Evidence for a Role of Smad3 and Smad2 in Stabilization of the Tumor-derived Mutant Smad2.Q407R*

Received for publication, December 9, 2002, and in revised form, April 8, 2003
Published, JBC Papers in Press, April 16, 2003, DOI 10.1074/jbc.M212496200

Emmanuelle Dumonté, François Lallemand‡, Céline Prunier‡, Nathalie Ferrand‡, André Guillouzo‡, Bruno Clément‡, Azeddine Atfi§, and Nathalie Théret‡

From INSERM U456, University of Rennes I, 35043 Rennes, France and INSERM U482, Hôpital Saint-Antoine, 75571 Paris Cedex 12, France

Transforming growth factor β (TGF-β) is a potent inhibitor of cell proliferation and the loss of responsiveness to TGF-β may contribute to the development of human cancers. In hepatocellular carcinomas, the potential role of TGF-β signaling as a tumor suppressor pathway can be illustrated by the presence of mutations in genes encoding TGF-β receptors or downstream components of this signaling such as Smad2. Although Smad2 is mutated in hepatocellular carcinomas, the alteration of TGF-β signaling with respect to tumor progression remains to be established. Using the HepG2 hepatoma cells, we showed here that expression of Smad2.Q407R, a missense mutation found in human hepatocellular carcinoma, was less effective than expression of wild-type Smad2 in enhancing the ability of TGF-β to induce transcription from the Mix.2 promoter. This effect was specifically associated with a decrease in the steady-state level of Smad2.Q407R, presumably because of an enhancement of its ubiquitination and degradation through the proteasome machinery. More importantly, we found that the instability of Smad2.Q407R was reversed when this mutant undergoes homo-oligomerization with wild-type Smad2 or hetero-oligomerization with Smad3 within the cells. Therefore, our findings allowed us to propose a novel mechanism for suppression of the deleterious effect of a tumor-derived mutation of Smad2, which loss may lead to dysregulated cell proliferation during tumorigenesis.

Transforming growth factor β (TGF-β) regulates a broad range of cellular functions, including cell cycle arrest, regulation of extracellular matrix production, and induction of programmed cell death. TGF-β signals through a heteromeric complex of two types of transmembrane serine/threonine kinases, the type I (TβRI) and type II (TβRII) receptors. TGF-β binding to TβRII induces recruitment and phosphorylation of TβRI that transduces signals to downstream intracellular substrates, the Smad proteins (2, 3). The interaction of receptor complex and receptor-regulated Smads (R-Smads), Smad2 and Smad3, is facilitated by SARA (Smad anchor for receptor activation) (4). Once phosphorylated, Smad2 or Smad3 dissociate from SARA, a prerequisite for their hetero-dimerization with the common partner Smad (Smad4) (5). Then, the complexes move into the nucleus where they regulate the transcription of TGF-β-target genes, most of which remained to be identified (6, 7). In contrast to Smad2 and Smad3, the inhibitory Smads, Smad6 and Smad7, block signal transduction by preventing access and phosphorylation of endogenous Smad2 or Smad3 by the activated TβRII (8, 9). Recently, it has been shown that Smad7 can also function to target the ubiquitination and the proteasome-dependent degradation of the activated TβRI by recruiting the ubiquitin ligases Smurf1 and Smurf2 (10, 11).

Smad proteins share two domains of highly conserved sequence in their amino and carboxyl termini, termed mad homology domain MH1 and MH2, respectively, linked by a proline-rich region of variable length. In the basal state, association of Smad proteins in homo-oligomer involves intramolecular interactions between MH1 and MH2 domains, and this interaction is believed to keep Smad protein in the inactive state (12, 13). However, once signaling has commenced, the MH1 domain dissociates from the MH2 domain, resulting in the hetero-oligomerization with Smad4. In the nucleus, the activated Smads can bind to TGF-β-responsive promoter DNA either directly through the MH1 domain or by associating through its MH2 domain with other sequence-specific DNA binding partners such as the Fast proteins (7, 14). Via the MH2 domain, Smads can also bind the coactivators p300/CBP, which have histone acetylase activity, or the corepressors TGIF, Ski, and SnoN, resulting in the recruitment of a histone deacetylase and the formation of transcriptional repressor complexes (15, 16).

As potent inhibitor of cell growth (17) or inducer of apoptosis in hepatocyte (18), TGF-β controls liver growth. Loss of TGF-β responsiveness in hepatocarcinogenesis has been suggested to be associated with mutations that can occur in TGF-β cell surface receptors or downstream effector components of the TGF-β signaling. Thus, TGF-β type II receptor mutations were found with microsatellites instability (19, 20). In addition, defective receptor processing (21) and allelic loss (22) have also been described, providing further evidence for a role of TGF-β in suppressing liver tumorigenicity. Important support for a more general role of TGF-β signaling in liver tumor development came with the finding that human Smad2 and Smad4 (also called DPC4, for “deleted in pancreatic cancer”) are mutated in human liver tumors (23). However, the physiological relevance of alterations of Smad2 functions in promoting liver tumorigenesis is still unknown.

In addition to hepatocellular carcinoma, several mutations in Smad2 and Smad4, either in MH1 or MH2 domains, were found in a variety of human tumors (24–27). Such mutations

* This work was supported by INSERM and Association pour la Recherche sur le Cancer. 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: INSERM U456, 2 Avenue Léon Bernard, 35043 Rennes, France. Tel.: 33-2-23-23-48-11; Fax: 33-2-23-23-47-94; E-mail: nathalie.theret-bdioui@rennes.inserm.fr.
‡ The abbreviations used are: TGF-β, transforming growth factor-β; MEF, mouse embryonic cells; MH, Mad homology domain; ARE, activin response element.
Evidence for a Role of Smad3 and Smad2 in Mutant Smad2.Q407R

MATERIALS AND METHODS

Plasmids—Expression vectors for HA-Smad4, FLAG-Smad2, and 6×Myc-Smad3 were described previously (29, 30). The activin response element (ARE)-Luc reporter construct was a gift from Dr. J. Wrana and expression vectors for myc-Fast1 was provided by Dr. M. Whitman. The TGF-β-responsive promoter, Gsc-lux, and expression vectors for the Smad2.R133C and the Smad2.L440R mutations of Smad2 were provided by Dr. L. Attisano. Myc-His-tagged ubiquitin and the dominant negative version of E2 ligases were generously provided by Dr. F. Soud. Expression vectors for the MH1 and MH2 domains of Smad2 were obtained by PCR using the expression vector for Smad2 as a template. The Q407R mutation of Smad2 was obtained by mutagenesis using the GeneEditor™ in vitro Site-directed Mutagenesis System™ (Promega, Madison, WI). Mutated cDNAs were checked by sequencing (ABI310, PerkinElmer Life Sciences).

Cell Culture and Transfection—The human embryonal kidney cell line (293T) and COS7 cells were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum and 10 units/ml penicillin-streptomycin. The human hepatoma cell line (HepG2) was maintained in Williams’ E medium containing 0.1% albumin, 1 mM l-glutamine, 5 μg/ml insulin, 5 units/ml penicillin-streptomycin, hydrocortisone, and supplemented with 10% fetal calf serum. HepG2 cells were transfected using LipofectAMINE Plus (Invitrogen) and HepG2 cells were transfected by electroporation with a 1 s pulse from a 1800 μF capacitor charged to 230 V.

The immortalized mouse embryonic cells (MEF) from Smad2 wild-type or knock-out mice were a generous gift from Dr. E. Bottiger. MEFs were maintained in Dulbecco’s modified essential medium supplemented with 10% heat-inactivated feto bovine serum, 100 units/ml penicillin, and 50 μg/ml streptomycin.

Transcriptional Response Assays—Expression plasmids for Smads were cotransfected with the ARE luciferase reporter and myc-Fast1 plasmid into HepG2 cells. At about 30 h post-transfection, cells were incubated in medium containing 0.5% fetal calf serum and treated overnight with 2 ng/ml human TGF-β (Sigma). Cell extracts were assayed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega). The luciferase activities were normalized on the basis of Renilla luciferase expression from pRL-TK control vector. Experimental points were realized in triplicates in at least two independent transfections.

Immunoprecipitation and Immunoblotting—293T cells were plated to semi-confluence and 24 h later were transfected with expression vectors by the LipofectAMINE method. The total DNA amount in each transfection was kept constant by the addition of pCMV5 empty vector. A pCMV5 LacZ vector that expresses β-galactosidase under the control of the cytomegalovirus promoter was included in all transfections to allow normalization of transfected proteins. After one night in serum-starved medium, 293T cells were treated for 1 h with 5 ng/ml TGF-β and then lysed at 4 °C in lysis buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM MgCl2, 10% glycerol, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin). Aliquots of cell lysates were either analyzed by direct immunoblotting or subjected to immunoprecipitation with the monoclonal anti-FLAG M2 antibody (Sigma) for 2 h, followed by adsorption to Sepharose-4B for 1 h. The beads were washed five times in lysis buffer and samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Proteins were electrophotically transferred to nitrocellulose membrane (Amersham Biosciences) and probed with the appropriate antibodies: anti-FLAG M2 antibody (Sigma), 9E10 anti-Myc antibody (Roche Diagnostics), or anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA), as indicated. To control the amount of proteins loaded in the gel, all blots were probed with anti-actin antibody. The bound antibodies were visualized with horseradish peroxidase-conjugated antibodies to rabbit or mouse IgG (Sigma) using an enhanced chemiluminescence system.

RESULTS

Transcriptional Activation of a TGF-β Responsive Element Is Decreased in Smad2.Q407R Mutant—Mutation of glutamine to arginine at codon 407 in MH2 domain of Smad2 (Smad2.Q407R) has been described in hepatocellular carcinoma (23) and was suggested to be deleterious for the TGF-β signaling pathway. Indeed, the tumor-derived mutation Q407R maps to the MH2 domain in the three-helix bundle at helix H4 that is involved in the homotrimer formation (31). To investigate whether the mutation Q407R interferes with the ability of Smad2 to mediate TGF-β transcriptional responses, we focused our analysis on Xenopus mix.2 promoter as a target of Smad2 because activation of mix.2 by TGF-β requires the formation of a Smad2-Smad4-Fast complex that binds to a sequence promoter known as the TGF-βARE. To evaluate the consequence of the missense mutation Q407R on transcriptional activity, the TGF-β responsive HepG2 cell line was cotransfected with the wild-type Smad2 or Smad2.Q407R and the forkhead-containing DNA binding protein Fast-1 and the pARE-Lux reporter, which contains a luciferase reporter gene under the control of three copies of ARE from the Mix.2 Xenopus promoter (6, 7). As expected, expression of the pARE-Lux construct alone had minimal basal activity in HepG2 cells, but co-transfection with Fast-1 yielded a TGF-β-dependent induction of luciferase activity that was further enhanced by coexpression of cells with wild-type Smad2. Under these experimental conditions, we found that transfection of equivalent amounts of transfected Smad2.Q407R mutant could not disrupt the TGF-β-dependent transcriptional activation, but the mutant Smad2.Q407R was less effective than wild-type Smad2 in enhancing the ability of TGF-β to induce transcription from the Mix.2 promoter (Fig. 1A, left panel). Similar results were obtained either by transfecting HepG2 cells by the TGF-β-responsive promoter Gsc-lux, which, like ARE-lux, contains activin responsive elements known to bind the complex Smad2-Smad4-Fast (Fig. 1A, left panel, inset) or by transfecting the 293T cell line with the pARE-Lux reporter (Fig. 1A, right panel).

A growing body of evidence indicates that the alteration of the Smad steady-state levels is one of the mechanisms by which tumor cells may escape to the growth inhibitory effect of TGF-β (32, 33). Therefore, we reasoned that the decrease of Smad2 transcriptional activity upon introduction of Q407R may be due, at least in part, to a reduction in the stability of Smad2 protein. To address this issue, we first investigated the level of Smad2.Q407R or wild-type Smad2 proteins in transiently transfected HepG2 (Fig. 1B, left panel) and 293T cells, (Fig. 1B, right panel) by direct Western immunoblotting. A dramatic decrease in the amount of Smad2.Q407R compared with wild-type Smad2 was detected irrespective of whether cells were

Steady-state Protein Level Assays and Smad Ubiquitination—For Smad stability, transiently transfected COST cells were metabolically labeled for 30 min with 100 μCi/ml Redivue Pro-mix I-35S (Amersham Biosciences) in methionine and cysteine-free medium, and then chased in Dulbecco’s modified essential medium containing 10% fetal calf serum and 5 μg/ml actinomycin D. Cell lysates were subjected to immunoprecipitation, and proteins were resolved by SDS-PAGE and visualized by autoradiography. The involvement of the ubiquitin-dependent proteasome pathway was realized by transfecting 293T cells with combinations of FLAG-tagged Smads and the dominant negative of E2 ligases UbcH5a, UbcH5b, UbcH6c, or 20K. In some cells, experiments were transfected with wild-type FLAG-Smad2 or FLAG-Smad2.Q407R were treated for 6 h with the proteasome inhibitor MG-132. Lysates from those cells were subjected to immunoblotting with anti-FLAG antibody. For direct ubiquitination assay, 293T cells were transfected with wild-type FLAG-Smad2 or FLAG-Smad2.Q407R and Myc-His-ubiquitin. Lysates were immunoprecipitated with anti-FLAG M2 before being immunoblotted with anti-Myc antibody.
Evidence for a Role of Smad3 and Smad2 in Mutant Smad2.Q407R

TGF-β-Smad2. About 40 h post-transfection, cells were treated with 5 ng/ml transfected with either FLAG-tagged Smad2.Q407R or wild-type (WT). 293T-transfected cells with [35S]methionine showed that to a diminution of mRNA translation, because labeling of blot/H9251 TGF-β the tumor-derived mutant Smad2.Q407R (Smad2.Q407R). Another with Fast-1 in the presence or absence of wild-type Smad2 (right panel) was examined with a reporter gene driven by ARE-binding site. Cells were transfected with pARE-Lux alone or Smad2.Q407R transcriptional activity in HepG2 cells (left panel) and 293T cells (right panel) showed that Smad2.Q407R and wild-type Smad2 were neo-synthesized at approximately similar levels (see Fig. 2A, chase 0 h).

Together, the above observations strongly suggested that Smad2.Q407R might induce transcriptional activation less efficiently than its wild-type counterpart because of the reduced level of the mutated protein. If this prediction was true, then transfecting increasing amounts of Smad2.Q407R should produce a corresponding increase in transcriptional activation of the pARE-Lux promoter. Indeed, transfection of 20 μg of Smad2.Q407R was able to activate transcription with an efficiency approaching that elicited by cotransfection of 5 μg of wild-type Smad2, which is consistent with the hypothesis that introduction of the missense mutation Q407R in the background of wild-type Smad2 did not disrupt the function of the Smad2 protein as a transcriptional factor (Fig. 1C). Further
Evidence for the functionality of Smad2.Q407R was provided by our finding that this tumor-derived mutation retains its ability to be phosphorylated by the activated type I receptor (Fig. 1D) and to form hetero-oligomers with Smad4 upon TGF-β stimulation (see Fig. 5B).

The Mutation Q407R Increased Degradation of Smad2 through the Ubiquitin/Proteasome Pathway—Having shown a decrease in the steady-state level of Smad2.Q407R, we next sought to determine whether this mutation leads to an altered turnover of the protein. To test this possibility, we performed a pulse-chase experiment with a [35S]methionine/cysteine mixture using transfected 293T cells (Fig. 2A). As indicated above, the amounts of neo-synthesized Smad2.Q407R proteins were similar to that of wild-type Smad2, which is consistent with the hypothesis that mutation Q407R may accelerate the turnover of the protein. Further evidence was provided by our observation that newly synthesized Smad2.Q407R disappeared more rapidly compared with wild-type Smad2. Taken together with the Western blot analysis, the data suggested that the mutation Q407R did not appreciably affect Smad2 synthesis but rather enhanced its degradative process.

The ubiquitin-proteasome system is a common pathway for the degradation of a wide range of cellular proteins, including Smad2. To approach the question of how Smad2.Q407R undergoes rapid turnover, we evaluated the involvement of the ubiquitin-proteasome-dependent pathway in this process by investigating the effect of the proteasome inhibitor MG-132 on the Smad2.Q407R stability or by cotransfecting 293T cells with dominant-negative forms of ubiquitin-conjugating enzyme (UbcH5a also named E2 enzymes). As shown in Fig. 2B, the steady-state level of Smad2.Q407R was dramatically increased in cells treated with the proteasome inhibitor MG-132. A similar increase was observed with transfected 293T cells containing dominant-negative forms of ubiquitin-conjugating enzyme (Ubcs also named E2 enzymes). As shown in Fig. 2C, middle panel), these mutant enzymes contain catalytic site mutations and have been shown to behave in a dominant-negative manner (34) to inhibit the degradation of Smad2 mediated by the ubiquitin-proteasome dependent pathway (Ref. 32 and Fig. 2C, left panel). This effect was specific to the UbcH5 family because cotransfection of the inactive mutant 20K did not appreciably affect the overall pattern of Smad2.Q407R expression. In addition the data confirmed that the dominant-negative forms of UbcH5a, UbcH5b, and UbcH5c (Fig. 2C, middle panel). These mutant enzymes contain catalytic site mutations and have been shown to behave in a dominant-negative manner (34) to inhibit the degradation of Smad2 mediated by the ubiquitin-proteasome dependent pathway (Ref. 32 and Fig. 2C, left panel). This effect was specific to the UbcH5 family because cotransfection of the inactive mutant 20K did not appreciably affect the overall pattern of Smad2.Q407R expression. In addition the data confirmed that the dominant-negative forms of UbcH5a, UbcH5b, and UbcH5c, but not 20K can block the degradation of Smad2.R133C, a tumor-derived mutant with an accelerated level of protein degradation (Ref. 32 and Fig. 2C, right panel).

Next, we sought to directly demonstrate that the mutation Q407R induced a constitutive degradation of Smad2 through the ubiquitin/proteasome system. For this, 293T cells were transfected with either FLAG-Smad2.Q407R or FLAG-wild-type Smad2 and Myc-His ubiquitin in the absence or presence of MG-132 (Fig. 2D). Consistent with published results (2, 35), little or no ubiquitinated forms of wild-type Smad2 were detected (lane 1). However, exposure of cells to MG-132 led to a marked accumulation of high molecular mass Smad2 forms corresponding to ubiquitin-conjugated protein (lane 2). More importantly, introducing Q407R into Smad2 leads to a 3-fold increase in the ubiquitination of Smad2 protein (lane 4). Taken together with the dominant-negative mutants of UbcH5a, UbcH5b, and UbcH5c ligases data, these finding strongly suggest that Smad2.Q407R is degraded through the proteasome pathway more rapidly than wild-type Smad2.

Increased Expression of the Wild-type Smad2 Overcomes the Degradation of the Mutant Smad2.Q407R—Because Smad2 can form homo-oligomers, we next investigated whether the stability of the wild-type Smad2 may be affected by its association with the mutant Smad2.Q407R. To explore this hypothesis, 293T cells were co-transfected with wild-type 6×Myc Smad2 and either FLAG-Smad2.Q407R or FLAG-wild-type Smad2, and the steady-state levels of transfected proteins were analyzed by Western blotting. As shown in Fig. 3A, the level of 6×Myc wild-type Smad2 was not affected by coexpressing FLAG-Smad2.Q407R, demonstrating an inability of Smad2.Q407R to enhance the degradation of its wild-type counterpart. In contrast we found that Smad2.Q407R was stabilized when wild-type Smad2 was cotransfected. This effect occurs independently of TGF-β signaling because exposure of cells to TGF-β did not synergize with wild-type Smad2 to increase the steady-state level of Smad2.Q407R. During the course of the analysis we also examined the effects of wild-type Smad2 on the level of two other tumor-derived mutations localized either in the MH2 domain (Smad2.L440R) or MH1 domain (Smad2.R133C). As shown in Fig. 3A, an overexpression of wild-type Smad2 failed to overcome the degradation of
Evidence for a Role of Smad3 and Smad2 in Mutant Smad2.Q407R

To investigate the stabilization of Smad2.Q407R by wild-type Smad2, we first examined whether the stabilization of Smad2.Q407R occurred through the formation of wild-type Smad2-Smad2.Q407R complex. To address this issue, cell lysates from transiently transfected 293T cells were subjected to immunoprecipitation with anti-FLAG antibody directed toward tagged Smad2 mutant, followed by immunoblotting with anti-Myc antibody for the presence of wild-type Smad2 (Fig. 3B). Analysis of wild-type FLAG-Smad2 coprecipitating with wild-type 6×Myc-Smad2, showed that overexpression of Smad2 lead to a ligand-independent homo-oligomerization of Smad2, confirming published results (13). A similar result was obtained with FLAG-Smad2.Q407R and wild-type 6×Myc-Smad2 supporting the hypothesis that the formation of complexes between Smad2.Q407R and wild-type 6×Myc-Smad2 may protect the mutant from the ubiquitin-dependent degradation. To demonstrate this directly, we used the tumor missense mutation Smad2.D450E (24, 36) that completely disrupts the homo-oligomerization of the Smad2 protein (37) (Fig. 3C). In contrast to wild-type Smad2, coexpression of Smad2.D450E failed to stabilize Smad2.Q407R, providing direct evidence that the homo-oligomerization process plays an important role in stabilization of Smad2 mutant protein.

To investigate the role of endogenous wild-type Smad2 in stabilizing the tumor-derived mutant Smad2.Q407R, MEFs from Smad2 wild-type or knock-out mice were transfected with the expression vectors for FLAG-Smad2.Q407R and wild-type 6×Myc-Smad2 supporting the hypothesis that the formation of complexes between Smad2.Q407R and wild-type 6×Myc-Smad2 may protect the mutant from the ubiquitin-dependent degradation. As shown in Fig. 4, little or no Smad2.Q407R protein in MEF Smad2−/− (lanes 1 and 2) was detected, whereas in MEF Smad2+/+, a large amount of the tumor-derived mutant Smad2.Q407R was observed (lanes 1 and 2), consistent with the hypothesis that endogenous wild-type Smad2 might prevent the degradation of Smad2.Q407R. Consistent with this, we showed that coexpression of wild-type Smad2 in MEF Smad2−/− prevented the degradation of Smad2.Q407R (lanes 5 and 6). As with 293T cells, these stabilization processes were totally independent of TGF-β cell stimulation. Accordingly with our biochemical analyses, these data indicated that wild-type Smad2 can restore the transcriptional function of the tumor-derived mutant Smad2.Q407R by preventing its ubiquitin-dependent degradation potential.

**Smad3 but Not Smad4 Stabilizes the Tumor-derived Mutant Smad2.Q407R**—The phosphorylation of Smad2 by the activated type I TGF-β receptor and its subsequent heterodimerization with Smad4 and translocation to the nucleus form the basis for a model of how Smad proteins work to transmit TGF-β signals from the plasma membrane to the nucleus. Therefore, we investigated whether the TGF-β-dependent association of Smad2.Q407R and wild-type Smad4 may result in an increase in the steady-state levels of Smad2.Q407R (Fig. 5A). In contrast to wild-type Smad2, we were unable to see any stabilization of Smad2.Q407R by wild-type Smad4 even in the presence of TGF-β. A similar conclusion could be drawn when the mutant Smad2.R133C was used instead of Smad2.Q407R. The absence of stabilization of Smad2.Q407R by wild-type Smad4 was not because of a lack of hetero-oligomerization because...
hetero-oligomers of FLAG-Smad2.Q407R/HA-Smad4 were detectable in 293T cells transfected with FLAG-Smad2 Q407R and HA-Smad4 in the presence of a constitutively active TβRII mutant, which is able to signal in the absence of TβRII and ligand (Fig. 5B).

Because Smad2 and Smad3 are structurally highly similar and can form heteromers, the effect of overexpression of Smad3 in the transfected 293T cells was investigated. Similar to wild-type Smad2, we found that the steady-state level of Smad2.Q407R was increased (Fig. 5C) in cells cotransfected with wild-type Smad3 and this stabilization effect was associated with the formation of hetero-oligomer complexes (Fig. 5D). This effect was specific to the Smad2.Q407R mutant because increased expression of wild-type Smad3 did not protect Smad2.R133C from undergoing extensive degradation, as it has been shown for wild-type Smad2 (Fig. 3A). Thus, it is likely that Smad3 is involved in maintaining the integrity of Smad2 signaling by preventing the degradation of the tumor-derived mutation of Smad2, i.e., Smad2.Q407R, found in human liver cancer.

DISCUSSION

The Smad2 protein plays an essential role in the TGF-β signaling pathway, which mediates growth inhibitory signals from the cell surface to the nucleus in a variety of cells, including hepatocytes. Because hepatoma cells were found to develop resistance to the negative growth-regulatory effects of TGF-β, it is tempting to speculate that one of the mechanisms whereby cells undergo neoplastic transformation and escape from normal growth control involves an altered response to TGF-β. A missense mutation (Q407R) in the MH2 domain of Smad2 has been identified in human hepatocellular carcinoma (23), raising the interesting possibility that Smad2 might play a critical role in tumor progression of the liver. In the present study we show that the tumor-derived mutation Q407R can confer a decreased stability of Smad2 through increased ubiquitination-dependent degradation, resulting ultimately in a corresponding decrease in the ability of Smad2 to activate gene expression in response to TGF-β signaling. Our analysis of the steady-state levels of Smad2.Q407R strongly suggests that the primary defect caused by the Q407R mutant is to induce targeting of Smad2 for ubiquitin-mediated proteolysis. Consistent with this, transfection of higher amounts of Smad2.Q407R enhanced activation of the ARE-lux close to levels obtained with wild-type Smad2. Further evidence for the functionality of Smad2.Q407R is provided by our finding that Smad2.Q407R is phosphorylated by the activated type I receptor at the carboxyl-terminal serines that serve as TGF-β receptor phosphorylation sites. This phosphorylation event is important to Smad2 function because the nonfunctional form of Smad2 (Smad2.D450E) found in human colorectal and lung cancers is not phosphorylated (24).

Extensive analysis of the TGF-β signaling pathway has clearly indicated that regulated proteolysis plays an important role for proper regulation of TGF-β signaling by targeting different components of this pathway for destruction by the 26 S proteasome. For example, the pool of activated Smad2 in a TGF-β-treated cell is targeted in the nucleus for ubiquitination-dependent degradation, thus providing a mechanism to reset the Smad2 pathway for interpretation of subsequent TGF-β signals (35). The ubiquitination-dependent degradation may also play a role during tumor progression because the steady-state level of Smad2 can be decreased in a Smad2 mutant found in colorectal cancer (32) and harboring an arginine to cysteine mutation at position 133 in the MH1 domain. A similar mechanism has been proposed to explain the inability of a tumor-derived mutation detected in the MH2 domain of Smad2 (Smad2.L440R) (24, 32) to mediate the TGF-β response, supporting the general notion that the loss of Smad2 function may contribute to the development of tumors. In these respects, Smad2.Q407R was closely similar to Smad2.L440R, as the finding outlined in the present data strongly suggests that the Q407R mutation in Smad2 generates a protein with increased susceptibility to degradation through the proteasome pathway. Consistently, degradation of the Smad2.Q407R was prevented by the proteasome inhibitor MG-132 and the level of Smad2.Q407R was stabilized by overexpression of catalytically inactive versions of the E2 ubiquitin-conjugating enzymes UbcH5a, UbcH5b, and UbcH5c. These mutants, which harbor an active Cys to Ala mutation, behave in a dominant-negative manner and have been shown to suppress the degradation of Smad2 protein by the proteasome (34).

In the present study, we also provide evidence demonstrating that the wild-type allele can suppress the ubiquitin-dependent degradation of some tumor-derived mutation of Smad2 (i.e., Smad2.Q407R and Smad2.L440R). This protective effect may occur in the absence of Smad2 phosphorylation by the activated type I receptor and its subsequent hetero-oligomerization with Smad4. Of note, the homo-oligomerization of Smad2 was independent of TGF-β signaling in 293T cells, like in other overexpression systems such as COS cells, suggesting the possibility that the homo-oligomerization of Smad2.Q407R with wild-type Smad2 may play a critical role in suppressing the ability of the mutation Q407R to enhance the degradation of Smad2 through the ubiquitin-proteasome pathway. Consistent with this interpretation, we observed that coexpression of Smad2.D450E, a tumor-derived mutation unable to form homomer complexes, failed to stabilize Smad2.Q407R compared with wild-type Smad2. The physiological relevance of this phenomenon was demonstrated by showing that endogenous wild-type Smad2 prevent the degradation of the tumor-derived mutant Smad2.Q407R, using immortalized mouse embryonic cells from Smad2 wild-type (Smad2+/+) or knock-out (Smad2−/−) mice.

Mutations in Smad2 and Smad4 genes, but not that of Smad3, have been detected in several carcinomas, suggesting that Smad2 and Smad4 function as tumor suppressor genes. Cells deficient in Smad3, or expressing a mutant dominant-negative Smad3 in which its three carboxyl-terminal serines are changed to alanines, are unable to induce transcription from TGF-β-responsive promoter constructs (38, 39), and are resistant to the antiproliferative effect of TGF-β. Furthermore, mice with homozygous inactivating mutations of Smad3 have been shown to develop colon carcinoma (40), consistent with its role as mediator of TGF-β growth inhibitory responses. Although a role of Smad3 as a tumor suppressor gene is conceivable, no inactivating Smad3 mutations have been observed in human tumors. In the present study, we show that expression of Smad3 can block the degradation of the missense mutation Smad2.Q407R through the ubiquitin-proteasome pathway. At present, the molecular mechanisms whereby Smad3 protect the mutation Smad2.Q407R are unknown. Given the similarity in terms of sequence between Smad2 and Smad3 (over 91% identity), and the observation that Smad2 and Smad3 can form heteromers, one attractive possibility is that Smad3 might associate with Smad2.Q407R, resulting in a decreased accessibility to this mutant by the ubiquitin proteasome system. Regardless of the mechanism, the results outlined in the present study provide the first evidence that Smad3 can overcome at least one genetic defect observed in the related suppressor gene Smad2 during tumor development. We believe that this newly identified function of Smad3 is very interesting, because this mechanism would allow cells to display the antiproliferative
Evidence for a Role of Smad3 and Smad2 in Mutant Smad2.Q407R

REFERENCES

1. Massague, J. (1998) Annu. Rev. Biochem. 67, 753–791
2. Attisano, L., and Wrana, J. L. (2000) Curr. Opin. Cell Biol. 12, 235–243
3. Zimmerman, C. M., and Padgett, R. W. (2000) Gene (Amst.) 249, 17–30
4. Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998) Cell 95, 779–791
5. Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996) Nature 383, 832–836
6. Chen, X., Rubock, M. J., and Whitman, M. (1996) Nature 383, 691–696
7. Chen, X., Weinberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Whitman, M. (1997) Nature 386, 85–89
8. Nakao, A., Afraktibie, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawahata, M., Heldin, C. H., and ten Dijke, P. (1997) Nature 389, 631–635
9. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawahata, M., and Miyazono, K. (1997) Nature 389, 622–626
10. Ebisawa, T., Fukuichi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001) J. Biol. Chem. 276, 12477–12480
11. Kavask, P., Rasmusson, R. K., Causin, C. G., Bonni, S., Zhu, H., Tel, G. H., and Wrana, J. L. (2000) Mol. Cell 6, 1365–1375
12. Wu, R. Y., Zhang, Y., Feng, X. H., and Derynck, R. (1997) Mol. Cell Biol. 17, 2521–2528
13. Hata, A., Lo, R. S., Wotton, D., Lagna, G., and Massague, J. (1997) Nature 388, 82–87
14. Derynck, R., Zhang, Y., and Feng, X. H. (1998) Cell 95, 737–740
15. Liu, X., Sun, Y., Weinberg, R. A., and Lodish, H. F. (2001) Cytokine Growth Factor Rev. 12, 1–8
16. Moustakas, A., Soucchelnytskii, S., and Heldin, C. H. (2001) J. Cell Sci. 114, 4359–4369
17. Faust, N., Laird, A. D., and Webber, E. M. (1995) FASEB J. 9, 1527–1536
18. Grossner, A. M., Lahme, B., Mannherr, H. G., and Polzar, B. (1997) J. Hepatol. 26, 1079–1092
19. Furuta, K., Misao, S., Takahashi, K., Tagaya, T., Fukuwata, Y., Ishikawa, T., Yoshikawa, K., and Kakunuma, S. (1999) Int. J. Cancer 81, 851–853
20. Salvucci, M., Lemoine, A., Safray, R., Azoulay, D., Leper, B., Gaillard, S., Bismuth, H., Reynes, M., and Debuire, B. (1999) Oncogene 18, 181–187
21. Bedossa, P., Peltier, E., Terris, B., Franco, D., and Poynard, T. (1995) Hepatology 21, 760–766
22. Kawate, S., Takenoshita, S., Ohwada, S., Mogi, A., Fukuwata, T., Makita, F., Kusano, H., and Morishita, Y. (1999) Int. J. Oncol. 14, 327–331
23. Yakinic, M. C., Irmak, M. B., Romano, A., Kew, M., and Ozurtk, M. (1999) Oncogene 18, 4879–4883
24. Eppert, K., Scherer, S. W., Oncelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L., Thomsen, G. H., Wrana, J. L., and Attisano, L. (1996) Cell 86, 543–552
25. Le, J., Lau, T. T., Shi, Y. Q., Zhou, X., Smolinski, K. N., Yin, J., Souza, R. F., Appel, R., Wang, S., Cymes, K., Chan, O., Abraham, J. M., Harpur, N., and Meltzer, S. J. (1996) Oncogene 13, 2459–2462
26. MacGregor, D., Pegram, M., Slamov, D., and Bookstein, R. (1997) Oncogene 15, 1111–1114
27. Schutte, M., Hruban, R. H., Hedrick, L., Cho, K. R., Nadasdy, G. M., Weinstein, C. L., Bova, G. S., Isaacs, W. B., Cairns, P., Navroz, H., Sidransky, D., Casero, R. A., Jr., Meltzer, P. S., Hahn, S. A., and Kern, S. E. (1996) Cancer Res. 56, 2527–2530
28. Hata, A., Shi, Y., and Massague, J. (1998) Mol. Med. Today 4, 257–262
29. Attisano, L., Busine, M., Mazars, A., and Gaspach, C. (1997) J. Biol. Chem. 272, 24731–24734
30. Prunier, C., Mazars, A., Nae, V., Brunoel, E., Mareel, M., Gaspach, C., and Attisano, L. (1999) J. Biol. Chem. 274, 22919–22922
31. Wu, J. H., Hu, M., Chai, J., Seane, J., Huse, M., Li, C., Roty, D. J., Kyn, S., Muir, T. W., Fairman, R., Massague, J., and Shi, Y. (2001) Mol. Cell 8, 1277–1289
32. Xu, J., and Attisano, L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4820–4825
33. Maurice, D., Pierreux, C. E., Howell, M., Wilentz, R. E., Owen, M. J., and Hill, C. S. (2001) J. Biol. Chem. 276, 34175–34181
34. Genes, H., Bercovich, B., Orian, A., Carrano, A., Takizawa, C., Yamanaka, K., Pagano, M., Iwai, K., and Cicchonan, A. (1999) J. Biol. Chem. 274, 14823–14830
35. Le, R. S., and Massague, J. (1999) Nat. Cell Biol. 1, 472–478
36. Uchida, K., Nagatake, M., Osada, H., Yatabe, Y., Kondo, M., Miyazaki, T., Masuda, A., and Takahashi, T. (1996) Cancer Res. 56, 5583–5585
37. Prunier, C., Perraud, N., Fournier, B., Pessah, M., and Attisano, L. (2001) Mol. Biol. Cell 21, 3302–3313
38. Datto, M. B., Frederick, J. P., Pan, L., Bortol, A. J., Zhang, Y., and Wang, X. F. (1999) Mol. Cell. Biol. 19, 2485–2504
39. Goto, D., Yagi, K., Inoue, H., Iwamoto, I., Kawabata, M., Miyazono, K., and Kate, M. (1998) FEBS Lett. 430, 201–204
40. Zhu, Y., Richardson, J. A., Parada, L. F., and Graff, J. M. (1998) Cell 94, 763–774
Evidence for a Role of Smad3 and Smad2 in Stabilization of the Tumor-derived Mutant Smad2.Q407R
Emmanuelle Dumont, François Lallemand, Céline Prunier, Nathalie Ferrand, André Guillouzo, Bruno Clément, Azeddine Atfi and Nathalie Théret

J. Biol. Chem. 2003, 278:24881-24887.
doi: 10.1074/jbc.M212496200 originally published online April 16, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212496200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 11 of which can be accessed free at http://www.jbc.org/content/278/27/24881.full.html#ref-list-1