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Loss of Smad4 Function in Pancreatic Tumors

C-TERMINAL TRUNCATION LEADS TO DECREASED STABILITY*

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At early stages of tumorigenesis, the transforming growth factor-β (TGF-β) signaling pathway is thought to have tumor suppressor activity as a result of its ability to arrest the growth of epithelial cells. Smad4 plays a pivotal role in the TGF-β signaling pathway and has been identified as a tumor suppressor, being mutated or deleted in ~50% of pancreatic carcinomas and 15% of colorectal cancers. A nonsense mutation generating a C-terminal truncation of 38 amino acids in the Smad4 protein has been identified in a pancreatic adenocarcinoma (Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996) *Science* 271, 350–353), and here we investigate the functional consequences of this mutation. We demonstrate that the C-terminal truncation prevents Smad4 homomeric complex formation and heteromeric complex formation with activated Smad2. Furthermore, the mutant protein is unable to be recruited to DNA by transcription factors and hence cannot form transcriptionally active DNA-binding complexes. These observations are supported by molecular modeling, which indicates that the truncation removes residues critical for homomeric and heteromeric Smad complex formation. We go on to show that the mutant Smad4 is highly unstable compared with wild type Smad4 and is rapidly degraded through the ubiquitin-proteasome pathway. Consistent with this, we demonstrate that the pancreatic adenocarcinoma harboring this mutated allele, in conjunction with loss of the other allele, expresses no Smad4 protein. Thus we conclude that these tumors completely lack Smad4 activity.

Transforming growth factor-β (TGF-β)[1] ligands require serine-threonine kinase receptors (type II and type I) to signal. The ligand brings the two receptors together in a complex, and the constitutively active type II receptor kinase phosphorylates and activates the type I receptor kinase. The signal is transmitted to the nucleus by the Smads (1). In response to TGF-β or activin, receptor-regulated Smads (R-Smads) Smad2 and Smad3 are phosphorylated and activated by the type I receptor and form complexes (probably trimeric) with a co-Smad (Smad4) (1, 2). The Smad complexes accumulate in the nucleus where they are directly involved in the regulation of transcription of target genes. Smad3-Smad4 complexes bind DNA directly (3), but Smad2-Smad4 complexes require specific transcription factors, *e.g.* Fast-1 or Mixer, to recruit them to specific promoter elements (4, 5).

R-Smads and co-Smads contain two well conserved domains, the N-terminal MH1 domain and the C-terminal MH2 domain, which are separated by a more divergent linker region. The MH1 domain is required for DNA binding in Smad3 and Smad4 and contains a nuclear localization signal (6–10), whereas the MH2 domain mediates many of the biological effects including interaction with other Smad proteins, transcription factors, co-activators, and co-repressors (reviewed in Ref. 11).

Aberrant TGF-β signaling has long been implicated in cancer (12, 13). At early stages TGF-β acts as a tumor suppressor, presumably as a result of its ability to growth arrest epithelial cells from which the majority of tumors are derived. At late stages of tumorigenesis, TGF-β has tumor promoting activities, in particular causing an epithelial to mesenchymal transition in tumor cells and enhancing invasiveness and malignancy (12, 13). Although many tumor-derived cell lines are resistant to the anti-proliferative effects of TGF-β, only in pancreatic and colorectal cancers is it clear that this is caused by inactivating mutations in components of the TGF-β signaling pathway (12, 14, 15). The major components to be affected are Smad4 and the type II receptor. Mutations or deletions in *Smad4* have been found in about half of pancreatic cancers and in about 15% of colorectal cancers (16–18). Germline mutations in *Smad4* have also been reported in juvenile polyposis families, which predispose the affected individuals to hamartomatous polyps and gastrointestinal cancer (19–21). Mutations in the type II receptor are estimated to occur in about 28% of colon adenocarcinomas, usually associated with defects in DNA mismatch repair (22).

Smad4 (also called *DPC4*, for deleted in pancreatic carcinoma locus 4) has the characteristics of a classical tumor suppressor gene (23). About half of pancreatic cancers contain either homozygous deletions of Smad4 or inactivating mutations in one allele associated with loss of the other allele (loss of

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[1] The abbreviations used are: TGF-β, transforming growth factor β; MH1, Mad homology domain 1; MH2, Mad homology domain 2; ARE, activin-responsive element; DE, distal element; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; ARF, activin responsive factor; PCNA, proliferating cell nuclear antigen.

heterozygosity) (23). The majority of the inactivating mutations, which are usually missense or nonsense mutations occur in the MH2 domain, although several have been identified in the MH1 domain and in the linker region (14, 24–26). Many mutations in the MH2 domain have been shown to prevent homo- and/or hetero-oligomerization with other Smads (26, 27), whereas those in the MH1 domain were thought to increase autoinhibition of the Smads by stabilizing intramolecular interactions between the MH1 and MH2 domains (25). Recently, however, mutations in the MH1 domain have also been shown to target the proteins for degradation via the ubiquitin-proteasome pathway (28).

A nonsense mutation at residue 515 of one allele of Smad4 accompanied by loss of heterozygosity in the other has been identified in a pancreatic adenocarcinoma (23). The mutation generates a protein (Smad4 ΔC) that has a deletion of 38 amino acids at the C terminus. To understand how this naturally occurring mutation in Smad4 might contribute to tumor formation, we have analyzed its effect on Smad4 function. Using both *in vitro* and *in vivo* model systems, we demonstrate that Smad4 ΔC is unable to form homo or hetero-oligomeric interactions with other Smads in response to ligand. Furthermore, Smad4 ΔC is unable to be incorporated into a transcriptionally active DNA-binding complex with the transcription factors Fast-1 or Mixer. Most importantly, we show that Smad4 ΔC is a highly unstable protein that is rapidly degraded via the ubiquitin-proteasome pathway. We go on to demonstrate that the pancreatic adenocarcinoma harboring this mutated Smad4 allele, associated with loss of the other allele, expresses no Smad4 protein, consistent with this truncated Smad4 being highly unstable *in vivo*. Our findings contrast with previous data that concluded that this mutant Smad4 had dominant negative activity (29, 30). However, our data provide an explanation for at least some of the apparent dominant negative activity of this mutant, because we demonstrate that in certain conditions, the mutant Smad4 can target co-expressed Smad2 for degradation. These results provide important new insights into the mechanism of inactivation of the tumor suppressor Smad4 in cancer.

MATERIALS AND METHODS

Plasmids—The following plasmids have been described previously: Mixer, Fast-1, XSmad2, hSmad4 and ALK5 (TD) in FLAG- and HA-containing EF expression vectors (5, 31), ARE- and DE-driven luciferase reporter genes (8), XSmad2 in pFTX5 (31), and EF-lacZ (32). Human Smad4 was subcloned into pFTX5. The mutant hSmad4, Smad4ΔC was constructed by polymerase chain reaction and subcloned into FLAG- and HA-containing EF expression vectors and pFTX5.

Cell Culture and Transfections—NIH-3T3 and MDA-MB-468 cells were maintained in DMEM containing 10% fetal calf serum. NIH-3T3 cells were transfected using LipofectAMINE (Life Technologies, Inc.), and MDA-MB-468 cells were transfected using Superfect reagent (QIAgen).

Transcriptional Activation Assays, Immunoprecipitation / Western Blotting, and Bandshift Assays—Transcription assays and immunoprecipitation assays followed by Western blotting were performed as described previously (8). The antibodies used for immunoprecipitations were either anti-FLAG-coupled M2 agarose affinity gel (Sigma) or anti-HA rabbit polyclonal antibody (Roche Diagnostics) coupled to protein A beads (Amersham Pharmacia Biotech). The following antibodies were used for Western blotting: anti-HA (as above); anti-Smad4 (B8, Santa Cruz); anti-Smad2/Smad3 (Transduction Laboratories); anti-FLAG (M2, Sigma); anti-Myc (9E10, (33)); anti-PCNA (34). Bandshift assays using the ARE probe were as described previously (5). The antibodies used for the supershifts were against HA or Myc as described above.

Protein Stability Assays—To measure the rate of degradation of Smad4 and Smad4 ΔC, HA-tagged versions were transfected into NIH-3T3 cells that were treated with 20 μg/ml cycloheximide 48 h after transfection to prevent further protein synthesis in the presence or absence of the proteasome inhibitor, MG-132 (Calbiochem; final concentration, 50 μM). Whole cell extracts were prepared from samples taken at different time points, and the amounts of the two Smad4s were determined by Western blotting using the anti-HA antibody. For the pulse-chase experiment, NIH-3T3 cells were transfected with FLAG-tagged versions of Smad4 and Smad4 ΔC. 24 h after transfection, the cells were incubated for 1 h in methionine-minus DMEM/1% dialyzed fetal calf serum and then pulsed for 30 min with 250 μCi/ml [^{35}S]methionine in the same medium. The cells were then washed three times with DMEM and incubated in DMEM with 1% fetal calf serum in the presence or absence of lactacystin (Calbiochem; final concentration, 30 μM) for the times indicated in Fig. 4. Whole cell extracts were prepared, and the FLAG-tagged proteins were immunoprecipitated as described previously (8).

Embryo Manipulations and RNase Protection Assays—The production, maintenance, and manipulation of *Xenopus* embryos was described previously (31). mRNA for microinjection was generated *in vitro* (31) and injected at the single-cell stage: 1 ng of mRNA encoding Myc-hSmad4, 1 ng of Myc-XSmad2, and 1.5 ng of Myc-Smad4 ΔC. RNA isolation and RNase protection assays were performed as described (31). Antisense probes for *EF-1α*, *Xbra*, *Mix.2*, and *XFKH1* were as described (34).

Immunohistochemistry—Unstained 5-mm sections were cut from a paraffin block containing duct adenocarcinoma and normal pancreatic parenchyma (specimen PX101) (23). The slides were deparaffinized and labeled with a monoclonal antibody against Smad4 (B8, Santa Cruz) exactly as described previously (35). The sections were counterstained with hematoxylin.

RESULTS

Smad4 ΔC Does Not Associate with Endogenous Smad4 or Activated Smad2—In response to TGF-β stimulation, complexes containing activated Smad2 and Smad4 accumulate in the nucleus (1). The interaction of Smad4 with other Smads is mediated through its MH2 domain (1). We first tested whether the mutant Smad4 deleted in the C-terminal 38 amino acids (Smad4 ΔC) could form homomeric Smad4 complexes (36) or heteromeric complexes with activated Smad2.

HA-tagged Smad4 ΔC or Smad2 (as a positive control) was transfected into NIH-3T3 cells (which contain endogenous Smad4 and Smad2), in the presence or absence of the constitutively active TGF-β receptor ALK-5 (TD) to mimic TGF-β signaling (37) (Fig. 1*A*). Smad4 ΔC or Smad2 were immunoprecipitated with the anti-HA antibody, and the immunoprecipitates were blotted with an anti-Smad4 antibody recognizing both endogenous Smad4 and Smad4 ΔC. Smad4 ΔC did not associate with endogenous Smad4 in the presence or absence of TGF-β signaling; Smad2, however, readily associated with endogenous Smad4 upon TGF-β signaling (Fig. 1*A*, *top panel*). The expression of all the proteins was confirmed by Western blotting of whole cell extracts with anti-Smad4 and anti-HA antibodies (*middle* and *bottom panels*).

We then determined whether Smad4 ΔC could associate with Smad2 in a signal-dependent manner. Smad4 and Smad4 ΔC were immunoprecipitated with the anti-FLAG antibody, and the immunoprecipitates were blotted with an anti-HA antibody to detect co-precipitating Smad2 in the presence or absence of constitutively active TGF-β receptor ALK-5 (TD) (Fig. 1*B*, *top panel*). In contrast to Smad4, Smad4 ΔC did not interact with Smad2 in a signal-dependent manner. A control experiment indicated, however, that Smad4 ΔC was efficiently immunoprecipitated (*middle panel*). These results indicate that the C-terminal 38 amino acids of Smad4 are absolutely required for the formation of Smad4-Smad2 heteromeric and Smad4-Smad4 homomeric complexes.

Smad4 ΔC Is Not Incorporated into the DNA-bound ARF Complex—Activated Smad2-Smad4 complexes are predominantly recruited to promoter elements through their interaction with other transcription factors (1, 38, 39). The best characterized is the *Xenopus* winged helix/forkhead transcription factor, Fast-1, which forms an activin-induced complex with Smad2 and Smad4 called ARF at the ARE of the *Xenopus* Mix.2 promoter (4, 40, 41).

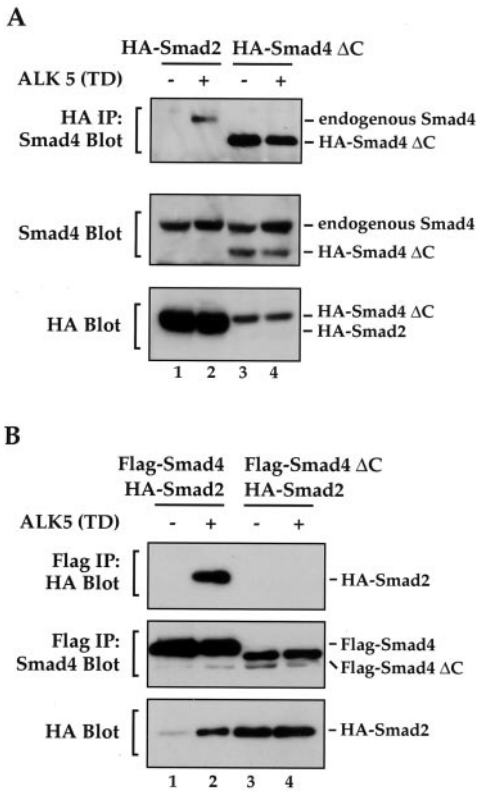

FIG. 1. **Loss of the 38 C-terminal residues of Smad4 prevents formation of homomeric complexes with endogenous Smad4 or heteromeric complexes with co-expressed Smad2.** *A*, NIH-3T3 cells were transfected with plasmids expressing HA-Smad2 or HA-Smad4 ΔC with or without the constitutively active type I receptor ALK5 (*ALK5 (TD)*). Extracts were assayed by immunoprecipitation (*IP*) of complexes with anti-HA antibody followed by Western blotting with anti-Smad4 antibody recognizing both endogenous Smad4 and mutant Smad4 ΔC (*top panel*) or by Western blotting whole cell extracts with antibodies against Smad4 (*middle panel*) or HA (*bottom panel*). *B*, extracts were prepared from NIH-3T3 cells transfected with plasmids expressing either FLAG-Smad4 and HA-Smad2 or FLAG-Smad4 ΔC and HA-Smad2, with or without ALK5 (TD), as indicated. Extracts were assayed by immunoprecipitation of complexes with anti-FLAG antibody followed by Western blotting with anti-HA antibody (*top panel*) or Western blotting with anti-Smad4 antibody (*middle panel*). As a control for HA-Smad2 expression, whole cell extracts were Western blotted with an anti-HA antibody (*bottom panel*).

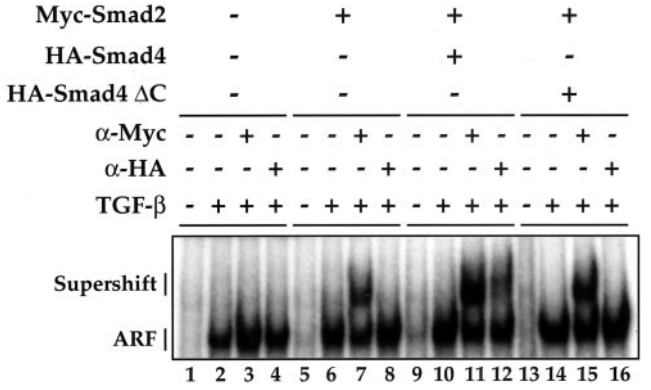

FIG. 2. **Smad4 ΔC does not interact with Smad2 and Fast-1 in the ARF complex on the ARE.** Whole cell extracts were prepared from NIH-3T3 cells transfected with FLAG-Fast-1 and Myc-Smad2, HA-Smad4, or HA-Smad4 ΔC, as indicated, that were either untreated or stimulated with TGF-β1 (2 ng/ml) for 1 h. The extracts were analyzed by bandshift using the radiolabeled ARE as a probe in the presence or absence of anti-HA or anti-Myc antibodies as indicated. The Fast-1-Smad2-Smad4 complex, ARF is indicated, as is antibody supershifted ARF.

Smad4 ΔC cannot directly hetero-oligomerize with Smad2. However, it remained possible that it might still be recruited into the ARF complex with Fast-1 on the ARE, because this complex is stabilized not only by interactions between the Smad2 and Smad4 MH2 domains but also by the binding of the Smad4 MH1 domain to DNA (9, 42). We therefore investigated whether Smad4 ΔC could be incorporated into the ARE-bound ARF complex upon TGF-β signaling. Extracts were prepared from NIH-3T3 cells transfected with Fast-1, Smad2 and Smad4, or Smad4 ΔC; the cells were either unstimulated or stimulated for 1 h with TGF-β.

An ARF complex containing endogenous Smad2 and Smad4 was detected upon TGF-β stimulation when Fast-1 was expressed (Fig. 2, *lanes 1–4*). When Myc-tagged Smad2 was overexpressed, Myc-tagged Smad2 clearly replaced some of the endogenous Smad2, because the ARF complex formed in these conditions could be partially supershifted with the anti-Myc antibody (*lane 7*). When both Smad2 and Smad4 were overexpressed, both proteins were detected in the ARF complex by antibody supershifts (*lanes 11* and *12*). In contrast, when Smad4 ΔC and Smad2 were overexpressed, although Smad2 was clearly incorporated into the complex (*lane 15*), Smad4 ΔC was not, as demonstrated by the lack of a supershift with the anti-HA antibody (*lane 16*). Thus Smad4 ΔC cannot be incorporated into the ARF complex upon TGF-β stimulation.

Smad4 ΔC Has No Intrinsic Transcriptional Activity and Does Not Interfere with Wild Type Smad4 Activity in Transcriptional Activation Assays—Previous work suggested that this truncated Smad4 mutant had dominant negative activity in certain situations (29, 30). We investigated this further by testing its ability to interfere with Smad4-mediated TGF-β-responsive transcription. We also assessed its ability to mediate TGF-β-responsive transcription itself. The cell line MDA-MB-468 was used, which responds to TGF-β and contains Smad2 but lacks Smad4 (24). The experiments could therefore be performed in the presence or absence of wild type Smad4. Two different TGF-β-responsive reporter genes were used. The ARE-luciferase reporter is driven by three AREs and requires Fast-1 to recruit active Smad2-Smad4 complexes to DNA (see above) (4, 9). The DE-luciferase reporter is driven by four DEs from the *Xenopus goosecoid* promoter. The DE binds the paired-like homeodomain protein Mixer, which recruits the Smad2-Smad4 complexes to DNA (5).

Both reporters are TGF-β-inducible in MDA-MB-468 cells when the appropriate transcription factor is co-expressed, as previously demonstrated (Fig. 3) (8). Transfection of increasing amounts of Smad4 ΔC had no effect on either the basal or induced level of either reporter gene (Fig. 3). In contrast, transfection of wild type Smad4 increased the induced level of both reporters and, in the case of DE-luciferase, also increased the uninduced level. However, Smad4 ΔC had no inhibitory activity on the Smad4-dependent transcription, when it was co-transfected in increasing amounts (up to 10-fold excess over wild type Smad4). These data indicate that Smad4 ΔC is not functional in transcription assays and does not act as a dominant negative in model tissue culture systems.

Smad4 ΔC Is a Highly Unstable Protein That Is Rapidly Degraded via the Proteasome Pathway—The observed low level of expression of Smad4 ΔC relative to wild type Smad4 (Fig. 1) suggested that the C-terminal truncation might lead to an increased turnover of the protein. To investigate this possibility, NIH-3T3 cells were transfected with both wild type Smad4 and Smad4 ΔC. Forty-eight hours after transfection, cycloheximide (20 μg/ml) was added to prevent further Smad synthesis. Whole cell extracts were prepared from the cells at different

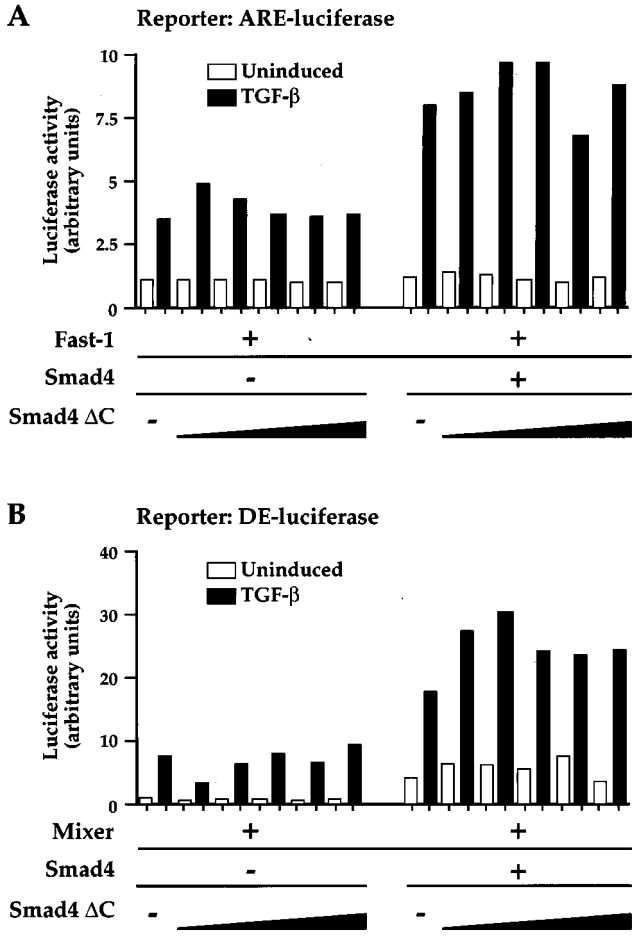

FIG. 3. **Smad4 ΔC has no dominant negative effect on Smad-mediated transcription from ARE or DE-driven reporter genes.** MDA-MB-468 cells were transiently co-transfected with ARE- or DE-luciferase reporters, together with plasmids expressing transcription factors Fast-1 or Mixer, Smad4, and Smad4 ΔC as indicated. The ratios of Smad4 ΔC to Smad4 were 0.5:1, 1;1, 2.5:1, 5:1, and 10:1. The cells were cultured with or without TGF-β1 (2 ng/ml) for 6 h. The cells were harvested, and luciferase activity was measured relative to β-Gal activity from the internal control. A representative experiment from at least three independent experiments giving similar results is shown. The data were normalized by setting the basal level in the absence of TGF-β to 1.

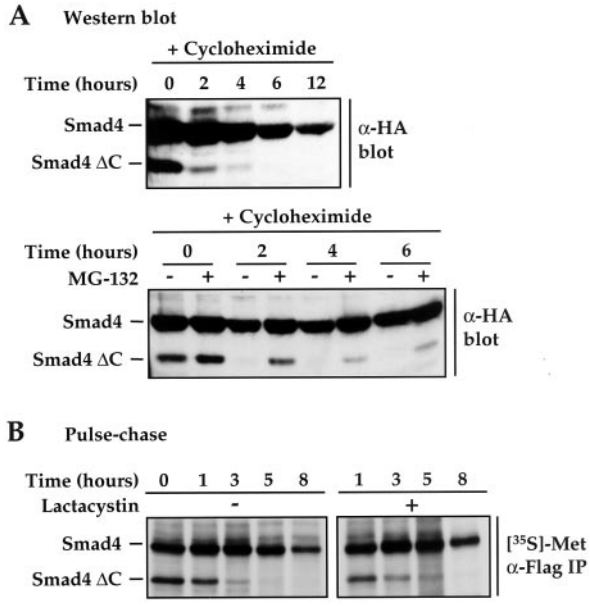

FIG. 4. **Smad4 ΔC is highly unstable and rapidly degraded via the ubiquitin-proteasome pathway.** *A*, *upper panel*, NIH-3T3 cells were co-transfected with HA-Smad4 and HA-Smad4 ΔC. After 48 h cycloheximide (final concentration, 20 μg/ml) was added to the culture to prevent any further Smad synthesis. Whole cell extracts were prepared at different time points and assayed by Western blotting with an anti-HA antibody recognizing both the full-length and truncated Smad4 proteins. *Lower panel*, NIH-3T3 cells were transfected and treated as above, except that the inhibitor of the 26S proteasome subunit, MG-132 (final concentration, 50 μM) was added at the same time as cycloheximide as indicated. Whole cell extracts were assayed by Western blotting using an anti-HA antibody. *B*, NIH-3T3 cells were co-transfected with FLAG-tagged Smad4 and Smad4 ΔC. After 24 h, the cells were pulsed with [^{35}S]methionine for 30 min and then chased with unlabeled methionine for the times shown, in the presence or absence of 30 μM lactacystin. Whole cell extracts were immunoprecipitated and analyzed by SDS gel electrophoresis and autoradiography.

times after cycloheximide addition, and the Smad4s were visualized by Western blotting.

The level of Smad4 ΔC rapidly decreased after the addition of cycloheximide, and the protein disappeared after 4 h (Fig. 4*A*, *upper panel*). In contrast, full-length Smad4 was considerably more stable and was still present 12 h after cycloheximide addition (Fig. 4*A*, *upper panel*). To determine whether Smad4 ΔC is targeted for degradation via the ubiquitin-proteasome pathway (43), we examined the effect of MG-132, a highly specific inhibitor of the 26S proteasome complex, on the stability of Smad4 ΔC. MG-132 treatment of NIH-3T3 cells co-transfected with wild type Smad4 and Smad4 ΔC decreased the amount of Smad4 ΔC degradation at each time point compared with untreated cells (Fig. 4*A*, *lower panel*), with the effects being more pronounced at the early time points.

These results were confirmed by a pulse-chase experiment. FLAG-tagged Smad4 and Smad4 ΔC were co-transfected into NIH-3T3 cells that were pulsed for 30 min with [^{35}S]methionine and chased with unlabeled methionine in the presence or absence of another specific inhibitor of the 26 S proteasome complex, lactacystin (Fig. 4*B*). Again it is clear that Smad4ΔC is much more unstable than wild type Smad4. Smad4 ΔC is virtually undetectable 3 h after the [^{35}S]methionine pulse, whereas wild type Smad4 levels are undiminished. The presence of lactacystin clearly stabilizes Smad4 ΔC at the 3- and 5-h time points (Fig. 4*B*). Thus Smad4 ΔC is an unstable protein that is rapidly degraded via the ubiquitin-proteasome pathway.

Smad4 ΔC Inhibits Smad2-mediated Induction of Mesodermal Genes—One of the systems in which Smad4 ΔC has been shown to have dominant negative activity is the early *Xenopus* embryo (29). During early embryogenesis, activin-related ligands induce mesodermal gene expression via activated complexes of Smad2 and Smad4β, a novel *Xenopus* Smad4 (44). This process can be recapitulated in animal cap explants from stage 8 embryos, either by treating the caps with activin for 6 h or by overexpressing Smad2 and/or Smad4 (34, 45, 46). It was previously reported that Smad4 ΔC overexpression could completely inhibit mesodermal gene expression activated by overexpression of Smad2 and weakly inhibit activin-induced mesodermal gene expression (29). Using the same experimental system, we investigated this in more detail.

In vitro synthetized mRNAs encoding Smad2, Smad4, or Smad4 ΔC were injected into single-cell embryos. Animal cap explants from injected and uninjected embryos were dissected at stage 8 and cultured in the absence or presence of activin for 6 h until control embryos had reached stage 10.5. For each condition, 25 explants were used, and the transcriptional activation of mesodermal markers was monitored by RNase protection using probes against *Mix.2, Xbra*, *XFKH1*, and *EF-1α* as an internal control for equal loading (34).

The transcription of all three genes was induced by activin in

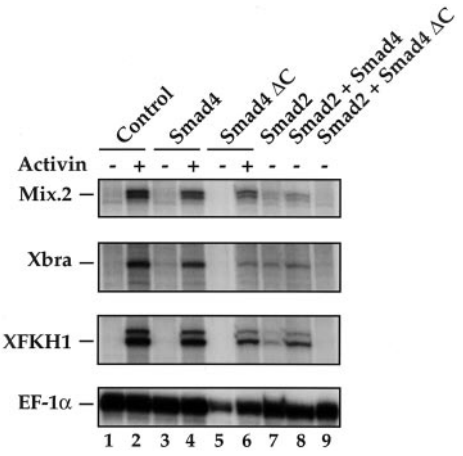

FIG. 5. **Smad4 ΔC inhibits Smad2-induced mesodermal gene expression but not activin-induced gene expression.** Single-cell *Xenopus* embryos were injected with mRNA encoding Myc-Smad4 (1 ng), Myc-Smad4 ΔC (1.5 ng), or Myc-Smad2 (1 ng) or combinations as indicated and cultured to stage 8. Animal cap explants were dissected and incubated in the absence or presence of activin 40 ng/ml until control embryos had reached stage 10.5. Total RNA from the animal cap explants was assayed by RNase protection for the transcription of *Mix.2*, *Xbra*, *XFKH1*, and *EF-1α*.

uninjected explants (Fig. 5, *lane 2*), and the activin-induced transcription was not significantly affected by overexpression of Smad4 or Smad4 ΔC (*lanes 4* and *6*). Overexpression of Smad2 induced low levels of *Mix.2*, *Xbra*, and *XFKH1* (*lane 7*) (34) and wild type Smad4 potentiated Smad2-induced transcription (*lane 8*). In contrast, and consistent with previous results (29), Smad2-induced activation of *Mix.2*, *Xbra*, and *XFKH1* was completely inhibited by overexpression of Smad4 ΔC (*lane 9*).

In the Animal Cap Assay, Overexpressed Smad4 ΔC Targets Exogenous Smad2 for Degradation—To better understand why Smad4 ΔC has an apparent dominant negative activity in *Xenopus* animal cap explants, we investigated the steady-state levels of the overexpressed Smads during *Xenopus* development in the same batches of injected embryos from which animal caps were dissected. The Myc-tagged Smads were visualized by Western blotting whole embryo extracts using a specific anti-Myc antibody (Fig. 6, *top panel*). Equal loading of protein extracts was confirmed by blotting with an antibody directed against the nuclear antigen, PCNA (*middle panel*).

When expressed individually, Smad4 ΔC levels were comparable with those of Smad4 and Smad2 at the 64 cell stage and stage 8 (the stage at which the animal caps were dissected) (*lanes 2–4* and *8–10*), whereas at stage 10.5, Smad4 ΔC was clearly present at a lower level (*lanes 14–16*). This presumably reflects the inherent instability of Smad4 ΔC. Most striking though, was the behavior of Smad4 ΔC when overexpressed with Smad2 (*lanes 6*, *12*, and *18*). When Smad4 ΔC was overexpressed with Smad2, both proteins were degraded. This was most obvious at stage 10.5 when no Smad4 ΔC and only relatively low levels of Myc-tagged Smad2 can be detected (*lane 18*). Wild type Smad4 did not have this effect. Its level of expression was the same whether it was expressed alone or with Smad2 (compare *lanes 2*, *5*, *8*, *11*, *14*, and *17*). It is also clear that Smad4 ΔC only targets overexpressed Smad2 for degradation. It had no effect on the levels of endogenous Smad2 (*bottom panel*, *lower band*, compare *lanes 1*, *3*, *7*, *9*, *13*, and *15*).

These data indicate that overexpressed Smad4 ΔC targets overexpressed Smad2 for destruction but has no effect on levels of endogenous Smad2. This explains why Smad4 ΔC inhibits Smad2-mediated mesodermal gene expression while having no effect on activin-induced mesodermal gene expression mediated by endogenous Smad2.

The Pancreatic Adenocarcinoma with a Nonsense Mutation at Codon 515 in One Allele of Smad4 Associated with Loss of the Other Allele Expresses No Smad4 Protein—We have demonstrated that Smad4 ΔC has no dominant negative activity in normal situations. Moreover, we have shown that it is a highly unstable protein. To prove that its instability is physiologically relevant, we asked whether this Smad4 mutant was unstable in the pancreatic adenocarcinoma (PX101) where it was first identified (23, 35). In this adenocarcinoma, one Smad4 allele contains a nonsense mutation at codon 515, giving rise to Smad4 ΔC, and there is loss of the other allele. A representative hematoxylin and eosin-stained section of the invasive pancreatic adenocarcinoma is shown (Fig. 7*A*). There are multiple irregular ducts (*black arrows*) with atypical nuclei and increased mitoses infiltrating the stroma. When a section of the same pancreatic adenocarcinoma was stained for the Smad4 protein, it is clear that the infiltrating adenocarcinoma, characterized by enlarged and irregular nuclei (blue counter stain), does not express any Smad4 protein (Fig. 7*B*) (35). This strongly suggests that the Smad4 ΔC protein is very unstable in the tumor cells and therefore does not accumulate. The normal islets of Langerhans (*red arrowheads*) and the normal stromal fibroblasts (*red arrows*) that have wild type *Smad4* stain strongly for Smad4 protein (*brown stain*), which provides a useful internal positive control. Thus we can demonstrate that not only is the truncated Smad4 protein (Smad4 ΔC) unstable when expressed in tissue culture cells, it is evidently also unstable *in vivo* in the pancreatic adenocarcinoma where the mutation was identified.

DISCUSSION

Smad4 ΔC Is a Nonfunctional Protein That Is Rapidly Degraded through the Ubiquitin-Proteasome Pathway—In this study, we have analyzed the effects of a somatic mutation of the Smad4 protein identified in a pancreatic adenocarcinoma. Our analysis of the mechanism of inactivation of the mutated Smad4 protein has identified two distinct defects. Firstly, we have shown that Smad4 ΔC is highly unstable and is targeted for degradation via the proteasome. This is demonstrated directly when the protein is overexpressed in tissue culture cells and inferred from the lack of Smad4 staining in the pancreatic adenocarcinoma. Secondly, we have shown that even when expressed at high levels in the cell, it is nonfunctional, because it is unable to form homomeric or heteromeric complexes with other Smads and is unable to be incorporated into transcriptionally active complexes on DNA with Fast-1 or Mixer.

Both of these observations are easily rationalized by analysis of the crystal structure of the Smad4 MH2 domain (26, 47). Deletion of the C-terminal 38 amino acids of Smad4 results in removal of α-helix 5 and β-strand 11 and half of the L3 loop (Fig. 8) (26). Loss of the β-strand 11 is likely to severely disrupt the overall structure of the Smad4 MH2 domain, because it is part of a β-sandwich that makes up the hydrophobic core of the molecule (26) leading to an unstable protein, as we observe. Interestingly, a point mutation in Smad2 in β-strand 11 (L440R) that destabilizes the protein by disrupting the packing of the hydrophobic core through the replacement of a critical hydrophobic residue with a charged residue (26) also results in a highly unstable protein that is ubiquitinated and rapidly degraded (27, 28).

The 38-amino acid truncation of Smad4 additionally results in loss of α-helix 5 (Fig. 8), which is predicted to have two important consequences. It will disrupt the three-helix bundle comprising α-helices 3, 4, and 5, leading to destabilizing of the overall structure of the Smad4 MH2 domain. Moreover, it will

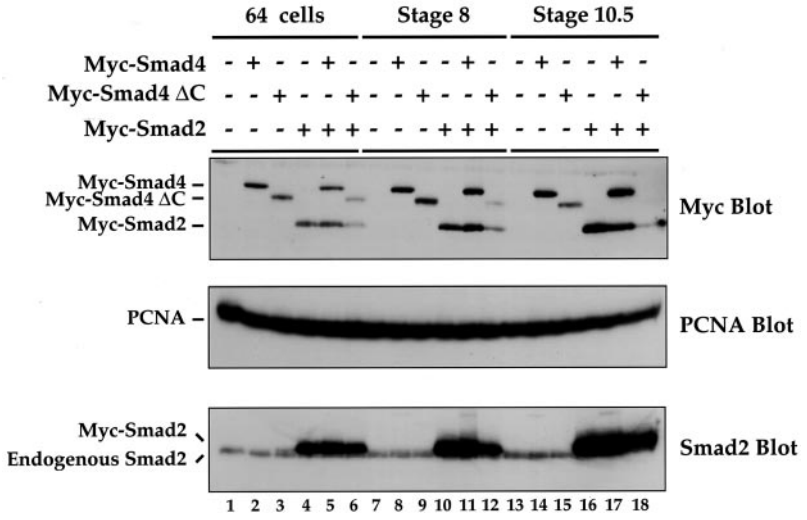

FIG. 6. **Smad4 ΔC targets overexpressed Smad2 for destruction.** Protein extracts derived from whole embryos injected with synthetic mRNAs as in Fig. 5 were prepared at three different time points of *Xenopus* development as indicated. Extracts were assayed by Western blotting using an anti-Myc antibody (*top panel*). The same blot was stripped and reprobed with a specific monoclonal antibody directed against Smad2 (*bottom panel*). Note that the level of exogenous Myc-tagged Smad2 is much higher than endogenous Smad2. Equal loading of the extracts was assessed by Western blotting with an antibody against PCNA (*middle panel*).

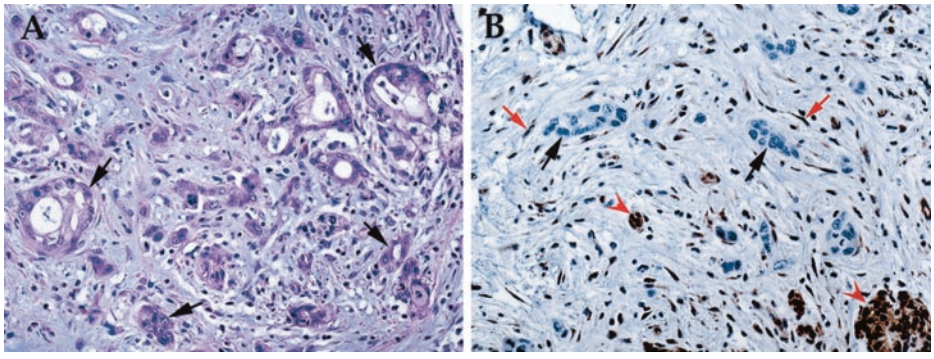

FIG. 7. **A pancreatic adenocarcinoma with a nonsense mutation at codon 515 in one allele of Smad4 and loss of the other allele expresses no Smad4 protein.** *A*, a representative hematoxylin and eosin-stained section of the invasive pancreatic adenocarcinoma (PX101) (23). There are multiple irregular ducts (*black arrows*) with atypical nuclei and increased mitoses infiltrating the stroma. *B*, a section of the same pancreatic adenocarcinoma stained for the Smad4 protein. This section shows invasive duct adenocarcinoma of the pancreas (*black arrows*) infiltrating among benign islets of Langerhans (*red arrowheads*). The benign islets, as well as the background benign stromal cells (*red arrows*), stain strongly for the Smad4 protein (*brown stain*). The infiltrating adenocarcinoma, evidenced by enlarged and irregular nuclei (*blue counter stain*), does not express Smad4 protein.

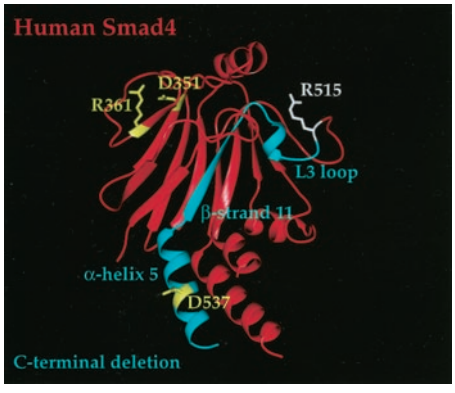

FIG. 8. **The structure of Smad4 indicating the region deleted in the 38-amino acid C-terminal truncation.** The crystal structure of amino acids 319–552 of Smad4 (26) in a ribbon representation with key elements labeled. The region colored *blue* is the region deleted in Smad4 ΔC. The residues colored *yellow* are those at the Smad trimer interface involved in Smad-Smad interactions (26,47). The residue colored *white* (Arg^{515}) has been implicated in binding the phosphorylated C terminus of R-Smads in Smad heterotrimeric complexes (2).

prevent homo- and heteromeric Smad complex formation, because residues in α-helix 5 are responsible for contacting other Smad subunits in homo- and hetero-Smad trimers (2, 26, 48). In particular, Asp^{537} in α-helix 5 contributes to a network of hydrogen bonds involving Asp^{351} and Arg^{361} in another Smad4 subunit of the Smad4 homotrimer (26, 48). Because these three residues are invariant among all Smads, the same hydrogen bonding network is thought to be critical for stabilization of R-Smad/co-Smad heterotrimers (48).

Finally, the 38-amino acid truncation of Smad4 results in the loss of approximately half of the L3 loop, which is also important for formation of heterotrimeric Smad complexes (Fig. 8). Arg^{515} in the L3 loop of Smad4 is essential for formation of trimeric complexes with activated R-Smads, thought to be through a direct electrostatic interaction between the phosphorylated C terminus of the R-Smad and the positively charged arginine residue (2). This critical residue is deleted in Smad4 ΔC (Fig. 8). The involvement of the L3 loop in formation of hetero-oligomeric Smad complexes has also been demonstrated by the fact that a mutation in *Drosophila Smad4* (*Medea*) that converts Gly^{727} to aspartate (equivalent to Gly^{508} in Smad4) prevents formation of complexes between Medea and Mad (49).

Smad4 ΔC Has Apparent Dominant Negative Activity Only under Certain Conditions—Previous reports suggested that the truncated Smad4 mutant had dominant negative activity (29, 30). We have shown that this is the case, but only under certain conditions. In *Xenopus* animal cap explants, overexpressed Smad4 ΔC can target co-expressed Smad2 for degradation, which results in a complete inhibition of Smad2-activated mesodermal gene expression. Because we demonstrate that overexpression of Smad4 ΔC has no effect on the levels of endogenous Smad2 and consequently cannot inhibit activin-induced mesodermal gene expression, we conclude that the apparent

dominant negative effect on Smad2-mediated gene expression is a consequence of greatly overexpressing Smad2 and Smad4 ΔC together. We surmise that in this artificial situation, a transient complex is formed between the two overexpressed proteins that is targeted for degradation via the Smad4 ΔC component. The apparent dominant negative effect of Smad4 ΔC is only observed in *Xenopus* embryos; we do not observe the same effect of overexpressed Smad4 ΔC on levels of co-expressed Smad2 in tissue culture cells. This may be due to differences in the way that the experiments are conducted. In tissue culture cells, plasmid DNA is transfected, and thus in the course of the experiments, the DNA will be continually transcribed and the mRNA translated, and the pools of Smad2 and Smad4 ΔC protein will be constantly renewed. In the *Xenopus* embryo experiments, however, synthetic mRNA is injected and efficiently translated. However, the mRNA has only a limited half-life in the embryo, and thus, once the pools of Smad2 and Smad4 ΔC have been targeted for degradation, they will not be effectively renewed.

Inactivation of Smad4 in Human Cancer—Our data give us new insights into the mechanism of inactivation of Smad4 in human cancer. Although Smad4 can be mutated in such a way that it has dominant negative activity, for instance by deleting its Smad activation domain (50), no naturally occurring Smad4 mutants characterized so far have dominant negative activity. This is supported by the observation that all mutations are biallelic. Smad4 inactivation occurs either by homologous deletion or by the acquisition of inactivating mutations in one allele, associated with deletion of the other allele (23, 51). The mutations have three possible consequences. They give rise to stable proteins that are unable to form homo- or heterotrimeric Smad complexes (*e.g.* D351H, R361C, V370D, and D537E) (26, 48), they promote autoinhibition and also affect protein stability (*e.g.* R100T) (25, 28), or, as we have shown in this work, they affect protein stability and Smad complex formation (R515X). We have gone on to demonstrate that in the pancreatic adenocarcinoma that contains this nonsense mutation at codon 515 in one allele in conjunction with loss of heterozygosity at the other allele, no Smad4 protein is detected. This indicates that any truncated protein that is expressed must be very rapidly degraded. Interestingly, it is becoming apparent that the instability of truncated Smad4 proteins is a general phenomenon in tumor cells harboring mutations in *Smad4*. In several different colorectal cell lines that contain mutations in *Smad4* that would encode truncated proteins, no Smad4 is detected by Western blotting, indicating that truncated Smad4s are highly unstable (51).

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Loss of Smad4 Function in Pancreatic Tumors: C-TERMINAL TRUNCATION LEADS TO DECREASED STABILITY

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