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The Integral Inner Nuclear Membrane Protein MAN1 Physically Interacts with the R-Smad Proteins to Repress Signaling by the Transforming Growth Factor-β Superfamily of Cytokines*

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Smad proteins are critical intracellular mediators of the transforming growth factor-β, bone morphogenic proteins (BMPs), and activin signaling. Upon ligand binding, the receptor-associated R-Smads are phosphorylated by the active type I receptor serine/threonine kinases. The phosphorylated R-Smads then form heteromeric complexes with Smad4, translocate into the nucleus, and interact with various transcription factors to regulate the expression of downstream genes. Interaction of Smad proteins with cellular partners in the cytoplasm and nucleus is a critical mechanism by which the activities and expression of the Smad proteins are modulated. Here we report a novel step of regulation of the R-Smad function at the inner nuclear membrane through a physical interaction between the integral inner nuclear membrane protein MAN1 and R-Smads. MAN1, through the RNA recognition motif, associates with R-Smads but not Smad4 at the inner nuclear membrane in a ligand-independent manner. Overexpression of MAN1 results in inhibition of R-Smad phosphorylation, heterodimerization with Smad4 and nuclear translocation, and repression of transcriptional activation of the TGFβ, BMP2, and activin-responsive promoters. This repression of TGFβ, BMP2, and activin signaling is dependent on the MAN1-Smad interaction because a point mutation that disrupts this interaction abolishes the transcriptional repression by MAN1. Thus, MAN1 represents a new class of R-Smad regulators and defines a previously unrecognized regulatory step at the nuclear periphery.

The transforming growth factor-β (TGFβ)[1] superfamily of cytokines, including TGFβs, BMPs, and activins, plays important roles in the regulation of various aspects of mammalian embryogenesis and carcinogenesis. The signals initiated by these cytokines are transduced by their receptors and the downstream Smad proteins (1–5). Upon ligand binding, activin or TGFβ receptor serine/threonine kinase phosphorylates and activates the type I receptor kinase, which then phosphorylates the downstream Smad proteins. The Smad proteins are critical mediators of TGFβ superfamily signaling. Upon phosphorylation by the activated type I receptor kinases, the receptor-associated R-Smads (Smad2 and Smad3 for TGFβ and activin; Smad1, Smad5, and Smad8 for BMPs) oligomerize with the common mediator Smad4, translocate into the nucleus where they interact with various transcription factors, bind to DNA, and regulate transcription of downstream genes.

The expression and activity of the Smad proteins can be modulated by interaction with various cellular proteins at the plasma membrane or in the cytoplasm and nucleus (2, 6, 7). For example, Smad proteins can interact with various transcriptional co-activators on promoter DNAs to regulate the activation of TGFβ, activin, or BMP target genes (2, 6). The activity or intracellular localization of the Smads can be modulated through binding to adaptors molecules such as SARA (8), Hgs (9, 10), chaperones (11), microtubules (12), or various co-repressors such as Ski, SnoN, SNIP, or SIP1 (13–24). Other post-translational modifications of the Smad proteins such as acetylation or sumoylation have also been demonstrated to occur through direct interactions with acetyltransferases or ubiquitin-protein isopeptide ligases for sumoylation and can affect their signaling activity (2, 25). Interaction of the Smad proteins with the nuclear transport machinery mediates their nuclear translocation (1, 26–35). Upon receptor activation, activated Smad proteins can also be degraded by the proteasome in a ubiquitination-dependent manner through direct association with the ubiquitin-protein isopeptide ligases (2, 25). Thus, identification and characterization of the Smad-interacting molecules hold the key to understanding how the Smad pathway is regulated and how it functions.

In this paper we report the identification and characterization of the interaction between an integral inner nuclear membrane protein, MAN1, and the R-Smads. MAN1 was originally identified as an antigen recognized by a self-antibody present in the serum of a patient with collagenosis (36). Cloning of MAN1 shows that it is an integral protein of the inner nuclear membrane with 910 amino acid residues. MAN1 contains an LEM (LAP2, Emerin, and MAN1) domain at the N terminus that is present in several proteins, including LAP2 and emerin, two inner nuclear membrane proteins (37), and is speculated to mediate protein-protein interactions with the components of the nuclear lamina and chromatin (38). Sequence analysis fur-

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[1] The abbreviations used are: TGFβ, transforming growth factor-β; HA, hemagglutinin; BMPs, bone morphogenic proteins; GST, glutathione *S*-transferase; siRNA, small interfering RNA; FBS, fetal bovine serum; RRM, RNA recognition motif; WT, wild type; HIV, human immunodeficiency virus; RNP, ribonucleoprotein.

ther indicates that MAN1 contains two putative transmembrane domains that may span the inner nuclear membrane twice, resulting in a protein with N- and C-terminal nucleoplasmic domains. The N-terminal nucleoplasmic domain and the first transmembrane domain of MAN1 have been shown to be necessary for the inner nuclear envelope localization of MAN1 (39). In addition, MAN1 contains one RNA recognition motif (RRM) in the C-terminal domain. The function of this RRM is unclear.

The function and regulation of MAN1 have not been defined. In *Caenorhabditis elegans*, Ce-MAN1 interacts with lamin and BAF and may play a role in maintaining nuclear integrity during mitosis and cytokinesis as well as promoting nuclear re-assembly (38, 40). When overexpressed in *Xenopus* embryos, MAN1 induces the formation of a secondary neural axis, suggesting that it may antagonize BMP signaling (41). Indeed, *Xenopus* MAN1 was found to interact with Smad1 (41). In this report, we show that MAN1 interacts with all R-Smad proteins in mammalian cells and represses TGFβ, activin, and BMP signaling. This interaction reveals a previous unrecognized step in the regulation of Smad activity at the inner nuclear membrane.

MATERIALS AND METHODS

Cells, Antisera, and Constructs—The human hepatoma Hep3B cell line was maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS). 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. C2C12 cell were grown in Dulbecco's modified Eagle's medium supplemented with 20% FBS. Antisera against HA and FLAG peptides were purchased from Sigma. An anti-MAN1 rabbit polyclonal antibody was raised against a MAN1 N-terminal fragment (residues 1–329). Antibodies for phospho-Smads 1–3 were generous gifts from Dr. Aristidis Moustakas. Antisera against Smad3 (FL-425 and I-20), Smad1 (T-20 and H-465), and Smad4 (B-8) were purchased from Santa Cruz Biotechnology. MAN1 mutants, constitutively active receptors, and Smad mutants were generated by PCR. An oligonucleotide pair encoding an siRNA specific for human MAN1 (5′-gatccccTGAGTCTAGAGTTCGCACGttcaagagaCGTGCGAACTCTAGACTCAtttttggaaa-3′) was cloned into the pSUPER vector (Oligoengine) and used to reduce endogenous MAN1 expression. Stable Hep3B cell lines expressing the siRNA for MAN1 were generated by co-transfection of Hep3B cells with the pSUPER vector encoding the siRNA duplex together with a vector expressing the puromycin resistance gene. Transfected cells were selected in minimal essential medium supplemented with 10% FBS containing 2 μg/ml puromycin.

Transfection and Luciferase Assay—293T and Hep3B cells were transiently transfected using Lipofectamine Plus (Invitrogen) following the manufacturer's recommendations. C2C12 cells were trypsinized and transiently transfected in suspension using Lipofectamine Plus (Invitrogen) (42). For the luciferase assay, a total of 2.5 μg of DNA was transfected into the Hep3B cells. For the TGFβ response, 0.5 μg of p3TP-lux or pLUC800 was co-transfected with or without FLAG-MAN1. For the BMP response, 0.75 μg of 15XGCCG-lux was co-transfected with 0.25 μg of Smad1, 0.25 μg of Smad4, and 0.5 μg of constitutively active Alk3 (Alk3*) in the presence or absence of FLAG-MAN1. For the activin response, 0.75 μg of 3ARE-lux was co-transfected with 0.01 μg of Fast1, 0.25 μg of Smad2, and 0.25 μg of Smad4. Luciferase activity was measured 16 h after stimulation with 50 pM of TGFβ1 or 48 h after the initial transfection.

Immunoprecipitation and Western Blotting—FLAG- and/or HA-tagged Smads and MAN1 were isolated from transfected 293T by immunoprecipitation with anti-FLAG and/or anti-HA beads, followed by elution with the FLAG and HA peptide, and analyzed by Western blotting as described previously (17). Endogenous Smads were isolated by immunoprecipitation using anti-Smad3 antibody (FL-425) and anti-Smad1 antibody (H-465).

Glutathione S-Transferase (GST) Pull-down—Recombinant fragments of MAN1 were expressed in and purified from *Escherichia coli* as GST fusion proteins. Recombinant Smad2 and Smad2C (residues 264–467) were similarly purified as GST fusion proteins followed by thrombin cleavage. For binding assays, 1.5 μg of GST-MAN1 immobilized on glutathione-Sepharose were incubated with 2 μg of purified recombinant Smad proteins for 1 h at 4 °C (14). After extensive washing, the MAN1-bound Smads were eluted by glutathione and detected by immunoblotting with anti-Smad2 (S-20).

Immunofluorescence—C2C12 and Hep3B were grown on glass coverslips, fixed with ice-cold methanol for 20 min, permeabilized with 0.1% Triton X-100 for 5 min, and stained with the appropriate primary and secondary antibodies. Nuclei were detected by 4,6-diamidino-2-phenylindole staining. Cells were visualized by a Zeiss 510 confocal microscope. For detergent extraction of the soluble proteins, C2C12 were incubated in 0.1% Triton X-100 for 30 s just before fixation.

Northern Blotting—Hep3B cells were serum-starved for 16 h and then stimulated with 200 pM TGFβ1 for the indicated period. Total RNA was prepared from these cells using an RNeasy kit (Qiagen), and 15–20 μg RNA was resolved on a 1% formaldehyde gel, transferred to a nylon membrane, and analyzed by Northern blotting.

Growth Inhibition Assay—5×10^4 Hep3B cells were incubated with various concentrations of TGFβ1 for 4 days. Cell growth was determined by cell counting and compared with that in the absence of TGFβ1 (100%).

Pulse-Chase Assay—Transfected 293T cells growing in 6-well plates were washed with 2 ml of Dulbecco's modified Eagle's medium lacking Met and Cys, pulsed with 0.4 mCi/ml ^{35}S-express (Roche Applied Science) for 30 min, and chased for different periods of time as described previously (14).

RESULTS

Identification of MAN1 as an R-Smad Partner—To identify Smad3-associated cellular proteins, FLAG-tagged Smad3 was introduced stably into the TGFβ-responsive Hep3B and RIE-1 cells. Cellular proteins bound to Smad3 were purified by affinity purification with anti-FLAG-agarose and eluted and analyzed by silver staining (Fig. 1*A*). Among the proteins that co-precipitated specifically with Smad3, a protein with an apparent molecular mass of 110 kDa was detected in both Hep3B/Smad3 (Fig. 1*A*, *lane 2*) and RIE-1/Smad3 cells (data not shown) but not in untransfected or vector control cells (*lane 1*). By both peptide sequencing and mass spectrophotometry, the protein was identified as MAN1, an 110-kDa integral protein of the inner nuclear membrane (37). To investigate whether MAN1 interacts with other Smad proteins, HA-MAN1 was co-transfected into the 293T cells together with FLAG-tagged Smad1–5. HA-MAN1 associated with the Smad proteins was isolated by immunoprecipitation with anti-FLAG followed by Western blotting with anti-HA. MAN1 was found to interact with all R-Smads tested, Smad1, Smad2, Smad3, and Smad5, but not with the Co-Smad Smad4 (Fig. 1*B*). The interaction is predominantly mediated by the C-terminal domain (amino acid residues 699–910) of MAN1 and the MH2 domains of the R-Smads (Fig. 1, *B* and *C*). A weak interaction between the N-terminal fragment of GST-MAN1-N (residues 1–329) and Smads was detected (Fig. 1*C*), suggesting a possible second lower affinity binding site, at least *in vitro*. Because of a lower expression of FLAG-Smad2C in this experiment (*middle panel*, Fig. 1*B*), the interaction between MAN1 and Smad2C appeared to be weak. However, upon expression and purification from *E. coli*, recombinant Smad2C associated with MAN1 as efficiently as full-length Smad2 (Fig. 1*C*), confirming that the interaction is mediated by the MH2 domain of Smad2. In addition, recombinant Smad2 and MAN1 fragments purified from *E. coli* readily bound to each other *in vitro* (Fig. 1*C*), indicating that the interaction is direct.

To investigate how the MAN1-R-Smad interaction is regulated by activation of TGFβ, activin, or BMP signaling, a constitutively active type I receptor for TGFβ (Alk5*, T204DAlk5), activin (Alk4*, T206DAlk4), or BMP (Alk3*, Q233DAlk3) was co-transfected with HA-MAN1 and various FLAG-Smad proteins. As shown in Fig. 1*D*, activation of TGFβ, activin, or BMP signaling had no effect on the MAN1-Smad interaction. In addition, both phosphorylated (in the presence of receptor) and unphosphorylated Smad proteins associated with MAN1 (Fig. 1*F*), indicating that MAN1 does not distinguish between the active and inactive forms of Smad proteins under this overex-

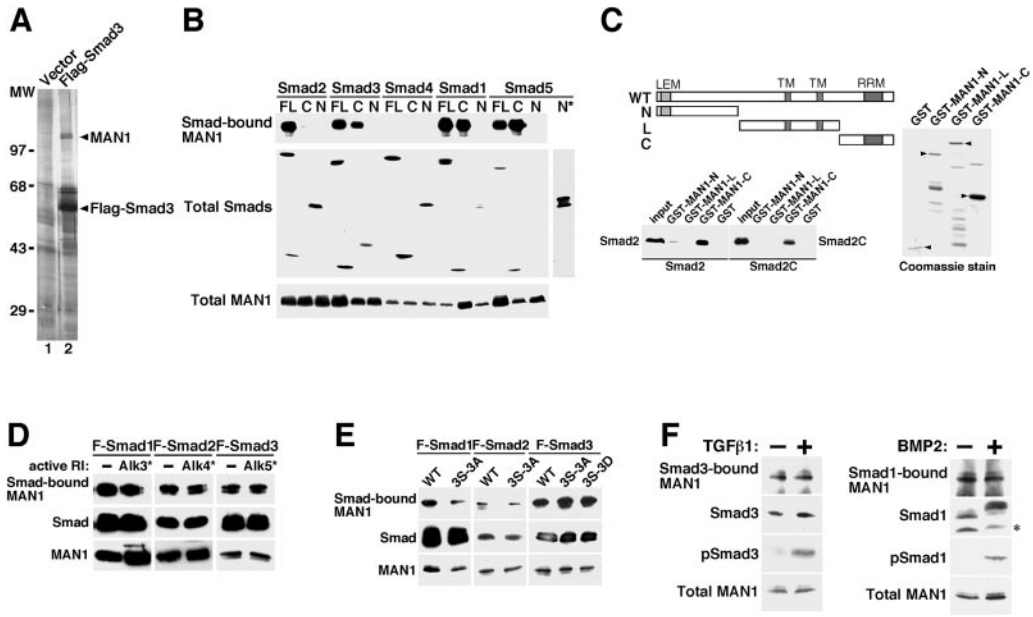

FIG. 1. **R-Smads associate with the integral inner nuclear membrane protein MAN1.** *A*, cell lysates were prepared from control Hep3B cells or Hep3B cells stably expressing FLAG-Smad3, and Smad3-associated cellular proteins were isolated by affinity purification using anti-FLAG-agarose followed by elution with the FLAG peptide as described under "Experimental Procedures." The purified Smad3 complexes were visualized by silver staining. The 110-kDa protein indicated by an *arrowhead* was digested with trypsin, and 11 tryptic peptides were microsequenced. The protein was positively identified as the human integral inner nuclear membrane protein MAN1. *B*, MAN1 binds to the MH2 domains of R-Smads. FLAG-tagged full-length (*FL*), C-terminal MH2 (*C*), or N-terminal MH1 (*N*) domains of the Smad proteins were co-transfected with HA-tagged MAN1 into 293T cells. MAN1 that associated with the Smad proteins were isolated by immunoprecipitation with anti-FLAG and detected by Western blotting with anti-HA (*top*). Total amounts of Smads in the immunoprecipitates were measured by Western blotting with anti-FLAG (*middle*), and MAN1 level was examined by Western blotting of total cell lysate with anti-HA (*bottom*). *N**, a longer exposure of the Smad5N lane to show that the protein was expressed. Upon a similar long exposure, no binding of this Smad5N with MAN1 was detected. *C*, MAN1 binds to Smad2 directly. *Top left*, WT and fragments of MAN1. *N*, residues 1–329; *L*, 297–698; and *C*, 699–910. GST-fused MAN1 fragments were purified from *E. coli*, immobilized and incubated with recombinant Smad2 or Smad2C, and also purified from *E. coli* as GST fusion proteins followed by thrombin cleavage. Smad2 or Smad2C that bound to MAN1 fragments was eluted with glutathione and detected by Western blotting with anti-Smad2 (*left bottom*). The amounts of GST-MAN1 fragments used in the experiment were quantified by Coomassie stain (indicated by *arrows*, *right panel*). *D*, interaction of MAN1with the R-Smads is not affected by activation of TGFβ, activin, or BMP2 signaling. FLAG-tagged Smads were co-transfected in 293T cells together with HA-tagged MAN1 in the presence or absence of constitutively active receptors. MAN1 that associated with Smads was isolated by immunoprecipitation with anti-FLAG and detected by Western blotting with anti-HA. The immunoprecipitates were subjected to Western blotting with anti-HA to detect Smad bound MAN1 (*top*) or with anti-FLAG for R-Smads (*middle*). Cell lysates were blotted directly with anti-HA for expression of the HA-MAN1 (*bottom*). *E*, interaction of MAN1 with the R-Smads is independent of receptor-mediated phosphorylation of R-Smads. HA-MAN1 was co-transfected in 293T cells together with WT or mutant FLAG-Smads. MAN1 that associated with Smads was isolated by immunoprecipitation with anti-FLAG and detected by Western blotting with anti-HA. The immunoprecipitates were blotted with anti-FLAG for Smad expression (*middle*). Cell lysates were blotted directly with anti-HA for expression of the HA-MAN1 (*bottom*). *F*, endogenous MAN1 and Smads interact. C2C12 cells were stimulated for 1 h with 200 pM of TGFβ1 or 200 ng of human recombinant BMP2. Endogenous MAN1 that associated with Smads was examined by immunoprecipitation of endogenous Smad proteins followed by Western blotting with anti-MAN1 antibody. *, a nonspecific band.

pression condition. Consistent with this, Smad3 mutants that either lack the C-terminal serine phosphorylation sites (3S-3A) or simulate phosphorylation (3S-3D) bound to MAN1 to a similar extent as WT Smad3 (Fig. 1*E*).

To investigate whether endogenous MAN1 and the R-Smads also interact and whether this interaction is subjected to regulation by the TGFβ family of growth factors, we generated a polyclonal antibody against the N-terminal part of MAN1 (residues 1–329 or GST-MAN1-N). In several human cell lines, this antibody, but not a pre-immune serum, specifically recognizes a 110-kDa protein that can be competed away effectively with GST-MAN1-N (data not shown). To examine the interaction of endogenous MAN1 and Smad proteins, the BMP2- and TGFβ-responsive C2C12 cells were stimulated with BMP2 or TGFβ1 for 1 h. MAN1 that associated with Smad1 or Smad3 was isolated by immunoprecipitation with anti-Smad1 or anti-Smad3 and detected by Western blotting with anti-MAN1. As shown in Fig. 1*F*, endogenous MAN1 bound to both phosphorylated and unphosphorylated Smad1 and Smad2/Smad3. Most interestingly, the MAN1-Smad1 or MAN1-Smad2/3 interaction was not affected by BMP2 or TGFβ1. Taken together, our results suggest that MAN1 interacts with the R-Smads proteins, and this interaction is independent of TGFβ, activin, or BMP stimulation.

MAN1 and Smad3 Interact at the Inner Nuclear Membrane—Because MAN1 is an inner nuclear membrane protein, but the R-Smads are usually cytoplasmic and translocate into the nucleus upon activation, we next examined the intracellular location where the MAN1-R-Smad interaction occurs by indirect immunofluorescence in C2C12 cells. As reported before, endogenous MAN1 is an integral inner nuclear membrane protein and displayed a ring-like localization around the nucleus as visualized by confocal microscopy (Fig. 2, *A* and *B*). Whereas Smad1 (Fig. 2*A*) and Smad3 (Fig. 2*B*) are predominantly distributed in the cytoplasm in the absence of BMP2 or TGFβ1, respectively, a ring-like perinuclear staining is present. This nuclear membrane staining is more apparent when the cells were extracted briefly with Triton X-100 to get rid of soluble proteins (Fig. 2, *A* and *B*, *3rd row* of each *panel*), suggesting that a small population of the R-Smads may associate with the nuclear membrane. Most importantly, this R-Smad staining co-localized with MAN1 staining.

Upon TGFβ1 or BMP2 stimulation, the R-Smads readily translocate into the nucleus (Fig. 2, *A* and *B*). Most interest-

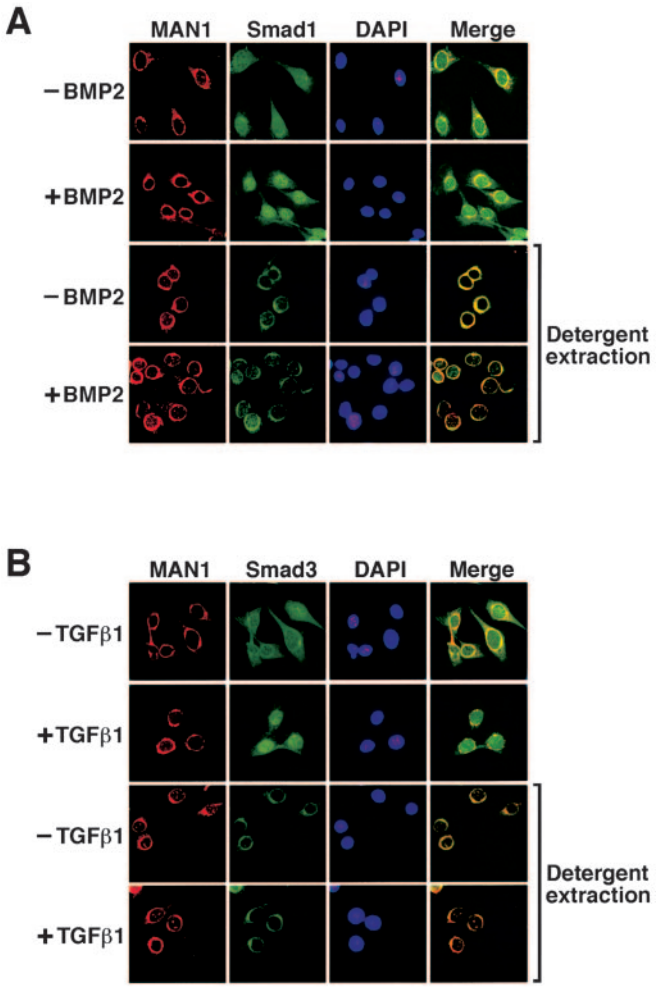

FIG. 2. **MAN1 and R-Smads co-localize at the inner nuclear membrane.** The BMP2- or TGFβ1-responsive C2C12 cells were stimulated with or without BMP2 (*A*) or TGFβ1 (*B*) for 1 h as indicated. Localization of endogenous MAN1 and Smad1 or Smad3 was detected by indirect immunofluorescence staining using polyclonal antisera specific for these proteins as described under "Experimental Procedures." 4,6-Diamidino-2-phenylindole (*DAPI*) stain for DNA was used to indicate the position of nuclei. *Bottom two rows* of each section showed staining of these proteins after the cells were extracted for 30 s with 0.1% Triton X-100 prior to fixation.

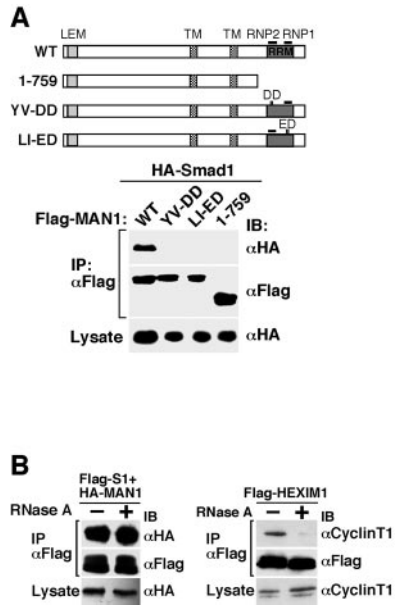

FIG. 3. **MAN1 interacts with the Smad proteins through the RRM domain.** *A*, *upper panel*, domain structure of WT and mutant MAN1. The positions of the LEM domain, two putative transmembrane domains (*TM*), and the RRM domain with two RNPs are indicated. *Lower panel*, co-immunoprecipitation assays. FLAG-tagged WT and mutant MAN1 were co-transfected in 293T cells together with HA-tagged Smad1 and isolated by immunoprecipitation (*IP*) with anti-FLAG antibody. The immunoprecipitates were subjected to Western blotting with anti-HA to detect MAN1-bound Smad1 (*top*) or with anti-FLAG for MAN1 (*middle*). *IB*, immunoblot. Cell lysates were blotted directly with anti-HA for expression of the HA-Smad1 (*bottom*). *B*, the MAN1-R-Smad interaction does not require an RNA intermediate. Anti-FLAG immunoprecipitates from 293T cells co-transfected with FLAG-Smad1 and HA-MAN1 were treated with or without RNase A and subjected to Western blotting with anti-HA to detect Smad1-bound MAN1 or with anti-FLAG for Smad1 expression. As a positive control, interaction of stably expressed FLAG-HEXIM1 with the endogenous cyclin T1 in the absence or presence of RNase A was analyzed in parallel (*right panel*). The HEXIM1-bound cyclinT1 was visualized by Western blotting of the anti-FLAG immunoprecipitates with anti-cyclinT1. Cell lysates were blotted with anti-cyclinT1 antibody as a control for the expression of total cyclinT1.

ingly, there is still a detergent non-extractable fraction of the R-Smads that is localized at the nuclear membrane and co-localized with MAN1 (*bottom rows*, Fig. 2, *A* and *B*). The interaction between MAN1 and this insoluble population of R-Smads appeared to be independent of stimulation by TGFβ1 or BMP2. This is consistent with our results from the co-immunoprecipitation experiments (Fig. 1*F*). Taken together, these data indicate that MAN1 interacts with a detergent non-extractable fraction of the R-Smads at the inner nuclear membrane in a ligand-independent manner.

MAN1 Interacts with the Smad Proteins through the RRM Domain—To determine the amino acid residues in MAN1 that are required for binding to the Smads, GST fusion proteins of MAN1 containing either the N-terminal 329 amino acid residues (MAN1-N), the middle region with two putative transmembrane domains (MAN1-L, residues 297–698), or the C-terminal fragment (MAN1-C, residues 699–910) were generated and purified. When incubated with purified recombinant Smad2 or Smad2C, the C-terminal MAN1 fragment showed a strong binding for Smad2 and Smad2C (Fig. 1*C*), indicating that the major Smad-binding site resides in the C-terminal region of MAN1. Most interestingly, this region contains an RRM motif found in several RNA-binding proteins including a cellular co-factor for the HIV Tat protein, TAT-SF1 (43) (Fig. 3*A*). RRM is an evolutionarily conserved domain that mediates RNA-protein or protein-protein interactions. Structural analysis of a putative RRM reveals that most RRMs contain two highly conserved octamers, RNP-2 and RNP-1, that form two β-sheets and are required for the interaction of an RRM with RNA or protein partners (44). The conserved Tyr and Val residues in RNP-2 or Leu and Ile residues in the RNP-1 are critical for maintaining their structural integrity. To investigate whether the RNPs in MAN1 are required for binding to the Smad proteins and, if so, which of the RNPs is critical, point mutations altering the Tyr and Val in the RNP-2 to Asp (YV-DD mutant) or Leu-Ile in the RNP-1 to Glu and Asp (LI-ED mutant) were introduced into MAN1. A deletion mutant of MAN1-(1–759) lacking the RRM and the subsequent C-terminal residues was also generated and tested. In the co-immunoprecipitation assay, although WT MAN1 bound to Smad1 readily, all three mutants failed to do so (Fig. 3*A*). These results suggest that the RRM domain is required for the interaction of MAN1 with the R-Smads.

The involvement of an RRM in the MAN1-R-Smad interaction raises an interesting possibility that this interaction may involve an RNA molecule. To test this, RNase treatment was carried out. FLAG-MAN1 was co-transfected into 293T cells with HA-Smad1 and isolated by immunoprecipitation with an-

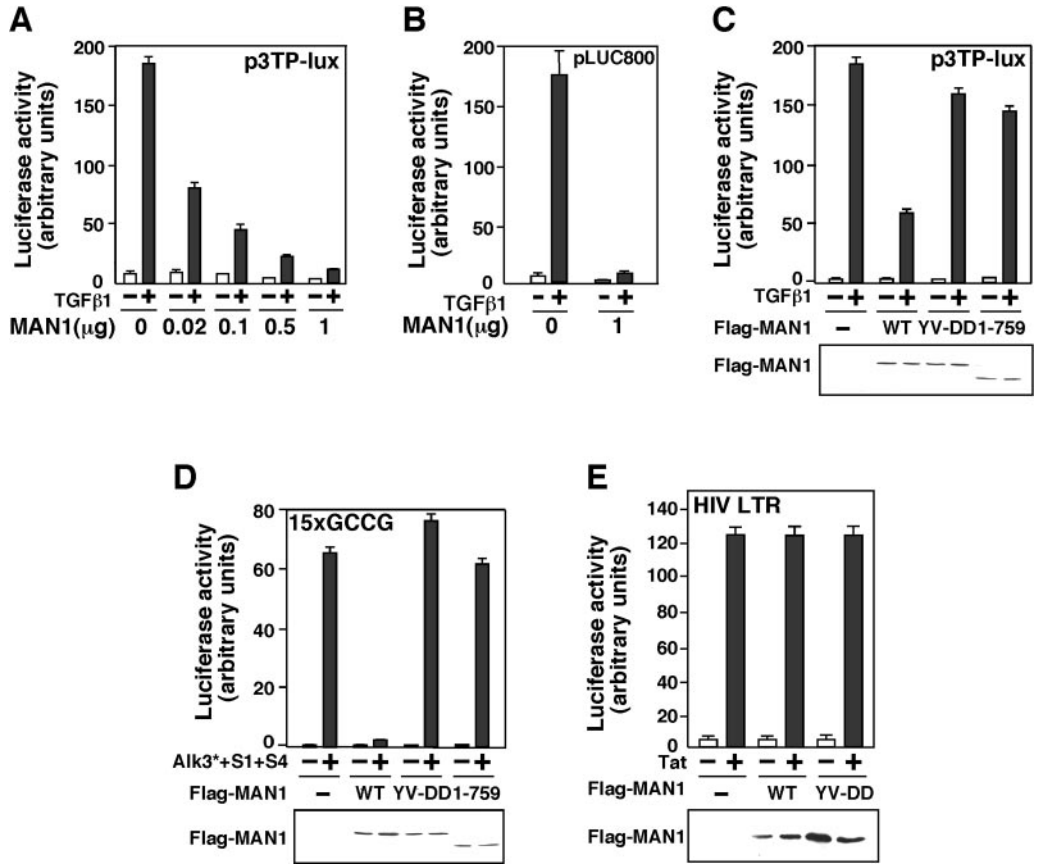

FIG. 4. **MAN1 represses transcriptional activation of TGFβ- and BMP2-responsive promoters.** Hep3B cells were transfected with p3TP-lux and increasing amounts of MAN1 (*A*) or the TGFβ-responsive region of the natural PAI-1 promoter pLUC800 and MAN1 (*B*). Luciferase activity was measured 20 h after stimulation with TGFβ1. *C*, wild type or mutant FLAG-MAN1 was co-transfected with p3TP-lux into the Hep3B cells. The expression levels of FLAG-MAN1 in these transfected samples were detected by Western blotting with anti-FLAG. *D*, WT or mutant FLAG-MAN1 was transfected together with the BMP-responsive 15XGCCG-luc, the constitutively active Alk3*, Smad1, and Smad4 into the Hep3B cells. The luciferase activity was measured 48 h later. The expression levels of MAN1 in the transfected samples were measured by Western blotting with anti-FLAG. *S1*, Smad1; *S4*, Smad4. *E*, MAN1 does not affect transcriptional activation induced by HIV Tat. pHIV-LTR-Luc was transfected into the Hep3B cells in the absence or presence of FLAG-MAN1 and/or Tat. Luciferase activity was measured 48 h later.

ti-FLAG. The immunoprecipitates were subjected to treatment with RNase A, and HA-Smad1 associated with MAN1 was detected by Western blotting with anti-HA. As a positive control for the dependence on RNA, we examined the interaction between FLAG-tagged HEXIM1 and the endogenous P-TEFb complex that is composed of CDK9 and cyclin T (Fig. 3*B*). This interaction has been shown previously to be dependent on the 7SK small nuclear RNA (45, 46). Indeed, treatment of the anti-FLAG immunoprecipitate with RNase A effectively blocked the interaction between FLAG-HEXIM1 and cyclin T (Fig. 3*B*, *right panel*). In contrast, RNase A treatment had no effect on the MAN1-Smad1 interaction (Fig. 3*B*, *left panel*), indicating that although the MAN1-R-Smad interaction is mediated by the RRM, it does not require an RNA intermediate.

MAN1 Inhibits Transcriptional Activation by the Smad Proteins—To investigate how MAN1 affects the signaling activity of the R-Smads, we examined the effects of MAN1 on the ability of Smads to activate transcription of the TGFβ, activin, and BMP-responsive promoters. Luciferase reporter constructs specific for TGFβ (p3TP-lux and pLUC800), activin (ARE-lux), or BMP2 (15XGCCG) were transfected into Hep3B cells in the absence or presence of MAN1. Activation of these reporter genes was measured 48 h later. As shown in Fig. 4, *A* and *B*, increasing concentrations of MAN1 induced a corresponding decrease in transcription from p3TP-lux in response to TGFβ stimulation. In addition, MAN1 strongly repressed activation of transcription from the natural PAI-1 promoter containing the TGFβ-responsive element (Fig. 4*B*) as well as the activin-(data not shown) and BMP-responsive promoters (Fig. 4*D*), with the fold of activation reduced by at least 10 times. As a control, transcription from another promoter unrelated to TGFβ signaling, pHIV-LTR-luc, either at basal level or activated by the HIV Tat protein, was not affected (Fig. 4*E*). This suggests that MAN1 can function as a specific repressor of TGFβ, activin, and BMP signaling, probably through its interaction with the R-Smad proteins.

If a direct interaction between MAN1 and the Smad proteins is required for the repression of TGFβ-, activin-, or BMP-responsive promoters, mutant MAN1 incapable of binding to the Smad proteins should be inactive in transcriptional repression. To test this, the point mutant YV-DD and the truncation mutant 1–759, lacking the first functional RNP-2 and unable to bind to the R-Smads (Fig. 3*A*), were co-transfected with the luciferase reporter constructs into the Hep3B cells. Both mutants failed to repress transcriptional activation in response to TGFβ1 (Fig. 4*C*), BMP2 (Fig. 4*D*), or activin signaling (data not shown). These data indicate that the MAN1-R-Smad interaction is indeed required for the repression of BMP, activin, or TGFβ signaling.

Reducing MAN1 Expression by siRNA Enhances TGFβ Signaling—Because high levels of MAN1 repress signaling by the TGFβ family of cytokines, reducing MAN1 expression should enhance transactivation induced by these cytokines. We therefore designed an oligonucleotide expressing an siRNA duplex specific for human MAN1 and cloned it into the pSUPER vector (47). In Hep3B cell lines stably expressing the siRNA, a signif-

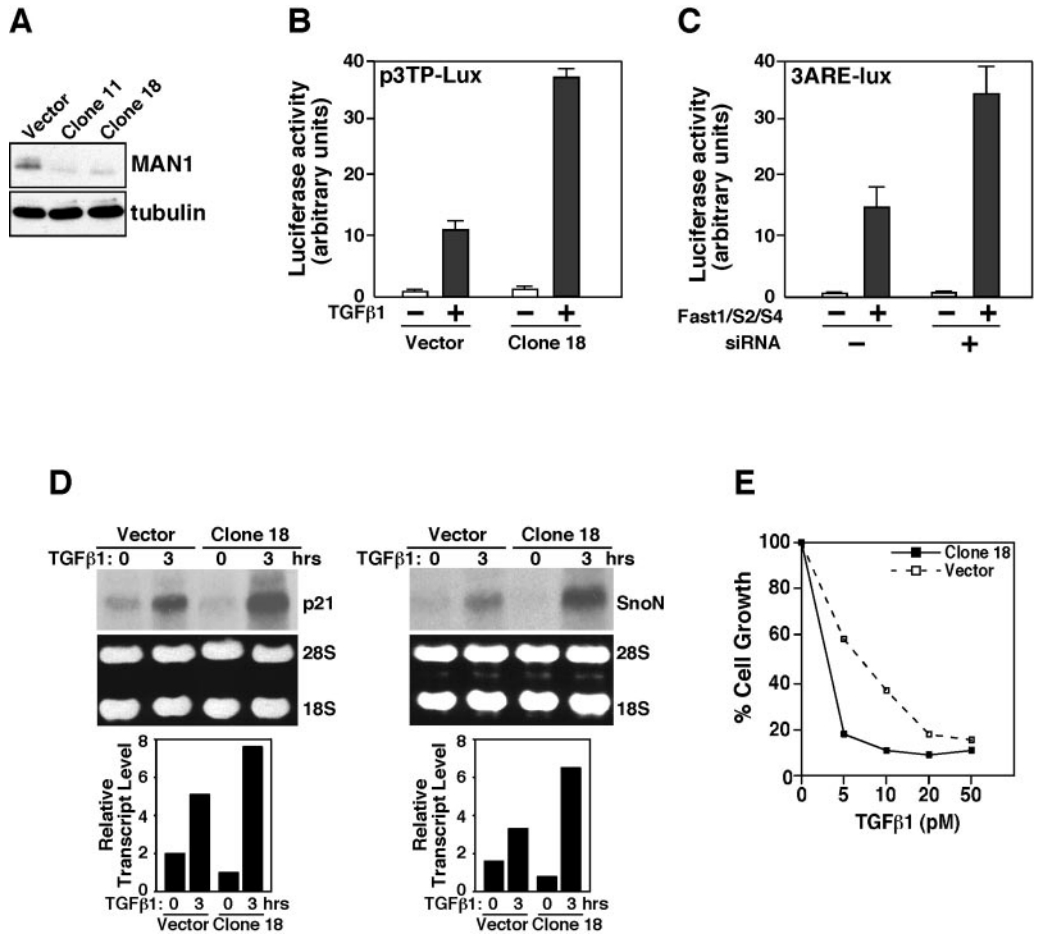

FIG. 5. **Reducing MAN1 expression enhances the responses of cells to TGFβ.** *A*, Hep3B clones expressing the pSUPER control vector or the siRNA duplex were analyzed by Western blotting with anti-MAN1 for the expression level of MAN1. Clones 11 and 18 displayed a marked reduction of MAN1 expression. The blot was stripped and reblotted with anti-tubulin to show equal loading of samples. *B*, clone 18 was transfected with p3TP-lux, and luciferase activity was measured after stimulation with 50 pM TGFβ1. *C*, Hep3B cells were co-transfected with 3ARE-lux, Fast1, Smad2, and Smad4 in the presence or absence of siRNA. Luciferase activity was measured 48 h after transfection. *D*, Northern blot analysis. Hep3B cells expressing the vector control or siRNA (clone 18) were serum-starved and stimulated with 200 pM TGFβ1 for 3 h. Expression of the TGFβ-responsive gene *snoN* and p21 was analyzed by Northern blotting. A representative set of experiments (out of three) is shown here. Ethidium bromide staining of 18 S and 28 S RNA was used to show loading of the samples. The levels of p21, *snoN*, and 28 S and 18 S RNAs were quantified by the ImageJ software. The relative transcript level was calculated by normalizing the p21 or *snoN* levels to the average of the levels of the 28 S and 18 S RNAs (in arbitrary units) and are shown in the accompanying graphs. *E*, growth inhibition assay. Hep3B cells expressing the vector control or siRNA (clone 18) were stimulated with various concentrations of TGFβ1. Cell growth was evaluated by cell counting and compared with that of cells grown in the absence of TGFβ1.

icant decrease in the MAN1 expression was detected in at least two of the clones (Fig. 5*A*), confirming that the siRNA is effective in reducing MAN1 expression. By using the clone 18, we next investigated the effect of a reduced endogenous MAN1 expression on activation of TGFβ-, activin-, or BMP2-responsive genes or promoters. In a luciferase reporter assay, decreasing MAN1 expression resulted in an increase in transcriptional activation of TGFβ- (Fig. 5*B*), activin- (Fig. 5*C*), or BMP (data not shown)-responsive promoters. To confirm that this increased transcription activation of TGFβ-responsive promoters also occurs with endogenous genes, total RNA was prepared from Hep3B cells expressing a vector control or siRNA before and after TGFβ treatment. Induction of endogenous TGFβ-responsive genes, such as *snoN* and p21, was analyzed by Northern blotting. As shown in Fig. 5*D*, in siRNA-expressing cells, a marked enhancement in the induction of endogenous *snoN* and p21 mRNA by TGFβ was detected, confirming that reducing MAN1 expression leads to enhanced TGFβ-induced transcription. Finally, to investigate whether a decreased MAN1 expression also affected the ability of cells to undergo growth inhibition in response to TGFβ, the vector control cell line and clone 18 were cultured in the presence of varying amounts of TGFβ1 for 4 days, and the growth of the cells was quantified. As shown in Fig. 5*E*, reducing MAN1 expression rendered cells more sensitive to TGFβ-induced growth inhibition. This enhanced growth inhibitory response to TGFβ1 was especially apparent at low concentrations of TGFβ1 (5 or 10 pM), and the cells reached a maximal level of growth arrest between 5 and 10 pM of TGFβ, whereas in control cells, the peak growth inhibition occurred between 20 and 50 pM. Taken together, our results suggest that MAN1 functions to repress TGFβ, activin, and BMP signaling.

Overexpression of MAN1 Results in Cell Death—In order to examine the effects of high levels of MAN1 on the growth inhibitory response of TGFβ and/or the ability of BMP2 to induce cell differentiation, we attempted to generate stable cell lines that overexpress WT MAN1 by either retroviral infection or transfection. However, in at least four epithelial or lymphoid cell lines, overexpression of MAN1 resulted in rapid cell death. As shown in Fig. 6, compared with the vector-expressing cells, Mv1Lu mink lung epithelial cells or Hep3B cells expressing MAN1 died within 6 days in culture. This activity appears to be independent of the ability of MAN1 to repress TGFβ, BMP, and activin signaling because

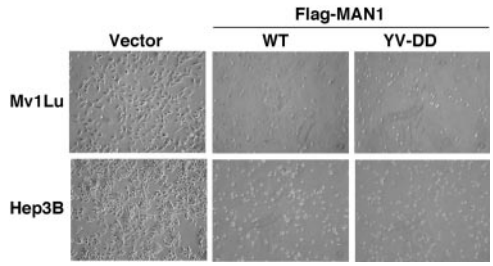

FIG. 6. **Overexpression of MAN1 induces cell death.** Mv1Lu mink lung epithelial cells or Hep3B cells were co-transfected with vector control, WT, or the YV-DD mutant MAN1 and a vector expressing the puromycin resistance gene. 48 h after transfection, cells were selected in the presence of puromycin for 2 days. An equal number of surviving cells were plated and grown for 4 additional days. Pictures of these cell plates were taken under the phase-contrast microscope.

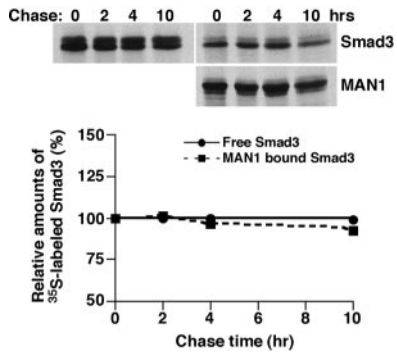

FIG. 7. **MAN1 does not affect the stability of Smad3.** 293T cells were singly transfected with FLAG-Smad3 or co-transfected with FLAG-MAN1 and HA-Smad3. Cells were pulse-labeled and chased as described under "Experimental Procedures." Immunoprecipitation was carried out with anti-FLAG to isolate free Smad3 or MAN1-bound Smad3 and analyzed by SDS-PAGE. Quantification of the bands was performed using the ImageJ software.

the YV-DD mutant that is defective in binding to the Smads was as potent as WT MAN1 in inducing cell death (Fig. 6). Attempts to reduce MAN1 expression by using an antisense construct also result in cell death (data not shown). These results suggest that a well controlled level of MAN1 expression is critical for the proper function and survival of the cell. Although the mechanism by which MAN1 causes cell death is not clear, it is conceivable that as an integral protein of the inner nuclear membrane, a proper level of MAN1 expression is probably critical for the integrity of nuclear membrane and its reassembly during cell division. This ability to induce cell death when overexpressed also made it impossible for us to evaluate the effects of MAN1 on TGFβ-induced growth inhibition and BMP2-induced cell differentiation.

MAN1 Does Not Affect the Stability of the Smad Proteins—MAN1 could repress Smad function by inducing degradation of the R-Smads. To investigate whether MAN1 affects the stability of the R-Smad proteins, pulse-chase assays were carried out to measure the half-lives of Smad3 in the presence or absence of MAN1. 293T cells transfected with FLAG-Smad3 or with FLAG-MAN1 and HA-Smad3 were pulse-labeled with ^{35}S-express and chased for various periods of time as indicated (Fig. 7). MAN1-associated Smad3 and free Smad3 were isolated by immunoprecipitation with anti-FLAG from co-transfected cells and singly transfected cells, respectively. As shown in Fig. 7, MAN1-associated Smad3 had a half-life that is similar to that of free Smad3. Similar results were obtained with other R-Smads (data not shown). Thus, expression of MAN1 had no effect on the half-lives of R-Smads.

Overexpression of MAN1 Results in a Decrease in Receptor-mediated Phosphorylation and Oligomerization of the R-Smads—Activation of the R-Smad proteins involves receptor-mediated phosphorylation and heterodimerization with Smad4 followed by nuclear translocation. To understand how MAN1 represses R-Smad signaling, we first examined whether MAN1 affected phosphorylation or hetero-oligomerization of the R-Smads in response to TGFβ signaling. To examine the phosphorylation of the R-Smads by the activated type I receptor in the presence of MAN1, HA-Smad1 was co-transfected into 293T cells together with the constitutively active type I BMP receptor (Alk3*) in the absence or presence of FLAG-MAN1. Smad1 that associated with MAN1 was isolated by immunoprecipitation with anti-FLAG, and free Smad1 was isolated by immunoprecipitation with anti-HA. The levels of their phosphorylation induced by the activated BMP type I receptors were then examined by Western blotting with an antibody specific for phospho-Smad1. As shown in Fig. 8*A*, although MAN1-associated Smad1 was still phosphorylated by the active type I receptor kinase (*lane 2*), the level of this phosphorylation was less than that of the free Smad1 (*lane 1*). This indicates that binding to MAN1 results in at least a partial reduction of receptor-mediated phosphorylation of the R-Smads.

To investigate whether overexpression of MAN1 affects heterodimerization of the R-Smads with Smad4, FLAG- or HA-Smad3 was co-transfected with HA-Smad4 and the constitutively active Alk5* into 293T cells in the absence or presence of FLAG-MAN1. Interaction of the MAN1-associated Smad3 with Smad4 was analyzed by a co-immunoprecipitation assay. Because MAN1 does not interact with Smad4 directly, any HA-Smad4 found in the anti-FLAG-MAN1 immunoprecipitate is mediated by heterodimerization with Smad3. In the absence of MAN1, Smad3 readily formed a heteromeric complex with Smad4, and this interaction was markedly enhanced by the activation of TGFβ signaling (Fig. 8*B*, *2nd lane*). In contrast, MAN1 associated Smad3 did not complex with Smad4 either in the absence of presence of active receptor signaling (Fig. 8*B*, *3rd* and *4th lanes*), indicating that MAN1 blocked the hetero-oligomerization of the Smad proteins.

Overexpression of MAN1 Blocks Nuclear Translocation of the R-Smads—Because MAN1 resides at the inner nuclear membrane and represses Smad-mediated transcriptional activation in mammalian cells, we next asked whether overexpression of MAN1 inhibits nuclear translocation of R-Smads in response to TGFβ. To do this, FLAG-tagged WT or the YV-DD mutant MAN1 was transfected into the Hep3B cells, and localization of endogenous Smad3 before and after TGFβ stimulation was examined by staining with anti-Smad3 (Fig. 8, *C* and *D*). In control experiments, overexpressed FLAG-MAN1 was found to co-localize with endogenous MAN1 (data not shown). Although lower levels of MAN1 expression did not significantly affect the nuclear translocation of Smad3 in response to TGFβ1, high levels of MAN1 protein effectively blocked Smad3 from entering the nucleus in the presence of TGFβ1 (Fig. 8, *C* indicated by *arrowhead*, and *E*). This inhibition of Smad3 translocation by MAN1 appears to require an interaction of MAN1 with the R-Smad because the YV-DD mutant, even when expressed at similar high levels, failed to block TGFβ-induced nuclear translocation of Smad3 (Fig. 8, *D* indicated by *arrowhead*, and *E*). Most importantly, this experiment also showed that similar to WT MAN1, the YV-DD mutant is also localized to the inner nuclear membrane predominantly. Thus, the inability of this mutant to bind to the R-Smads is not due to an aberrant localization of the mutant protein.

MAN1 Expression Is Not Altered in Mammalian Cancer Cells—The TGFβ-Smad pathway has tumor suppressive activity in normal and early stage tumor cells and has been found to

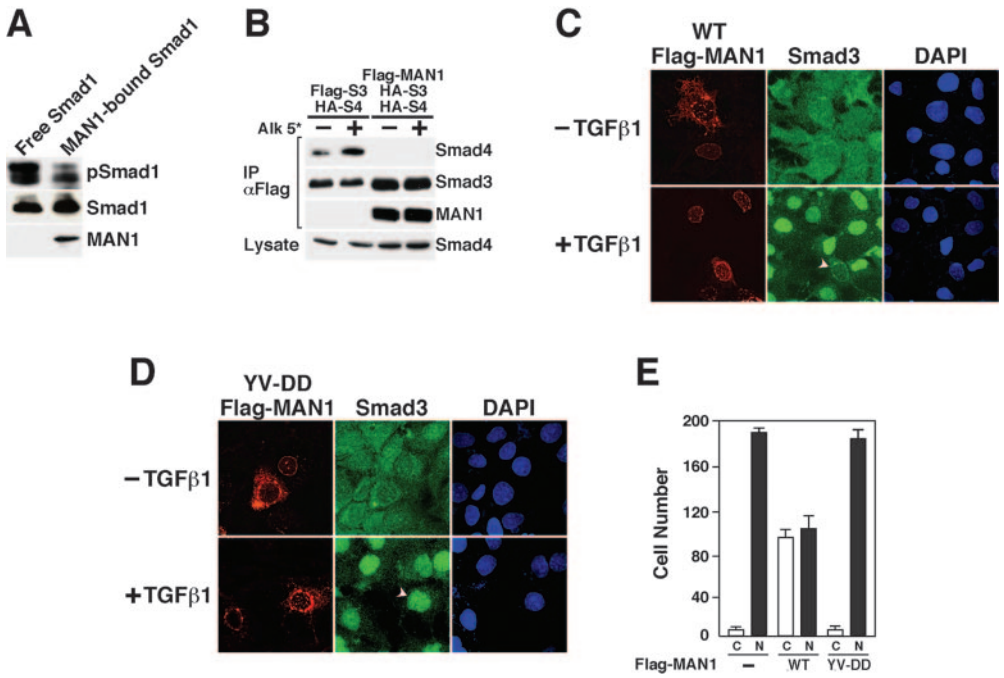

FIG. 8. *A*, overexpression of MAN1 results in decreased phosphorylation of Smad1. HA-Smad1 and the constitutively active Alk3* were transfected into 293T cells with or without FLAG-MAN1. Free Smad1 was isolated by immunoprecipitation with anti-HA and MAN1-bound Smad1 by anti-FLAG. Receptor-mediated phosphorylation of Smad1 was analyzed by Western blotting with an anti-phospho-Smad1 (*top*). The levels of Smad1 in the two samples were measured by Western blotting of the immunoprecipitates with anti-HA (*middle*). MAN1 expression was monitored by Western blotting with anti-FLAG (*bottom*). *B*, overexpression of MAN1 leads to inhibition of the heterodimerization of R-Smads with Smad4. 293T cells were co-transfected with HA-Smad3 and HA-Smad4 or FLAG-Smad3 and HA-Smad4 in the presence or absence of FLAG-MAN1 and the constitutively active Alk5* as indicated. In the absence of MAN1, Smad4 that associated with Smad3 was isolated by immunoprecipitation (*IP*) with anti-FLAG and detected by Western blotting with anti-Smad4 (*top panel*). Total level of Smad3 in the immunoprecipitates was measured by Western blotting with anti-Smad3 (*2nd panel*). When MAN1 was present, the MAN1-bound Smad complex was isolated by immunoprecipitation with anti-FLAG. Smad3 that associated with MAN1 was detected with anti-Smad3. Smad4 that associated with Smad3 in the MAN1 complex was analyzed by Western blotting with anti-Smad4. The levels of MAN1 and Smad4 proteins in the immunoprecipitates or cell lysates were analyzed by Western blotting using the appropriate antibodies as indicated (*bottom two panels*). *C* and *D*, overexpression of WT, but not mutant MAN1 defective in binding to the Smads, blocks nuclear translocation of the R-Smads. FLAG-tagged WT (*C*) or the YV-DD mutant MAN1 (*D*) was transfected into the Hep3B cells. After stimulation with TGFβ1 for 1 h, localization of endogenous Smad3 was determined by immunofluorescence staining with anti-Smad3. MAN1-transfected cells were detected by staining with anti-FLAG. Cells with both low and high MAN1 expression were shown. TGFβ1-induced nuclear translocation of Smad3 was abolished in cells expressing a high level of WT but not mutant MAN1 (indicated by *white arrowhead*). *E*, quantification of the number of cells displaying cytoplasmic and nuclear Smad3 as shown in *C* and *D*. The cell number counts include cells that highly overexpress MAN1, which blocks Smad3 nuclear translocation, as well as those that express a lower level of MAN1, which does not affect Smad3 translocation. *C*, cytoplasmic Smad3; *N*, nuclear Smad3.

be frequently inactivated in many human cancer cells. Because MAN1 is a repressor of this pathway, we asked whether MAN1 expression is up-regulated in human cancer cells. To do this, the level of MAN1 protein in untransformed human and rodent epithelial cell lines was compared with that in various cancer cell lines. Equal amounts of total cell lysates were prepared from two untransformed cell lines, the rat intestinal epithelial RIE-1 cell line and the human mammary epithelial HMT3522-S1 cell line (48), and nine various human and rodent cancer cell lines, including those derived from breast cancer, lung cancer, hepatoma, and colon cancer. MAN1 expression was analyzed by Western blotting with an anti-MAN1 polyclonal antibody. As shown in Fig. 9*A*, MAN1 was expressed at similar levels in both untransformed and malignant cancer cell lines. Thus, MAN1 expression is not significantly up-regulated in human cancer cells.

Next we examined whether MAN1 expression can be regulated by TGFβ. Hep3B cells were treated with 200 pM TGFβ1 for various periods of time, and the expression of MAN1 mRNA was examined by Northern blotting (Fig. 9*B*). No significant alteration in MAN1 mRNA expression was detected upon TGFβ stimulation, suggesting that MAN1 probably does not regulate TGFβ signaling in a negative feedback manner.

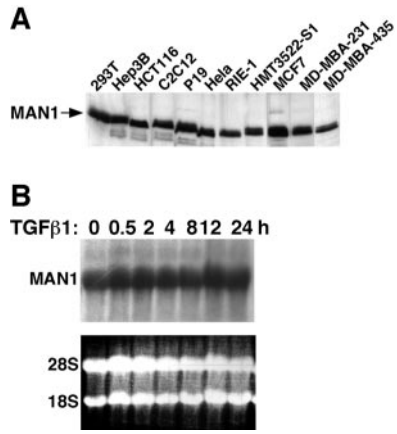

FIG. 9. *A*, expression levels of MAN1 in various cancer and untransformed cell lines. Equal amounts of total cell lysates were prepared from various cell lines, and the level of MAN1 expression was measured by Western blotting with an anti-MAN1 antibody. *B*, TGFβ1 does not affect MAN1 expression. Hep3B cells were serum-starved for 16 h and stimulated with 100 pM TGFβ1 for various amounts of time as indicated. Total cellular RNA was isolated from these cells, and MAN1 expression was analyzed by Northern blotting.

DISCUSSION

The TGFβ-Smad pathway has been shown to be regulated at multiple levels: extracellular ligand processing and availability (49), receptor binding and activation (49, 50), Smad phosphorylation and nuclear translocation, as well as DNA binding and transcriptional activation (1). Here we have identified a novel

step in the regulation of R-Smad activity at the inner nuclear membrane through a physical interaction between the R-Smads and the integral inner nuclear membrane protein MAN1. Our results have extended a previous study showing that *Xenopus* MAN1 can interact with Smad1 to induce neural cell fate in the *Xenopus* embryos (41) to TGFβ and activin signaling in mammalian cells. We found that MAN1 can physically interact with all R-Smads, but not Smad4, and repress transcriptional activation induced by TGFβ, activin, and BMP. We further showed that MAN1 interacts with a fraction of the R-Smads that may associate with the nuclear matrix and cannot be extracted by detergent. This indicates that upon entry into the nucleus, the activity of the R-Smad proteins can still be regulated at the inner nuclear membrane. This is the first example that such a regulatory step exists.

We found that the MAN1-Smad interaction results in repression of the transcription activity of the R-Smads. However, the mechanism of this repression is not entirely clear. Several possibilities exist. First, MAN1 could repress Smad function through inhibiting any or all of the steps involved in R-Smad activation, for example down-regulation of the R-Smad expression, inhibition of its phosphorylation, oligomerization with Smad4, nuclear translocation, or transactivation activity. We found that MAN1 does not affect the expression or stability of the R-Smad proteins. Most interestingly, MAN1-associated R-Smads appear to be phosphorylated to a lesser extent than free R-Smads upon receptor activation and failed to form a heteromeric complex with Smad4. Moreover, overexpression of MAN1 blocks nuclear translocation of the R-Smads. These effects of MAN1 on R-Smad phosphorylation and oligomerization are similar to those observed when SANE, a protein that shares homology with MAN1, was overexpressed in BMP-responsive cells (51). However, MAN1 differs from SANE in that it is localized to the inner nuclear membrane instead of the plasma membrane and cytoplasmic localization displayed by SANE. Because of the close proximity, SANE physically binds to the BMP receptor complex and may inhibit its ability to phosphorylate the R-Smads (51). It is not clear how MAN1 blocks phosphorylation of the R-Smads. The reduced phosphorylation of R-Smads in the presence of high levels of MAN1 could be caused by the sequestration of a fraction of the R-Smads by MAN1 at the inner nuclear membrane, blocking their access to the receptors. Because of the relative low stoichiometry of the MAN1-R-Smad interaction, this possibility seems unlikely. Alternatively, MAN1 may recruit a yet-to-be-identified serine/threonine phosphatase to the nuclear periphery to dephosphorylate the R-Smads. Whatever the mechanism, the reduced phosphorylation of the R-Smads in the presence of MAN1 can in turn lead to inhibition of hetero-oligomerization and nuclear translocation of the R-Smads. Because the inhibitory effect of MAN1 was only observed under a very high level of expression, it is also possible that when overexpressed MAN1 may be concentrated in the endoplasmic reticulum due to the inefficient retention of all the MAN1 at the inner nuclear membrane (39). The binding of the R-Smads with the MAN1 in the endoplasmic reticulum may indirectly lead to retention of the R-Smads in the cytoplasm and subsequent exclusion from the nucleus. This possibility does not seem likely because we did not observe a typical endoplasmic reticulum staining pattern of the R-Smad proteins in cells overexpressing MAN1 (Fig. 8*C*).

Second, given the unique inner nuclear membrane localization of MAN1, we speculate that MAN1 may also suppress R-Smad function through sequestration of the Smad proteins at the nuclear periphery, preventing their access to the downstream target promoters. This is consistent with the observation that at the endogenous level, a fraction of R-Smads colocalize with MAN1 at the inner nuclear membrane both before and after ligand stimulation. Third, MAN1 may recruit R-Smads and the associated chromatin to the perinuclear area. Because it is known that perinuclear localization of chromatin facilitates transcriptional silencing (52), the MAN1-Smad interaction may promote transcription silencing of the TGFβ-responsive genes. Future studies will determine which of these mechanisms are responsible for repression of TGFβ and BMP signaling by MAN1.

The MAN1-Smad interaction could serve several functions. It may help to maintain the repressed state of TGFβ target genes in the absence of ligand by sequestering the low level of R-Smads away from the nuclear cytoplasm. Because MAN1 binds to both phosphorylated and unphosphorylated R-Smads, it may also help to limit the amount of active R-Smad proteins in the nucleus, thereby regulating the threshold of activation for different TGFβ target genes. Finally, the MAN1-R-Smad interaction may help to maintain the proper localization of the phosphorylated, nuclear R-Smads after mitosis and during nuclear envelope reassembly.

It is interesting to note that MAN1 interacts with all the R-Smads but not Smad4. This binding specificity for R-Smads is reminiscent of that displayed by the type I TGFβ receptors and the plasma membrane-associated adaptor molecule SARA. Biochemical and structural analyses of the interactions between the above two proteins and the R-Smads indicate that two different domains in the R-Smads are involved in the specific interactions with type I TGFβ receptor and SARA, respectively. Although type I TGFβ receptor binds to the L3 loop and the helix 1 regions in the R-Smads (53), SARA interacts with a β-strand in the R-Smad MH2 domain (54). Because MAN1 is a membrane-associated molecule, similar to SARA, we wondered whether they may share common binding residues in the R-Smads. However, mutation of one of the critical residues in the Smad3 MH2 domain required for binding SARA (Asn-339) to serine did not affect the MAN1-Smad interaction (data not shown), whereas in the same reaction, this mutant failed to bind to SARA. Thus, MAN1 binds to a region in the R-Smad MH2 domain different from that employed by SARA. In addition, we showed that MAN1 does not distinguish between the phosphorylated and unphosphorylated R-Smads, indicating that the phosphorylated serine residues that are present only in R-Smads but not Smad4 do not mediate this interaction. Future biochemical and structural studies will determine the residues in R-Smads that mediate this interaction.

While this paper was in the review process, a new report was published showing that loss-of-function mutations in MAN1 are found in several related human diseases including osteopoikilosis, Buschke-Ollendorff syndrome, and melorheostosis (55). These mutations cause deletions of the C-terminal part of MAN1, including the RRM domain, and disrupt the interaction of MAN1 with the Smad proteins. Patients expressing the mutant MAN1 may display enhanced BMP, TGFβ, or activin signaling, and this is consistent with the observed increase in bone density in these patients. In addition to interaction with the R-Smads and repression of signaling by the TGFβ superfamily of cytokines, MAN1 may also have other cellular functions. As an integral inner nuclear membrane protein, it has been shown to play a key role in maintaining nuclear membrane integrity during mitosis and may be essential for chromosome segregation and cell division (37, 38). Indeed, we found that alteration of the MAN1 expression through overexpression (Fig. 6) results in cell death, and this activity appears to be independent of its ability to bind to the Smad proteins. This indicates that an appropriate level of MAN1 activity is required for maintaining the basic normal function and homeostasis of

cells. More studies are needed to fully determine the function of this important and interesting protein.

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