synthesis of proteins. At specific time points, remaining radiolabeled Smad7 protein was immunoprecipitated with an anti-FLAG antibody, resolved by SDS-PAGE, and quantified by phosphorimaging. Data are presented as mean from 3 experiments, and error bars represent S.D.

FIG. 5. Intracellular distribution of Smad7 phosphorylation site mutants. Mv1Lu cells were transiently transfected with FLAG-Smad7 (wild-type and S249A and S249D mutants) using FuGene6 reagent. After 48 h, cells were fixed and immunostained with anti-FLAG antibodies. Intracellular localization of Smad7 protein was visualized by immunofluorescence and is shown in A (wild-type), C (S249A mu-
ant), and E (S249D mutant). B, D, and F show counterstaining of nuclei with 4',6-diamidino-2-phenylindole in the same slide sections shown in A, C, and E, respectively.

DISCUSSION

Here we report the identification of Ser-249 as a major phosphorylation site in Smad7. Unlike the case for R-Smads, the phosphorylation of Smad7 was found not to be dependent on TGF-\textbeta treatment of cells. We show that phosphorylation at Ser-249 does not affect the stability or subcellular localization of Smad7, nor does it affect the ability of Smad7 to inhibit TGF-\textbeta signaling. Interestingly, however, the presence of the phosphorylatable Ser-249 in Smad7 was found to be important for a novel function ascribed to Smad7, \textit{i.e.} its ability to regulate transcription.

Ser-249 is one of the two major phosphorylation sites in Smad7 (Fig. 2); the second major site is currently under investigation. Ser-249 is located in the C-terminal part of the region corresponding to the linker in other Smads. Interestingly, a Smad activation domain (SAD) has been identified in the corresponding part of Smad4; SAD interacts with MSG-1, a tran-
scriptional coactivator of Smad4, and has an important role for the transcriptional activity of Smad4 (22, 23). We have not observed any homology between SAD and the corresponding

\textsuperscript{2} M. Landström, unpublished data.
region of Smad7; however, given the importance of SAD in Smad4 transcriptional regulation, it is interesting that phosphorylation in the corresponding region of Smad7 affects its transcriptional activity.

The nuclear localization (Fig. 5) is consistent with the possibility that Smad7 is involved in regulation of gene expression. R-Smads and Smad4 are known as potent transcriptional regulators, and they are translocated to the nucleus upon ligand addition. Their function is dependent on interactions with co-activators and corepressors and on the ability of Smads to bind DNA directly (1–3, 24). Recently, Bai et al. (25) reported that inhibitory Smad6 also can act as a transcriptional regulator through interaction with the homeobox (Hox)e8 protein, extending the suggestion of transcriptional regulator function to inhibitory Smads. In line with previous observations (26), we could detect only a weak effect of GAL4-FLAG-Smad7 on the activity of GAL4-binding sequence-containing reporters in Mv1Lu cells of epithelial origin (data not shown). However, in cells of mesenchymal origin, GAL4-FLAG-Smad7 significantly induced the GAL4-TGTA-luc reporter. Interestingly, a GAL4-binding sequence-containing reporter with an SV40 or TK minimal promoter was inhibited by the wild-type GAL4-FLAG-Smad7 fusion. Similar opposite effects on gene expression were also described for Smad3; Smad3 was found to potently induce CAGA12-luc (18) but inhibited stimulation of the goosecoid reporter (27). In similarity to Smad3, the stimulatory or inhibitory effects of Smad7 on the regulation of transcription may depend on the interaction between Smad7 and other proteins. Our data suggest that such interactions may be regulated by Smad7 phosphorylation, as the phosphorylation-deficient S249D mutant of Smad7 shows impaired effects when compared with the wild-type protein (Fig. 6). The S249D mutant of Smad7, partially mimicking the presence of a phosphoyryl group by introducing a negative charge, behaved similar to wild-type Smad7.

Our findings suggest that Smad7 is not only involved in control of signaling pathways of TGF-β family members, but may also have direct effects in the nucleas, possibly regulated by other regulatory pathways. To explore this possibility, it will be important to identify the kinase phosphorylating Smad7, components interacting with Smad7 in the nucleus, as well as target genes for Smad7 as a transcription factor. These studies are in progress.

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