Evidence That Smad2 Is a Tumor Suppressor Implicated in the Control of Cellular Invasion*

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The Smad2 protein plays an essential role in the transforming growth factor-β (TGF-β) signaling pathway. This pathway mediates growth inhibitory signals from the cell surface to the nucleus. Although Smad2 protein is significantly mutated in human cancers, there is no definitive evidence implicating Smad2 as a tumor-suppressor gene. Here we show that overexpression of the tumor-derived missense mutation Smad2.D450E, an unphosphorylatable form of Smad2 found in colorectal and lung cancers, did not abolish the TGF-β-mediated growth arrest, suggesting that resistance to the growth-inhibiting effects of TGF-β exhibited by human tumors cannot be linked to the inactivation of Smad2 protein. In contrast, overexpression of Smad2.D450E induces cellular invasion, and this effect was enhanced by TGF-β. A similar invasive phenotype was obtained in cells expressing another inactivating mutation in Smad2 (Smad2.P445H) found in colorectal cancer. These findings indicate that genetic defects in Smad2 are sufficient to confer the invasion-promoting effect of TGF-β and reveal that TGF-β acts through Smad2 to induce cellular invasion by a novel mechanism that is independent of Smad2 phosphorylation by the activated TGF-β type I receptor.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine involved in the regulation of many biological processes including cell proliferation, extracellular matrix formation, differentiation, and cell death (1–3). Extensive analysis of TGF-β signaling pathways has demonstrated that TGF-β responses are elicited by ligand-induced formation of a heteromeric complex containing two transmembrane serine/threonine kinase receptors, TβRI and TβRII (1). The signal is then transferred to the nucleus through the type I receptor kinase-mediated phosphorylation of Smad2 on the C-terminal serine residues. This phosphorylation event results in association of Smad2 with Smad4 and translocation to the nucleus where they function as transcriptional regulators via their interactions with DNA-binding proteins such as Fast-1 and Fast-2 (1, 4). Although TGF-β is one of the most potent inhibitors of normal cell growth, many malignancies of epithelial and hematopoietic origins are resistant to TGF-β, suggesting that mutations in signaling components of this pathway may allow tumor cells to escape from growth inhibition by TGF-β (1, 5, 6). Resistance to TGF-β has been found to be associated with mutations in TGF-β receptors or downstream signaling mediators (1). Studies on Smad2 protein in human tumors have previously identified mutations that block phosphorylation of Smad2 by the activated type I receptor (7–9). At present, functional analysis of mesoderm induction in Xenopus embryos suggests that these types of mutation yield biologically inactive Smad2 proteins (7). However, the importance of alterations or loss in Smad2 protein in promoting tumorigenesis is still unknown. Mice with inactivating mutation in the loci for Smad2 died in utero (10). This early lethality likely precluded tumor formation and, therefore, these knockout mice did not shed light on the role of TGF-β signaling in carcinogenesis. Using a collagen I invasion in vitro assay, we now present evidence that alterations of Smad2 play a significant role in TGF-β-mediated invasiveness and malignant progression.

EXPERIMENTAL PROCEDURES

Plasmids—Mammalian expression vectors for Smad2 mutants and pAR3-lux were a gift from Dr. J. Wrana. pcDNA3-Flag-Smad7 was a gift from Dr. P. ten Dijke. Expression vector for Fast-1 was a gift from Dr. M. Whitman. Expression vectors for Smad4, GAL4-Smad4, and G15E1b-lux have been described (11). Plasmids for GAL4-Smad2 and GAL4-Smad2.D450E were constructed using the pSG424 vector. For stable transfection, Smad2 mutants were subcloned into pEGFP in frame with an amino-terminal green fluorescent protein (GFP) tag.

Cell Line—The epithelial Madin-Darby canine kidney (MDCK) cells or MDCK cells stably expressing a constitutively activated mutant of phosphoisoitdine 3-OH kinase (p110α) were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum and 5 μg/ml gentamycin. Cells were transfected with expression vectors by the LipofectAMINE method (Life Technologies, Inc.) and selected in G418 or hygromycin. Cells were transfected with the appropriate vector alone and selected in parallel with the others cells. For double transfectants, stable cell lines were secondarily transfected with a vector encoding a hygromycin-resistant gene and selected in hygromycin-containing growth medium. For the clonal growth assays, hygromycin-resistant colonies were visualized with crystal violet 10–13 days after transfection.

Gene Expression Analysis—For gene expression analysis, cells were plated to semiconfluence and 24 h later were transfected with expression vectors by the LipofectAMINE method. Cells were subsequently incubated in the presence or absence of human TGF-β1 (2 ng/ml) for 16 h. Extracts were then prepared and assayed for luciferase activity using the luciferase assay system described by the manufacturer (Promega). The luciferase activities were normalized on the basis of β-galactosidase expression from pCMV5.LacZ control vector and protein content.

Invasion Assays—Invasion assays across dish coated with type I collagen were performed as described (12). Collagen G (type I solution, Seromed, Biochrom KG, Berlin, Germany) was dissolved at 0.22% (w/v) in bicarbonate buffer-containing Dulbecco’s modified Eagle’s medium, and 1.2 ml aliquots were poured into a 6-well plate and incubated
Resistance to the growth-inhibiting effects of TGF-β is not associated with genetic defects in Smad2 found in human cancers. A, effects of TGF-β on the proliferation of MDCK cells stably transfected with the empty vector pCMV5 (Neo), vector Smad2.D450E, or the dominant-interfering mutant of Smad2 (Smad2.S465A). Exponentially growing MDCK cells were cultured in serum-containing medium before being treated with various concentrations of TGF-β (0–5 ng/ml) in low serum. After 20 h, the cells were pulsed for 4 h with [3H]thymidine, and the amount of incorporated radioactivity was measured by scintillation counting as previously described (13). B, MDCK cells were transfected with the indicated vectors together with pEM4 vector encoding a hygromycin resistance gene and selected in hygromycin-containing growth medium. Hygromycin-resistant colonies were visualized with crystal violet 10–13 days after transfection. 

RESULTS AND DISCUSSION

Mutant Smad2 proteins carrying point mutations found in human cancers are no longer phosphorylated in response to TGF-β, suggesting that alteration of Smad2 may function to disrupt TGF-β signaling. To test this hypothesis, we generated pools of MDCK cells stably transfected with Smad2.D450E, a tumor-derived missense mutation found in colorectal and lung cancers (7, 8). Surprisingly, we found that expression of Smad2.D450E failed to block TGF-β-induced cell cycle arrest (Fig. 1A). As a control, expression of a dominant-negative mutant of Smad2 (Smad2.S465A), which contains a substitution of Ser at position 465 and cannot undergo TGF-β-induced phosphorylation (14, 15), suppressed the antiproliferative action of TGF-β (Fig. 1A). In a clonal growth assay, we found that overexpression of Smad2.D450E, but not wild-type Smad2, was unable to trigger growth arrest in MDCK cells (Fig. 1B), suggesting that the mutant protein is recessive in this assay. Smad2.D450E also failed to restore the number of surviving colonies from MDCK cells expressing Smad4 (Fig. 1B). A similar conclusion could be drawn when Smad2.D450E was co-transfected with wild-type Smad2 (Fig. 1B), suggesting that Smad2.D450E cannot act as a dominant-negative inhibitor to disrupt Smad signaling. From these results, it is becoming evident that the acquisition of TGF-β resistance in tumors is not associated with this mutation of Smad2 and may result from defects of other components in TGF-β-mediated responses.

To investigate the possibility that mutations of Smad2 found in human tumors could interfere with the function of wild-type Smad2 protein, we first tested the ability of Smad2.D450E to signal transcriptional responses that are typical of TGF-β. A fusion protein construct containing the full-length Smad4 and the DNA binding domain GAL4 (1-147) failed to stimulate transcription, but when it was cotransfected with Smad2, we observed a strong TGF-β-dependent induction of the heterologous promoter construct. In contrast, when Smad2.D450E was used, we observed a strong decrease in GAL4-Smad4 transcriptional activity (Fig. 2A). Similar results were obtained when GAL4-Smad2 fusion proteins containing either wild-type
Smad2 or Smad2.D450E were tested in this assay (Fig. 2B). However, quite different results were obtained in another assay using the pAR3-lux, which contains three activin responsive elements (AREs) linked to a basic TATA box and a luciferase reporter gene (16). As shown in Fig. 2C, expression of a pAR3-lux construct had minimal basal activity, but a strong TGF-β-dependent increase of transcriptional activity was detected in MDCK cells expressing both pAR3-lux and the forkhead-containing DNA-binding protein Fast-1, a transcription factor which associates with Smad2 and activates transcription from an ARE (16, 17). Interestingly, coexpression of Smad2 or Smad2.D450E along with Fast-1 resulted in ligand-independent synergistic induction of the reporter gene, although to a variable extent (Fig. 2C). Whereas Smad2 caused only a modest increase, overexpression of Smad2.D450E led to superinduction of the AR3 reporter. The induction of AR3 reporter by Smad2.D450E is not a general phenomenon because of the accumulation of unphosphorylated forms of Smad2 since expression of the dominant-negative mutant of Smad2 (Smad2.S467A), which contains a substitution of Ser at position 467 and cannot undergo TGF-β-induced phosphorylation (14, 15), showed a negligible effect in this assay. Identical results were obtained with the HepG2 cell line, suggesting that the effect of Smad2.D450E on the AR3 reporter may be a general phenomenon (data not shown). Taken together, our findings raise the interesting possibility that the tumor-derived missense mutation Smad2.D450E is constitutively active in regulating some physiological functions that do not depend on its phosphorylation by the activated TGF-β type I receptor.

Whereas the loss of growth inhibition by TGF-β frequently accompanies the progression of malignancy and may contribute to development of many types of human cancers, recent studies revealed that TGF-β showed a biphasic action during tumor progression, thereby acting as a tumor suppressor at early stages and as an enhancer of invasiveness at later stages (1, 18). To understand the functional significance of Smad2 alteration in tumors, we investigated the potential effects of Smad2.D450E on cellular invasion. As shown in Fig. 3A, expression of Smad2.D450E stimulates the invasion of MDCK cells in the absence of TGF-β, as detected by a dramatic increase of invasion into collagen I matrices. The induction of invasion by Smad2.D450E is not a general phenomenon because of the accumulation of unphosphorylated forms of Smad2 because expression of the dominant-negative mutant of Smad2 (Smad2.S467A), which cannot undergo TGF-β-induced phosphorylation (14, 15), is defective in inducing cellular invasion (Fig. 3A). More importantly, we find that TGF-β addition to MDCK cells transfected with Smad2.D450E, but not empty vector, further increased cellular invasion. Similar results were obtained with two independent populations of MDCK cells expressing Smad2.D450E, indicating that the invasion phenotype that we observed in MDCK-Smad2.D450E was not because of the selection of an aggressive line of G418 cells. These results indicate that the naturally occurring mutation of Smad2 is sufficient to confer the invasion-promoting effect of TGF-β, which is consistent with the interpretation that alterations of Smad2 in human tumors play a significant role in TGF-β-mediated malignant progression and invasiveness (Fig. 3A).

The effect of Smad2.D450E on cellular invasion is specific to Smad2 and TGF-β signaling because TGF-β addition had no effect on the invasion of MDCK cells transfected with a constitutively activated mutant of p110* (Fig. 3A), the prime mediator of cellular invasion (19, 20). Conversely, addition of hepatocyte growth factor (HGF), a potent inducer of cellular invasion (21), failed to increase invasion of MDCK cells expressing Smad2.D450E, although addition of HGF to cells expressing constitutively activated mutant of PI-3 kinase (p110*), or empty vector. Cells were transfected with expression vectors and selected in G418-containing growth medium and expanded as pools of stably transfected cells. Cells were seeded on top of the collagen gel and incubated at 37 °C. Where indicated, cells were treated with TGF-β (5 ng/ml) or HGF (10 units/ml). After 24 h, the percentage of cells that had invaded the gel was measured using an inverted microscope. The data shown are the mean value (± S.D.) of a representative experiment performed at least three times.

B, the tumor-derived mutants of Smad2 cooperate with the constitutively activated PI-3 kinase p110* in inducing cellular invasion. MDCK cells expressing p110* were secondarily transfected with Smad2.D450E, Smad2.P445H, or empty vector, and a vector encoding a hygromycin resistance gene and selected in hygromycin-containing growth medium. The invasion into collagen gel of pools of stably transfected cells was analyzed as described in panel A. C, phase-contrast images of single and double transfectants of MDCK cells.
Cell lysates prepared from cells stably expressing Smad7 protein were analyzed by immunoblotting using anti-Flag M2 antibody. Invasion into collagen gel of MDCK cells stably expressing Smad7, Smad2-D450E, or empty vector. Where indicated, cells were treated with TGF-β or HGF. After 24 h, the percentage of cells that had invaded the gel was measured using an inverted microscope. The data shown are the mean value (± S.D.) of a representative experiment performed at least three times.

reinforcing the conclusion that inactivation of Smad2 in cancers leads to increased cellular invasiveness (Fig. 2, A and B).

The phosphorylation of Smad2 and its association with the TGF-β receptor are essential for Smad2 function in TGF-β signal transduction (1, 6). Because Smad2-D450E interacts stably with TGF-β type I receptor (22), we generated independent clones of MDCK cells stably expressing Smad7 to determine whether Smad2-D450E induces cellular invasion by preventing access and phosphorylation of endogenous Smad2 protein to the receptor. As a direct antagonist of TGF-β signaling pathways, Smad7 functions by associating stably with the activated TGF-β type I receptor to block the interaction, phosphorylation, and nuclear translocation of Smad2 (16, 23). Western blot analysis of whole-cell lysates showed that two independent clones of MDCK cells express high levels of Flag-Smad7 fusion protein (Fig. 4A). In agreement with published results (16, 23), we find that expression of Smad7 blocks the majority of TGF-β-mediated growth arrest in MDCK cells (data not shown). By contrast, overexpression of Smad7 did not show any effect in the invasion assay irrespective of whether the cells were cultured in the presence or absence of TGF-β or HGF (Fig. 4B). These results indicate that Smad2-D450E enhances malignant progression by a mechanism that is independent of Smad2 phosphorylation by the activated type I receptor and disruption of TGF-β signaling pathways.

In numerous tumors, resistance to TGF-β is associated with inactivating mutations that can occur in components of this signaling pathway such as Smad2 protein (1). Although these tumor cells often harbor other genetic alterations as well, resistance to TGF-β-induced growth inhibition cannot be linked definitively to the mutations in Smad2 protein and may result from defects of other components in TGF-β signaling pathways (1). Consistent with this interpretation, recent studies indicated that the missense mutation D450E reconstructed on the corresponding residue of Smad3 suppressed TGF-β-mediated cell cycle arrest (24). Furthermore, mice with inactivating mutation in the loci for Smad2 develop colorectal cancer, reinforcing the role of Smad3 in mediating growth inhibitory signals (25). In contrast to the growth inhibitory effect of TGF-β, overexpression of Smad2-D450E induces cellular invasion, and this effect was enhanced by TGF-β. Taken together, the findings outlined in the present study provide strong evidence that Smad2 is a tumor suppressor that plays a critical role in malignant progression and the control of cell invasiveness. For instance, it is not clear whether TGF-β is directly or indirectly mediating the activation of these mutant proteins and their interaction with other TGF-β-related signal transducers, such as Smad3 and Smad4. Work is now in progress to address these issues. In any event, our study revealed that TGF-β enhances malignant progression through Smad2 by a novel intracellular mechanism that is independent of Smad2 phosphorylation by the activated type I receptor because Smad2 mutants are not phosphorylated in response to TGF-β (7). Thus, it will clearly be interesting in the future to determine whether, in addition to promoting invasiveness, this mechanism may fulfill other functions in cellular transformation and differentiation.

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