A single missense mutant of Smad3 inhibits activation of both Smad2 and Smad3, and has a dominant negative effect on TGF-β signals

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Abstract A missense mutation of Smad2 identified in cancer cells was reconstructed on the corresponding residue of Smad3. This mutant, Smad3D407E, was not phosphorylated by the constitutively active form of type I receptor for transforming growth factor-β (TGF-β), and inhibited the phosphorylation of co-expressed wild-type Smad2 and Smad3. This mutant also had a dominant negative effect on the growth inhibition of HaCaT cells and on the expression of p3TP-lux reporter gene induced by TGF-β. However, it did not alter the phosphorylation of Smad1 induced by the constitutively active form of the bone morphogenetic protein type IA receptor. These findings showed that a single missense mutation in Smad3 could specifically block TGF-β signals by preventing activation of both Smad2 and Smad3.

Key words: Transforming growth factor-β; Smad; Cancer; Dominant negative mutant

1. Introduction

Transforming growth factor-β (TGF-β) is a multifunctional peptide factor which regulates cell proliferation, differentiation, adhesion, apoptosis, and extracellular matrix accumulation [1]. TGF-β binds to type II and type I serine/threonine kinase receptors, and transduces intracellular signals through Smad proteins [2,3]. Of eight reported mammalian Smad proteins, Smad2 and Smad3 have been shown to be directly phosphorylated by TGF-β type I receptor (TβR-I) on the C-terminal SSXS motif, form hetero-oligomers with Smad4, translocate into nuclei, and serve as components of transcription factors upon TGF-β stimulation [4–13]. Overexpression of Smad2 and/or Smad3 together with Smad4 stimulates TGF-β-responsive gene expression, and C-terminally mutated Smads have dominant negative effects on TGF-β signals [5,6,14].

Many cancer cells exhibit deficient growth inhibitory responses to TGF-β [1,15]. Mutations of the TGF-β type II receptor (TβR-II) are a frequent known cause of this deficiency. More than 90% of cases of hereditary non-polypotic colon cancer had inactivating mutations in the *TβR-II* gene [16], and the loss of functional TβR-II was responsible for the loss of TGF-β responsiveness [17]. Mutations in the *Smad* genes have also been found in various cancers. The *DPC4/Smad4* gene was originally identified as a candidate tumor suppressor gene at 18q21.1 [18]. Homozygous deletions at 18q21 were found in about 30% of human pancreatic cancers, and potentially inactivating mutations in the *DPC4/Smad4* gene were identified in about 20% of pancreatic cancers. DPC4/Smad4 has been shown to act as a common mediator in the signaling pathways for the members in the TGF-β superfamily [7,19]. Mutations in the *DPC4/Smad4* gene have also been identified less frequently in colorectal cancers, lung cancers, breast cancers, ovarian cancers, and head and neck cancers [20–24], and the loss of DPC4/Smad4 has been shown to be responsible for the loss of TGF-β responsiveness in some cases [25,26]. Similar mutations were also detected in the *Smad2* gene in colorectal cancers and lung cancers [4,27].

Smad3 is also thought to play a role in the TGF-β-induced growth inhibitory signaling pathway [6,14]. However, the Smad3 mutation has not yet been identified in cancer cells [28,29]. It is unclear if Smad3 could also perturb TGF-β signaling by oncogenic mutations identified in Smad2 or Smad4. A missense mutation of Asp-450 to Glu in Smad2 was originally reported in a case of colorectal cancer [4]. This mutant was defective in TGF-β receptor-dependent phosphorylation. Another mutation was found on the same residue of Smad2 in a case of lung cancer [27]. The corresponding aspartic acid residue (Asp-537) was located at the trimer interface in the crystal structure of Smad4, and a Smad4D537E mutant was defective in oligomer formation [30]. In the present study, we introduced this mostly characterized oncogenic mutation of Smad2 into the corresponding residue of Smad3, and examined the role of a Smad3D407E mutant in the TGF-β signaling pathway.

2. Materials and methods

2.1. Constructs and reagents

Smad3 cDNA and Smad4 cDNA were provided by R. Derynck (University of California, San Francisco, CA) and M. Schutte (Johns Hopkins University, Baltimore, MD), respectively. Expression constructs for various Smad proteins and constitutively active forms of TGF-β type I receptor (TβR-I(TD)/HA) and BMP type IA receptor (BMPR-IA(QD)/HA) were previously described [9]. Mutations and sequences of the exchanged restriction fragments in Smad3 were confirmed by DNA sequencing. Recombinant human TGF-β3 was provided by A. Suter (Ciba-Geigy, Basel, Switzerland). Anti-Flag antibodies (M2), anti-Myc antibodies (9E10), anti-HA antibodies (12CA5), and anti-phosphoserine antibodies were purchased from Eastman Kodak (New Haven, CT), Santa Cruz Biotechnology (Santa Cruz, CA), Boehringer Mannheim (Indianapolis, IN), and Zymed Laboratories (South San Francisco, CA), respectively.

2.2. Cell culture and transfections

COS7 cells were obtained from American Type Culture Collection (Bethesda, MD) and R mutant cells of Mv1Lu (clone 4-2) were provided by J. Massagué (Memorial Sloan Kettering Cancer Center, NY). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 10 μg/ml

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gentamicin. HaCaT cells were provided by Norbert E. Fusenig (DKFZ, Heidelberg, Germany), and maintained in MCDB 153 medium (Sigma, St. Louis, MO) supplemented with 10% dialyzed FBS, epidermal growth factor (10 ng/ml) and gentamicin (10 μg/ml) [31]. For transient transfection, 60–80% confluent cells in 6-well plates were transfected using DMRIE-C (Life Technologies, Gaithersburg, MD) or FuGENE 6 (Boehringer Mannheim) transfection reagents following the manufacturer's recommendations. To establish stable transfectants from HaCaT cells, 300 μg/ml of G418 (Life Technologies) was used for selection.

2.3. Immunoprecipitation and immunoblotting

For determination of levels of phosphorylation of Smad proteins, cell lysates were subjected to immunoprecipitation with the anti-Flag antibody followed by adsorption to protein G Sepharose (Pharmacia Biotech, Uppsala, Sweden). Precipitates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Pro-Blott membranes (Applied Biosystems, Foster City, CA). After immunoblotting with anti-phosphoserine antibodies, the membranes were subjected to re-blotting with the anti-Flag antibody to confirm levels of expression of Flag-tagged Smad proteins. To determine levels of expression of the receptors and Smad3D407E, aliquots of cell lysates were subjected to SDS-PAGE and immunoblotting with anti-HA, anti-Myc or anti-Flag antibodies, as indicated.

2.4. Luciferase assay

R mutant Mv1Lu cells were transiently transfected with p3TP-lux in the presence of various combinations of Smad and the receptor constructs. After transfection, cells were incubated for 24 h, and luciferase activities in the cell lysates were measured with a dual luciferase reporter assay system (Promega Biotech, Madison, WI) according to the manufacturer's recommendations, using a luminometer (Lumat LB 9501, EG&G Berthold, Bad Wildbad, Germany).

2.5. Growth inhibition assay

HaCaT cells and the stable transfectants were seeded in 24-well plates at a density of 5×10^4 cells per well, and [^{3}H]thymidine incorporation was assayed as previously described [31].

3. Results

Asp-407 in Smad3 is conserved in all known Smad proteins (Fig. 1A). This aspartic acid, which is located at the possible homo-oligomerization interface in Smad4, was replaced by

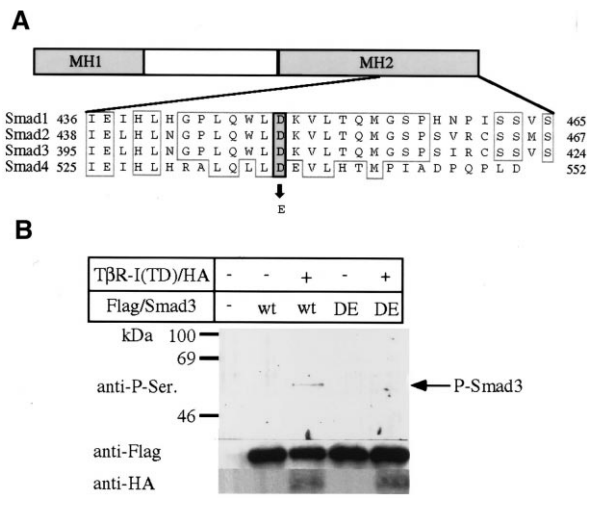


Fig. 1. A single missense mutant of Smad3 was defective in phosphorylation induced by TGF-β signaling. A: A schematic representation of Smad proteins and alignments of highly conserved C-terminal amino acid sequences of Smad1, Smad2, Smad3 and Smad4. Conserved amino acids are boxed. The location of the induced missense mutation in Smad3 and corresponding aspartic acid residues in other Smads is shaded. B: COS7 cells were transfected with wild-type (wt) or D407E mutant (DE) of Flag-tagged Smad3 in the presence or absence of a constitutively active form of TGF-β type I receptor (TβR-I(TD)/HA). Cell lysates were subjected to immunoprecipitation using the anti-Flag antibody, and precipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphoserine antibodies (anti-P-Ser). The membrane was re-blotted with the anti-Flag antibody to demonstrate equal levels of expression of Smad3 and Smad3D407E. Aliquots of cell lysates were also subjected to immunoblotting with anti-HA antibodies to detect expression of TβR-I(TD).

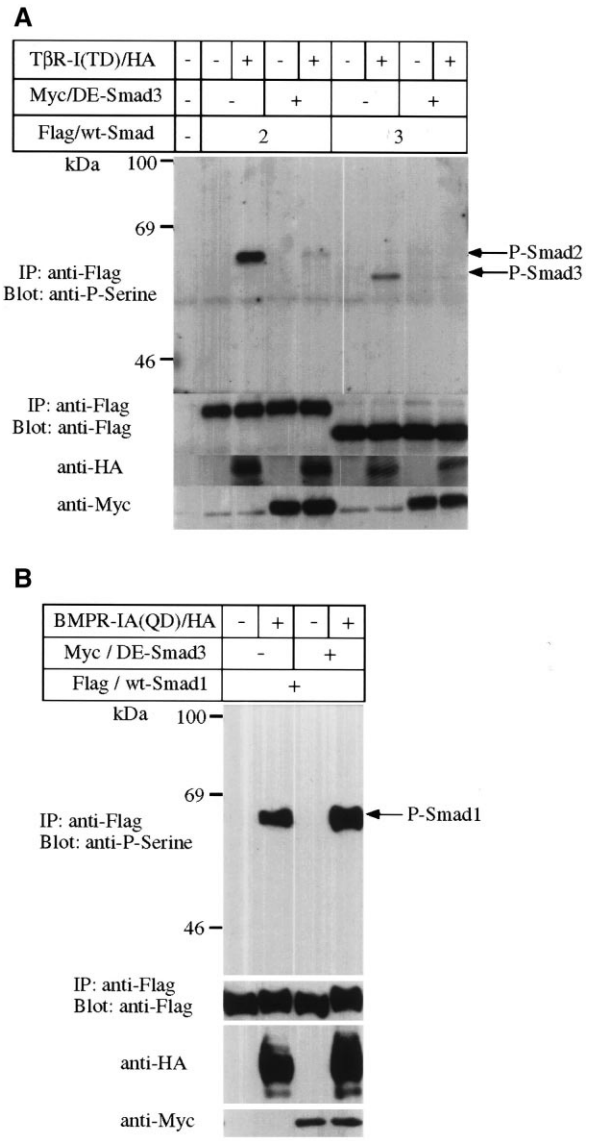
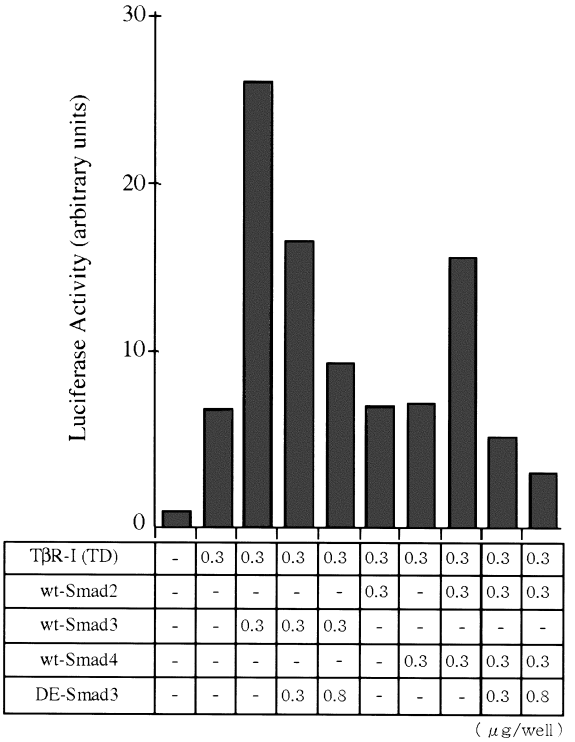


Fig. 2. Smad3D407E mutant dominantly blocked the phosphorylation of co-expressed wild-type Smads in a pathway-specific manner. A: COS7 cells were transfected with Flag-Smad2 (wt; 2) or Flag-Smad3 (wt; 3) in the presence or absence of Myc-Smad3D407E and TβR-I(TD)/HA, as indicated. B: COS7 cells were transfected with Flag-Smad1 in the presence or absence of Myc-Smad3D407E (DE-Smad3) and BMPR-IA(QD)/HA, as indicated. Cell lysates were subjected to immunoprecipitation using the anti-Flag antibody, and precipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphoserine antibodies (anti-P-Ser). The membrane was re-blotted with the anti-Flag antibody to demonstrate comparable levels of expression of wild-type Smad2, Smad3 (A) and Smad1 (B). Aliquots of cell lysates were also subjected to immunoblotting with anti-HA antibodies to detect similar levels of expression of TβR-I(TD)/HA (A) or BMPR-IA(QD)/HA (B). Smad3D407E was detected by anti-Myc antibodies.



TβR-I (TD)	-	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
wt-Smad2	-	-	-	-	-	0.3	-	0.3	0.3	0.3
wt-Smad3	-	-	0.3	0.3	0.3	-	-	-	-	-
wt-Smad4	-	-	-	-	-	-	0.3	0.3	0.3	0.3
DE-Smad3	-	-	-	0.3	0.8	-	-	-	0.3	0.8

(μg/well)

Fig. 3. Smad3D407E blocked TGF-β-induced gene expression. R mutant cells of Mv1Lu (clone 4-2) were transiently transfected with p3TP-Lux together with various combinations of TβR-I(TD)/HA, Smad2, Smad3, Smad4 and Smad3D407E, as indicated. The content of DNA was adjusted to 2 μg/well by pcDNA3 (Invitrogen). Luciferase activities were normalized to pRL-TK activities to control for transfection efficiencies. Duplicate experiments were repeated three times with similar results.

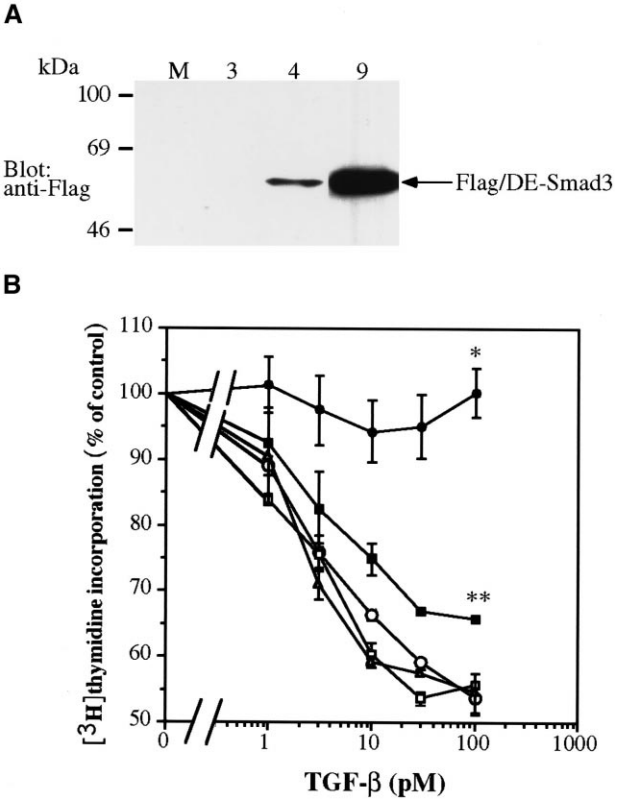


Fig. 4. Smad3D407E generates resistance to TGF-β-induced growth inhibition. A: HaCaT cells were stably transfected with the Flag-Smad3D407E expression construct, and the levels of expression in clones 3, 4, and 9 were assayed by immunoblotting using the anti-Flag antibody. A mock-transfected cell line (M) was used as a negative control. B: HaCaT cells (○), mock transfectant (□), and clone 3 (△), clone 4 (■) and clone 9 (●) of Smad3D407E transfectants established from HaCaT cells were subjected to [^{3}H]thymidine incorporation assay in the presence of various concentrations of TGF-β. Duplicate experiments were repeated three times with similar results. Error bars represent S.E.M. Inhibition of thymidine incorporation at 100 pM of TGF-β was significantly reduced in clone 4 and clone 9 compared to that in HaCaT and mock transfectant cells ($*P < 0.001$, $**P < 0.01$, Student's t-test).

glutamic acid to construct a Smad3D407E mutant. COS7 cells were transfected with the Smad3 or Smad3D407E expression constructs together in presence or absence of the constitutively active form of TβR-I, TβR-I(TD). Wild-type Smad3 and Smad3D407E had similar protein expression levels, and phosphorylation of the wild-type Smad3 was detected by immunoblotting using the antibodies to phosphorylated serine residues. However, Smad3D407E was defective in TGF-β-induced phosphorylation (Fig. 1B), as previously reported for the corresponding mutant of Smad2, Smad2D450E [4]. Smad3D407E also blocked the phosphorylation of co-expressed wild-type Smad2 and Smad3 (Fig. 2A). Phosphorylation of Smad1 by the constitutively active form of bone morphogenetic protein (BMP) type IA receptor was not affected by the mutant, suggesting that the inhibition was pathway-specific (Fig. 2B).

To determine the functional significance of Smad3D407E, we next assayed the effects of this mutant on TGF-β-induced transcriptional activation using the p3TP-lux promoter reporter construct. Luciferase activities were induced by TβR-I(TD), and were further enhanced by co-transfection of Smad3 or of Smad2 and Smad4. Expression of Smad3D407E reduced the luciferase activities induced by Smad3 or by Smad2 and Smad4 in a dose-dependent manner (Fig. 3).

We have also established HaCaT cells stably expressing Flag-tagged Smad3D407E. Expression levels of the mutant Smad3 in the stable transfectants were determined by immunoblotting using the anti-Flag antibody (Fig. 4A), and growth inhibition was measured by [^{3}H]thymidine incorporation assay. Expression of Smad3D407E clearly reduced the TGF-β-induced growth inhibitory response, and the degree of reduction of inhibition correlated with the levels of expression of the mutant protein (Fig. 4B). These findings again suggested the dominant negative function of Smad3D407E in the TGF-β signaling pathway.

4. Discussion

Among three Smad proteins involved in TGF-β signaling, mutations in the *Smad4* gene and *Smad2* gene have been identified in pancreatic cancers, colorectal cancers, lung cancers, breast cancers, ovarian cancers, and head and neck cancers [4,20–24,27]. Mutations in the *Smad3* gene have not yet been identified in any cancer [28,29]. However, the present findings showed that Smad3 could also perturb TGF-β signaling by a mutation identified in Smad2 in a case of colorectal cancer.

Smad3 is implicated in the TGF-β-induced growth inhibitory signaling pathway because mutant Smad3 proteins abrogate the growth inhibition [6,14]. The present study showed

that a single missense mutation in the *Smad3* gene could also result in perturbation of TGF-β signaling. However, this does not prove a non-redundant role for Smad3 in TGF-β signaling, since the Smad3D407E mutant inhibited the phosphorylation of both Smad2 and Smad3. Recently, the L3 loop of Smad proteins was found to determine the specificity of interaction between Smad proteins and receptors. Smad2 and Smad3 have identical amino acid sequences in the L3 loop, suggesting that they share the same binding site to the receptor. Smad1 and Smad5, which mediates BMP signals, also share identical sequences in the L3 loop, and their sequence differs at two positions from the Smad2/3 sequences. Swapping these two amino acids in Smad1 and Smad2 caused a switch in phosphorylation of Smad1 and Smad2 by the BMP and TGF-β receptors, suggesting that the L3 loops determine specificities of binding to the receptors [32].

Phosphorylation of Smad proteins has been suspected to reduce the affinity of Smads to the receptors, and stable binding of Smads to the receptors was obtained using kinase-defective type I receptors [5]. Similar stable binding was observed with phosphorylation site mutants of Smad2 as well as the inhibitory Smads, i.e. Smad6 and Smad7 [5,10,11,33–35]. In all these cases, Smad proteins bound to the receptors but were not phosphorylated. Smad3D407E also stably bound to the TGF-β type I receptor (data not shown), and as a result probably eliminated access of the wild-type Smads to the binding site. Since the D407E mutation in Smad3 does not affect the L3 loop, the specificity of binding to the receptor may not be modified, and thus the mutant may act in a lineage-specific manner.

Smad3D407E had dominant negative effects on growth inhibition of HaCaT cells and p3TP-lux gene expression by TGF-β. However, our findings do not show that either Smad2 or Smad3, or both Smad2 and Smad3 are required for growth inhibitory signaling by TGF-β. Further studies will be required to elucidate the different roles of Smad2 and Smad3 in the TGF-β signaling pathway.

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