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Journal

Human Molecular Genetics, 14(3)

ISSN

0964-6906

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Publication Date

2005-02-01

DOI

10.1093/hmg/ddi040

Peer reviewed

MAN1, an integral protein of the inner nuclear membrane, binds Smad2 and Smad3 and antagonizes transforming growth factor- β signaling

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Received November 9, 2004; Revised December 2, 2004; Accepted December 7, 2004

MAN1 (also known as LEMD3) is an integral protein of the inner nuclear membrane. Recently, mutations in MAN1 have been shown to result in osteopoikilosis, Buschke–Ollendorff syndrome and melorheostosis. We show that the nucleoplasmic, C-terminal domain of human MAN1 binds to Smad2 and Smad3 and antagonizes signaling by transforming growth factor- β (TGF- β). In a yeast two-hybrid screen using the C-terminal domain of MAN1 as bait, eight positive clones were obtained that encoded Smad3. In direct two-hybrid assays, this portion of MAN1 bound to Smad2 and Smad3. In glutathione-S-transferase precipitation assays, the C-terminal domain of MAN1 bound to Smad2 and Smad3 under stringent conditions. Antibodies against MAN1 were able to co-immunoprecipitate Smad2 from cells, demonstrating that they reside in the same complex *in vivo*. TGF- β treatment stimulated transcription from a reporter gene in control cells, but reporter gene stimulation was significantly inhibited in cells overexpressing MAN1 or its C-terminal domain but not its N-terminal domain. TGF- β -induced cell proliferation arrest was also inhibited in stable cell lines overexpressing MAN1. These results show that the nuclear envelope regulates a signal transduction pathway and have implications for how mutations in nuclear envelope proteins cause different human diseases.

INTRODUCTION

The nuclear envelope, including the nuclear lamins and integral proteins of the inner nuclear membrane, may function in the regulation of gene expression (1–9). This could have pathophysiological implications for the recent discoveries that a growing number of inherited diseases are caused by mutations in inner nuclear membrane proteins (2–9). Putative gene regulatory functions of inner nuclear membrane proteins have been inferred primarily from their interactions with DNA and proteins that function in gene regulation. For example, lamins A and C bind to DNA and histones (10–13) and retinoblastoma protein (14). The inner nuclear membrane protein LBR binds to mammalian orthologs of *Drosophila* heterochromatin protein 1 (15,16) and DNA (17,18). Emerin and lamina-associated polypeptide 2- β bind to the transcriptional

repressor germ cell-less (19,20). Despite these observations, however, there are few actual demonstrations that nuclear envelope proteins modulate gene regulation by interacting with transcription factors.

MAN1 (also known as LEMD3) is an integral protein of the inner nuclear membrane with a nucleoplasmic N-terminal domain, two transmembrane segments and a C-terminal domain (21). The N-terminal, nucleoplasmic domain contains a LEM domain, a small helix-turn-helix motif also present in emerin, lamin-associated polypeptide 2 and several other proteins (21–23). The C-terminal domain of MAN1 following the second transmembrane segment also faces the nucleoplasm (21). We now show that this domain of human MAN1 interacts with Smad2 and Smad3 and antagonizes signaling by transforming growth factor- β (TGF- β). Very recently, Hellems *et al.* (24) also showed that loss-of-function

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mutations in MAN1 result in osteopoikilosis, Buschke–Ollendorff syndrome and melorheostosis and that these mutations are associated with enhanced TGF- β activity.

RESULTS

MAN1 binds to Smad2 and Smad3

We performed a yeast two-hybrid screen of $\sim 10\,000\,000$ recombinants in a human skeletal muscle cDNA library using the nucleoplasmic, C-terminal domain of MAN1 as bait. The screen yielded eight positive clones that encoded portions of or full-length Smad3. To determine whether the C-terminal domain of MAN1 bound to other Smad proteins and which domains of the proteins mediate their interactions, we performed direct yeast two-hybrid assays. In this assay, the C-terminal domain of MAN1 interacted with Smad2 and Smad3, but not with Smad1 or Smad4 (Fig. 1A). The MH2 domains mediated the binding of these Smads to MAN1 (Fig. 1A). The smallest portion of MAN1 that could bind to Smad2 and Smad3 was from amino acid 730 to amino acid 910 (Fig. 1B). A RNA recognition motif, between amino acid residues 801 and 857 of MAN1, appears to be necessary but not sufficient for binding, as the polypeptide from amino acid 776 to amino acid 910, which contains this motif, did not bind.

To confirm the interactions between the C-terminal domain of MAN1 and Smad2 and Smad3 observed in the yeast two-hybrid assay, we performed glutathione-S-transferase (GST) precipitation assays. In these assays, [35 S]-labeled C-terminal domain of MAN1, synthesized by *in vitro* transcription–translation, was incubated with GST fusions of Smad2 and Smad3. The C-terminal domain of MAN1 bound to Smad2 and Smad3 under stringent conditions including 1% Nonident P-40 or 1.0 M NaCl (Fig. 2). Binding between the MH2 domains of Smad2 and Smad3 and the C-terminal domain of MAN1 was also confirmed in GST precipitation experiments (data not shown).

Co-immunoprecipitation of Smad2 with anti-MAN1 antibodies

To determine whether MAN1 and Smad2 or Smad3 reside in the same complex *in vivo* at endogenous cellular levels, we performed co-immunoprecipitation. Immunization of four rabbits with MAN1 fusion proteins or synthetic MAN1 polypeptides did not yield antibodies that were suitable for immunoblotting or immunoprecipitation (data not shown). However, we were able to use MAN antiserum (25) to immunoprecipitate MAN1. This well-characterized human serum contains high-titer autoantibodies against MAN1, lamina-associated polypeptide-2- β and an unidentified protein with a molecular mass of ~ 35 kDa (21,25,26). As a control, we used serum from a human subject with primary biliary cirrhosis, which contains autoantibodies against the E2-subunit of mitochondrial pyruvate dehydrogenase (27).

Immunoblotting of proteins separated by SDS–polyacrylamide gel electrophoresis showed that HeLa cell lysates contained MAN1, Smad2 and β -tubulin (Fig. 3A). As expected (21,25,26), MAN1 antiserum also recognized lamina-associated polypeptide-2- β (~ 58 kDa) and a lower

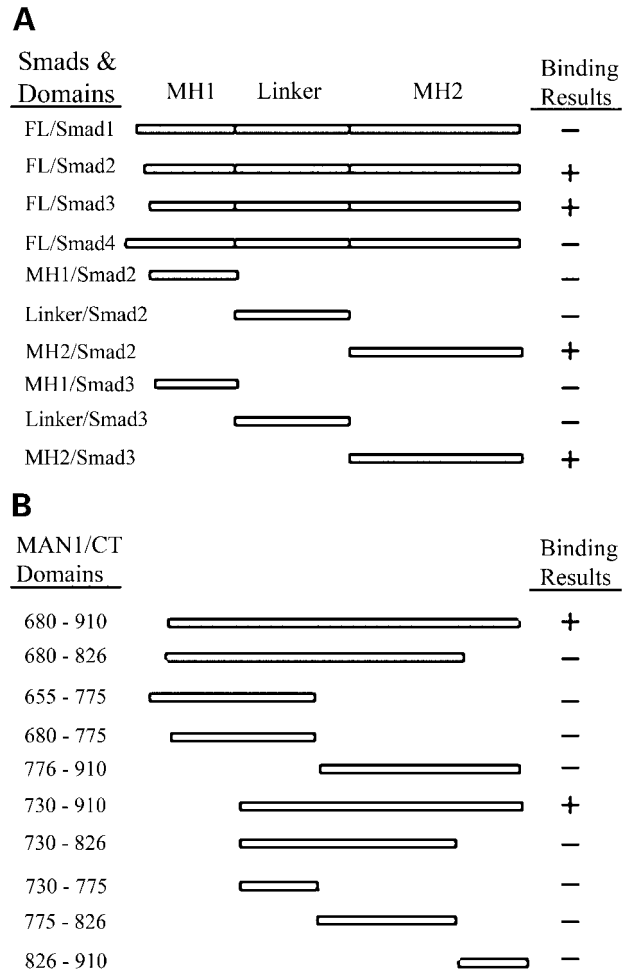


Figure 1. Domain-specific interactions between the C-terminal domain of MAN1 and the Smads in the yeast two-hybrid assay. **(A)** Smad1, Smad2 and Smad3 are divided into three domains, MH1, linker and MH2, as indicated schematically at the top of the diagram. In the yeast two-hybrid assay, the C-terminal domain of MAN1 interacted with full-length (FL) Smad2 and Smad3, but not with Smad1 or Smad4. The MH2 domains of Smad2 and Smad3 interacted with MAN1; the MH1 and linker domains did not. **(B)** Various portions of the MAN1 C-terminal domain were tested for their interactions with Smad2 and Smad3 in the yeast two-hybrid assay. Corresponding amino acid residues of full-length MAN1 are indicated at the left in the diagram. Portions of the MAN1 C-terminal domain from amino acid 680 to amino acid 910 and from amino acid 730 to amino acid 910 interacted with Smad2 and Smad3. Each direct two-hybrid experiment was performed three times; plus indicates three out of three experiments were positive for an interaction and minus indicates three out of three were negative for an interaction.

molecular mass protein (~ 35 kDa). The lysates, in buffer containing weak detergent to solubilize membrane proteins, were incubated with protein A-coupled beads to which antibodies in either MAN antiserum or control serum had been cross-linked. Beads were then extensively washed and bound proteins eluted by boiling in SDS sample buffer. Immunoblotting of proteins retained on the beads coupled to anti-MAN1 antibodies showed that MAN1 and Smad2, but not β -tubulin, bound to beads (Fig. 3B). In contrast, MAN1, Smad2 or β -tubulin bound to beads to which control antibodies in serum from a subject with primary biliary cirrhosis had been coupled (Fig. 3C). The E2-subunit of pyruvate dehydrogenase

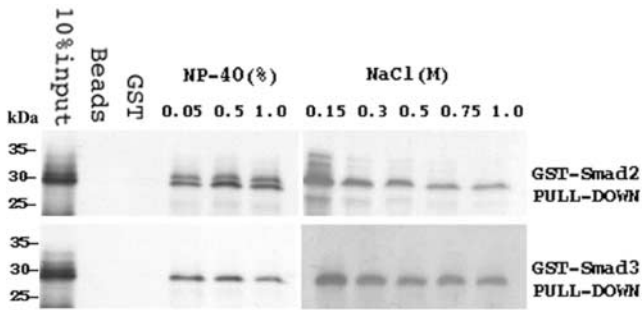


Figure 2. Binding of the C-terminal domain of MAN1 to Smad2 and Smad3 in GST precipitation assays. GST-fusions of Smad2 and Smad3 were incubated with ^{35}S -labeled C-terminal domain of MAN1 in buffers of increasing NaCl and detergent concentrations. GST-fusion proteins were precipitated using glutathione-Sepharose and, after washing with buffer, the bound proteins were stripped with SDS and analyzed by autoradiography of SDS-polyacrylamide slab gels. Upper panel shows autoradiogram from experiment with GST-Smad2 and lower panel with GST-Smad3. Migrations of molecular mass standards (kDa) are indicated at the left of the panels. First lanes show 10% of the ^{35}S -labeled MAN1 C-terminal domain used in each binding reaction. Lanes labeled 'Beads' and 'GST' show lack of binding of C-terminal MAN1 to glutathione-Sepharose alone or coupled to only GST. Lanes labeled 'NP-40%' show binding to GST-Smad2 and GST-Smad3 in the presence of increasing concentrations of Nonidet P-40. Lanes labeled 'NaCl (M)' show binding to GST-Smad2 and GST-Smad3 in increasing molar concentrations of NaCl. Representative autoradiograms shown are from one of three separate experiments.

did bind to these beads (data not shown). Unfortunately, two different commercially available antibodies against Smad3 were not of high enough quality to clearly detect this protein in cell lysates or immunoprecipitates. The anti-Smad2 and anti-Smad3 antibodies also could not immunoprecipitate their recognized antigens under the conditions used. Nonetheless, these results demonstrate that MAN1 and Smad2, at their endogenous levels in cells, are co-immunoprecipitated by anti-MAN1 antibodies.

MAN1 antagonizes transcriptional activation by TGF- β

Smad2 and Smad3 are intracellular mediators of signaling by TGF- β and other TGF- β superfamily members such as inhibin, activin and nodal (28–30). In the basal state, Smad2 and Smad3 are primarily cytoplasmic and translocated to the nucleus after phosphorylation by activated cell surface receptors for TGF- β or other ligands. In the nucleus, Smad2 and Smad3 interact with Smad4 and other co-factors to form a complex that regulates transcription from approximately 500 target genes. We therefore examined whether MAN1 affects TGF- β -activated transcription. HepG2 cells were transiently transfected to overexpress MAN1, its C-terminal nucleoplasmic domain or its N-terminal nucleoplasmic domain. HepG2 expressed endogenous MAN1 (Fig. 4A), and transfected cells expressed the appropriate plasmid-encoded proteins (Fig. 4B). Cells were co-transfected with reporter plasmid p3TP-LUX, which contains a TGF- β -activated plasminogen activator inhibitor-1 promoter that drives luciferase expression (31). Treatment of cells with TGF- β stimulated transcription from the reporter gene in control cells transfected with 'empty' vector and those overexpressing the N-terminal

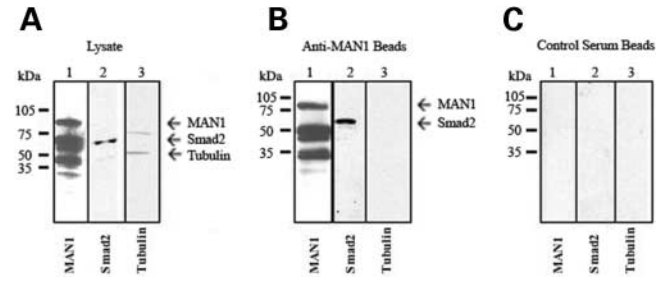


Figure 3. Co-immunoprecipitation showing that MAN1 and Smad2 interact *in vivo* at endogenous levels. (A) Immunoblots showing that HeLa cell lysates used for co-immunoprecipitation contained MAN1, Smad2 and β -tubulin. Proteins in cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose strips and probed with MAN1 antiserum (lane 1, MAN1), anti-Smad2 antibodies (lane 2, Smad2) or anti- β -tubulin antibodies (lane 3, tubulin). In addition to recognizing MAN1, MAN1 antiserum also recognized lamina-associated polypeptide- β -2 (~58 kDa) and a lower molecular mass protein (~35 kDa). Proteins recognized are indicated at the right and migrations of molecular mass standards are indicated at the left. (B) Immunoblots showing that MAN1 and Smad2 are co-immunoprecipitated from HeLa cell lysates. Cell lysates were passed over protein A-coupled beads to which antibodies in MAN1 antiserum had been cross-linked. Beads were extensively washed and bound proteins eluted by boiling in SDS sample buffer. Proteins eluted from beads with SDS were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose strips and probed with MAN1 antiserum (lane 1, MAN1), anti-Smad2 antibodies (lane 2, Smad2) or anti- β -tubulin antibodies (lane 3, tubulin). Proteins recognized are indicated at the right and migrations of molecular mass standards are indicated at the left. (C) Immunoblots showing that MAN1, Smad2 and β -tubulin were not precipitated by control antibodies. Cell lysates were passed over protein A-coupled beads to which antibodies in serum from a subject with primary biliary cirrhosis had been cross-linked. Beads were extensively washed and bound proteins eluted by boiling in SDS sample buffer. Proteins eluted from beads with SDS were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose strips and probed with MAN1 antiserum (lane 1, MAN1), anti-Smad2 antibodies (lane 2, Smad2) or anti- β -tubulin antibodies (lane 3, tubulin). Migrations of molecular mass standards are indicated at the left.

domain of MAN1, which does not bind to Smad2 or Smad3 (Fig. 4C). TGF- β stimulation of the reporter gene was significantly inhibited in cells overexpressing the C-terminal domain of MAN1 and full-length MAN1 (Fig. 4C). Although the results were less dramatic than that observed in HepG2 cells, p3TP-LUX reporter activity was also stimulated by 200 pM TGF- β in Mv1Lu cells transfected with 'empty' vector (28.3 ± 0.98 -fold; mean \pm standard error) and the stimulation was inhibited in Mv1Lu cells transiently transfected to overexpress MAN1 (21.2 ± 0.90 -fold; $P < 0.05$, $n = 2$). These results show that MAN1 antagonizes TGF- β activation of transcription mediated by the transcription factors Smad2 and Smad3.

MAN1 antagonizes TGF- β -induced cell proliferation arrest

TGF- β blocks the proliferation of mink lung epithelial cell line Mv1Lu (32,33). To examine the effects of MAN1 on inhibition of Mv1Lu cell proliferation by TGF- β , we established stable transfected cell lines that overexpress FLAG epitope-tagged full-length MAN1. These cell lines expressed MAN1

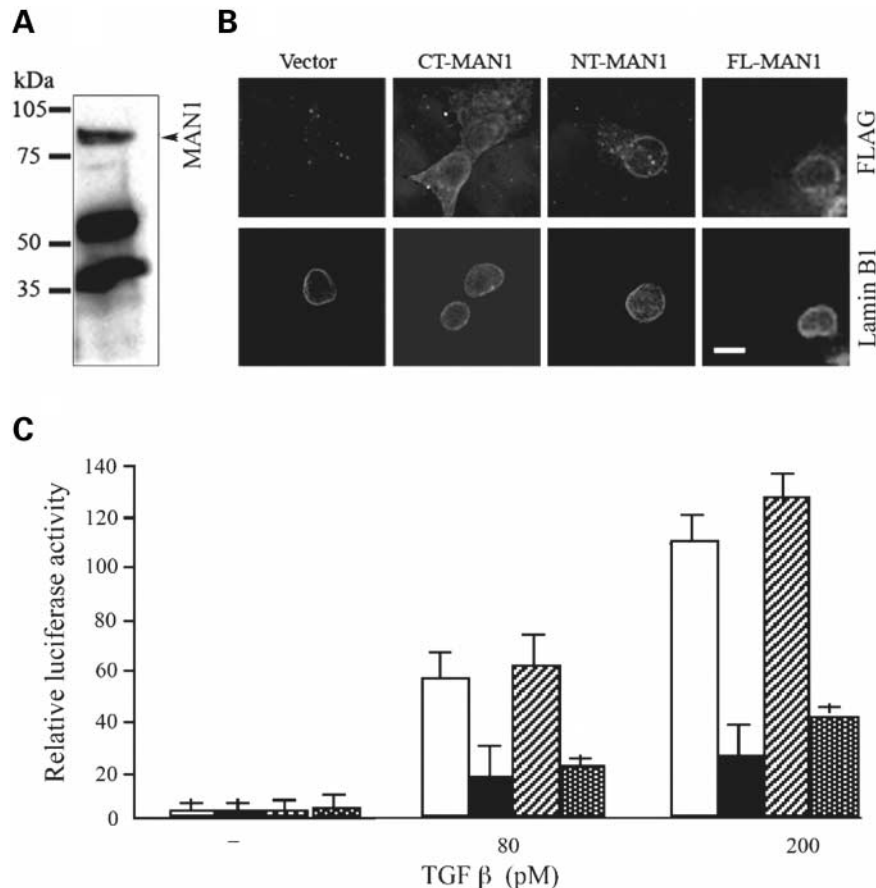


Figure 4. Overexpression of MAN1 inhibits TGF- β stimulated transcription of a plasminogen activator inhibitor-1 promoter-containing reporter gene p3TP-LUX. (A) Immunoblot showing that HepG2 cells express endogenous MAN1. Proteins in HepG2 cell lysates were separated by SDS-polyacrylamide electrophoresis, transferred to nitrocellulose strips and probed with MAN antiserum, which recognizes MAN1, lamina-associated polypeptide-2- β and an unidentified protein with an apparent molecular mass of ~35 kDa. Migration of MAN1 is indicated at right and migrations of molecular mass standards are indicated at left. (B) Immunofluorescence micrographs showing that HepG2 cells expressed FLAG-tagged proteins encoded by transfected plasmids. Transfected HepG2 cells were labeled with mouse anti-FLAG antibodies (top panels, FLAG), rabbit anti-lamin B1 antibodies (bottom panels, lamin B1) and appropriate secondary fluorescent antibodies. Cells were transfected with 'empty' vector (Vector) or plasmids that expressed FLAG-tagged C-terminal domain of MAN1 (CT-MAN1), N-terminal domain of MAN1 (NT-MAN1) and full-length MAN1 (FL-MAN1). Bar: 10 μ m. (C) Luciferase reporter gene activities in transfected HepG2 cells. Cells were co-transfected with p3TP-LUX, pSV- β -galactosidase and either 'empty' vector (white rectangles) or plasmids that expressed the C-terminal domain of MAN1 (black rectangles), the N-terminal domain of MAN1 (diagonal striped rectangles) or full-length MAN1 (spotted rectangles). Cells were treated with TGF- β for 24 h and relative luciferase activities measured in extracts and normalized to β -galactosidase activities. Activities (relative luciferase activity) are shown on the y-axis. Activities in the absence of TGF- β are shown at the left (-), after treatment with 80 pM TGF- β at the center (80) and after treatment with 200 pM TGF- β at the right (200). Values are means for $n = 3$ experiments (in which each sample was analyzed in triplicate); bars indicate standard errors. Stimulation of luciferase activities with 80 and 200 pM of TGF- β was significantly inhibited ($P < 0.01$) in cells overexpressing the C-terminal domain of MAN1 and full-length MAN1.

at the nuclear envelope (Fig. 5A), but cellular levels of Smad2 were not altered (Fig. 5B).

We treated native Mv1Lu cells and Mv1Lu cell lines overexpressing MAN1 with TGF- β . Proliferation of native Mv1Lu cells is inhibited by TGF- β in a concentration-dependent manner, with >50% inhibition of growth at 200 pM (Fig. 6). In contrast, proliferation of two Mv1Lu lines that stably overexpressed MAN1 was not significantly inhibited by TGF- β (Fig. 6).

DISCUSSION

The present results demonstrate that the inner nuclear membrane protein MAN1 is a negative regulator of Smad2 and

Smad3. MAN1 can bind to Smad2 and Smad3, potentially sequester them at the inner nuclear membrane and compete with other Smad-binding proteins for the formation of a transcription activation complex (Fig. 7). Other negative regulators of Smad transcriptional function include c-Ski (34,35), SnoN (36) and Evi-1 (37), which bind directly to Smad proteins. Some of these negative regulators of Smads function by competitive binding that prevents formation of an active transcriptional complex (29,30,38).

Recent reports have shown that two other LEM domain-containing proteins in *Xenopus* bind to Smad1 and antagonize bone morphogenetic protein signaling (39,40). One of these proteins appears to be the *Xenopus* ortholog of MAN1 in the inner nuclear membrane (39). The other, called SANE, lacks the inner nuclear membrane targeting sequences of

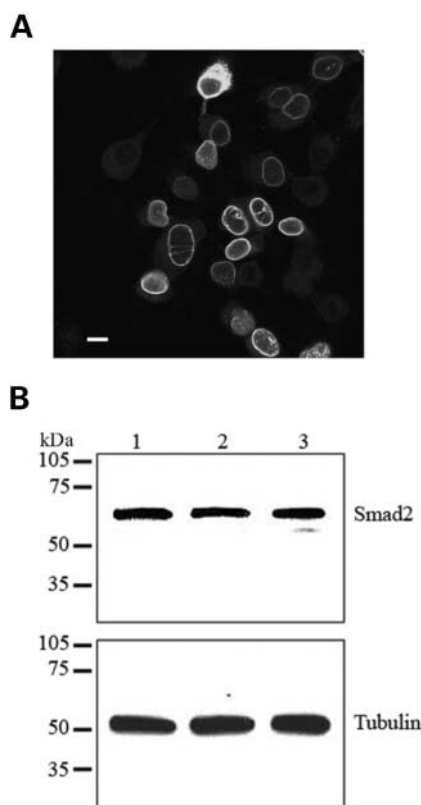


Figure 5. Immunofluorescence micrograph showing expression of FLAG-tagged MAN1 at the nuclear envelope in stably transfected Mv1Lu cells and no change in the cellular levels of Smad2. (A) A stably transfected Mv1Lu cells expressing MAN1 with a FLAG-epitope at the N-terminal. Cells were fixed and analyzed by immunofluorescence microscopy using mouse anti-FLAG antibodies and fluorescein-isothiocyanate conjugated rabbit anti-mouse secondary antibodies. Bar: 10 μ m. (B) Immunoblot showing no change in levels of Smad2 in control Mv1Lu cells and those shown in (A) that express MAN1. Proteins in Mv1Lu cell lysates were separated by SDS-polyacrylamide electrophoresis, transferred to nitrocellulose strips and probed with antibodies that recognize Smad2 (top panel, Smad 2) and β -tubulin (bottom panel, tubulin). Lane 1 shows proteins from untransfected Mv1Lu cells and lanes 2 and 3 show proteins from two different Mv1Lu cells stably transfected to overexpress MAN1 and used in the experiments in Figure 6. Migrations of molecular mass standards are indicated at left.

MAN1 identified by Wu *et al.* (41) and is likely associated with the plasma membrane (40). Although there are structural similarities between Smad1, Smad2 and Smad3, it is intriguing that, in amphibians, the same domain of MAN1 that we have shown binds to Smad2 and Smad3 in mammals appears to bind more strongly to Smad1. However, both Osada *et al.* (39) and Raju *et al.* (40) reported that *Xenopus* MAN1 and SANE bind 'weakly' to Smad2 or Smad3. It is likely that human MAN1 also binds to Smad1 (24); however, for various reasons, the interaction may not have been detected in the assays we used in the current study. These previous reports (24,39,40) and our current results suggest that depending upon the species and possibly other factors, MAN1-like proteins differentially regulate signaling by TGF- β , bone morphogenic protein and possibly other TGF- β superfamily members.

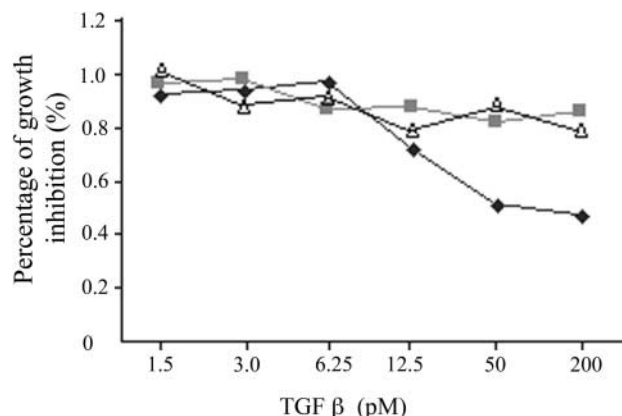


Figure 6. MAN1 inhibition of Mv1Lu cell proliferation arrest by TGF- β . Two Mv1Lu cells lines that stably overexpress MAN1 (rectangles and triangles) were treated with increasing concentrations of TGF- β (x-axis). Cell proliferation was measured and plotted as percent of growth inhibition compared to proliferation in the absence of TGF- β (y-axis). Similar data for untransfected Mv1Lu cells are shown for comparison (black diamonds). Results shown are means for cells grown in three separate wells on two different days for each condition ($n = 6$).

After the experimental work described in this paper was completed, Hellems *et al.* (24) reported that osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis are allelic variants caused by loss-of-function mutations in MAN1. These investigators also showed that MAN1 interacted with Smad1 and Smad2 in the yeast two-hybrid assay and reported that MAN1 overexpression inhibits TGF- β and BMP activation of gene expression (24). Fibroblasts from subjects with these disorders also showed enhanced expression of a TGF- β responsive gene (24). Osteopoikilosis is an autosomal dominant skeletal dysplasia characterized by multiple hyperostotic areas in different parts of the skeleton and can occur either as an isolated anomaly or in association with other abnormalities of skin and bone (42,43). Buschke-Ollendorff syndrome is an autosomal dominant disorder with the association of osteopoikilosis with disseminated connective-tissue nevi (44,45). Melorheostosis is characterized by hyperostosis of tubular bone cortex and is often accompanied by abnormalities of adjacent soft tissues such as joint contractures, scleroderma-like skin lesions, muscle atrophy and hemangiomas (46,47). Differences in the relative degrees of enhanced TGF- β and bone morphogenic protein signaling can explain most or all of these phenotypes.

In addition to bone and skin, signaling by TGF- β superfamily proteins controls specification of developmental state during embryogenesis and in a wide variety of mature tissues (29). In recent years, mutations in the inner nuclear membrane proteins lamins A and C have been linked to a wide range of inherited diseases including autosomal dominant Emery-Dreifuss and other muscular dystrophies, Dunnigan-type partial lipodystrophy, Charcot-Marie-Tooth type 2 peripheral neuropathy, madibuloacral dysplasia and Hutchinson-Gilford progeria syndrome (reviewed in 2-9). MAN1 is associated with the nuclear lamina (25) and nuclear lamins bind to emerin (48-50). These findings suggest the presence of an interconnected network of

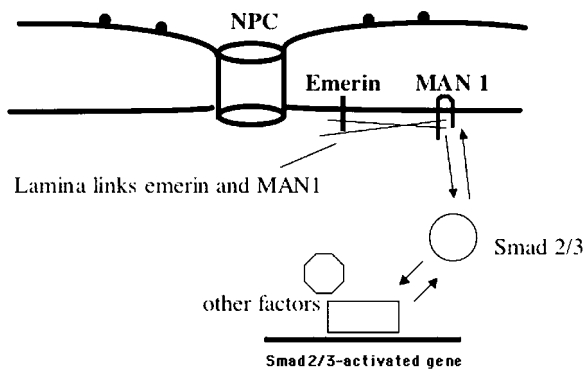


Figure 7. Schematic diagram showing how MAN1 may inhibit Smad2 and Smad3 function within the nucleus. MAN1 binds Smad2 and Smad3 and may compete with their binding to regulatory regions of Smad2/3-activated genes. Other factors are also necessary for transcriptional regulation of these genes. Inactivating mutations in MAN1 have been shown to result in osteopetrosis, Buschke–Ollendorff syndrome and melorheostosis (24). As MAN1 may be part of an interconnected network of MAN1, lamins and emerlin at the inner nuclear membrane, mutations in emerlin and lamins may also lead to alterations in MAN1 localization and its interactions with Smad2 and Smad3 in different tissues, such as striated muscle, and this could potentially play a role in diseases such as Emery–Dreifuss muscular dystrophy.

MAN1, lamins and emerlin at the inner nuclear membrane (Fig. 7). As MAN1 is a regulator of signaling by the TGF- β superfamily of cytokines and has physical connections to lamins and overlapping functions with emerlin (25,51), it is possible that mutations in lamins A and C and emerlin cause diseases by altering MAN1 function in certain cell types. This could lead to tissue-specific alterations in signaling by TGF- β superfamily members. Notably, myostatin, a TGF- β superfamily member, signals via Smad2 and Smad3 and decreases skeletal muscle mass by inhibiting myoblast proliferation and differentiation (52–54). Loss of MAN1 function at the inner nuclear membrane in striated muscle could therefore lead to an enhancement in myostatin signaling. This hypothesis warrants testing in X-linked Emery–Dreifuss muscular dystrophy, which is caused by mutations in emerlin (55), and autosomal dominant Emery–Dreifuss muscular dystrophy and limb girdle muscular dystrophy type 1 B, which are caused by mutations in lamins A and C (56,57).

MATERIALS AND METHODS

Yeast two-hybrid screening and assays

Human skeletal muscle pre-transformed Matchmaker cDNA library (HY4047AH; Clontech) was screened according to the manufacturer's instructions using the C-terminal domain of MAN1 as bait. To construct the bait plasmid, DNA encoding amino acid 681 to amino acid 911 of MAN1 was amplified by polymerase chain reaction using MAN1 full-length cDNA (21) as template. Amplified DNA was cloned in-frame into the GAL4 DNA binding domain fusion vector pGBKT7 (Clontech) to yield pGBKT7-MAN1-CT. *Saccharomyces cerevisiae* strain AH109 was transformed with this bait plasmid, and the

library recombinants in the GAL4 activation domain fusion vector pACT2 were pre-transformed in yeast host strain Y187. Before two-hybrid library screening using yeast mating, the optimal concentration of 3-aminotriazole was titrated to eliminate background selection, and the phenotypes and mating efficiencies were checked by controls. Positive pACT2-derived plasmids were rescued and used to co-transform yeast with pGBKT7-53 (Clontech) and pGBKT7 to confirm the specificities of the reactions. DNA inserts of positive clones obtained from library screening and the bait construct was performed on a 373 Sequencer (Applied Biosystems) at the Columbia University Cancer Center DNA Core Facility. Sequence analysis was performed using applications available via the Internet at the National Center for Biotechnology Information World Wide Website (<http://www.ncbi.nlm.nih.gov>).

For direct yeast two-hybrid interaction assays, DNAs encoding full-length Smad1, Smad2, Smad3, Smad4, various domains of Smad2 and Smad3 and portions of the C-terminal domain of MAN1 were generated by polymerase chain reactions and cloned into pGBKT7 and pACT2. To generate Smad1, Smad2 and Smad4 PCR products for cloning, plasmids containing the cDNA sequences, kindly provided by Dr Joan Massagué (Memorial Sloan-Kettering Cancer Center), were used as templates. The full-length Smad3 cDNA template for polymerase chain reaction was a positive pACT2 clone isolated in the two-hybrid screen. MAN1 cDNA (21) was used as template to generate polymerase chain reaction products encoding parts of the C-terminal domain. All plasmid constructs were confirmed by DNA sequencing. Yeast two-hybrid assays were performed according to the instructions of the manufacturer (Clontech).

GST precipitation assays

DNAs encoding Smad2, Smad3 and their various domains were generated by polymerase chain reaction as described earlier and cloned in-frame into appropriate pGEX plasmids (Amersham Pharmacia Biotech) to express GST fusion proteins. All constructs were confirmed by DNA sequencing. GST fusion proteins were expressed in *Escherichia coli* using standard methods (58). For *in vitro* transcription–translation of the C-terminal tail domain of MAN1, pGBKT7-MAN1-CT was used in the TNT T7 Coupled Reticulocyte Lysate System (Promega) containing L-[35 S]-methionine (Amersham Pharmacia Biotech). GST precipitation assays were performed as described previously (16,17).

Co-immunoprecipitation

Two 150 mm Petri dishes of 90% confluent HeLa cells were washed three times with phosphate-buffered saline and then scraped and collected in a buffer of 10 mM HEPES (pH 7.4), 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride and 1 mM MgCl₂. Cells were placed on ice for 15 min and

broken in a Dounce homogenizer. Broken cells were then centrifuged at 6000g for 20 min at 4°C in a J2-21M centrifuge (Beckman) with a J-20 rotor. The pellet was resuspended in 400 µl buffer containing 50 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (pH 8.0), 5 mM ethylenediaminetetraacetic acid, 0.2% bovine serum albumin, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride and 0.1% Nonident P-40. Cells were then sonicated with a tip sonicator and centrifuged at 4°C for 30 min at 13 000 rpm in a 5415D microcentrifuge (Eppendorf). The preparation was then incubated with antibody-coupled protein-A beads prepared according to the protocol provided with the Seize X Immunoprecipitation Kit (Pierce Biotechnology). Antibodies in human MAN antiserum (kindly provided by Dr Micheline Paulin-Levasseur) (21,25) or control human serum from a subject with primary biliary cirrhosis (27) were coupled to beads, and immunoprecipitation was performed according to the Seize X Immunoprecipitation Kit protocol. Proteins were eluted from the beads by boiling in denaturing SDS sample buffer, separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with diluted MAN antiserum, rabbit anti-Smad2 antibodies (Zymed Biotechnology) and rabbit anti-β-tubulin antibodies (Santa Cruz Biotechnology).

Transcription-response assays

Transcriptional-response assays were performed essentially as described by Tanaka *et al.* (59) except that LipofectAmine (Gibco BRL) was used instead of a calcium phosphate for cell transfection. HepG2 or Mv1Lu cells were seeded at a density of 10 000 per 60 mm plate and co-transfected 24 h later with 2 µg of the reporter plasmid p3TP-LUX (kindly provided by Dr Joan Massagué), 2 µg of pSV-β-galactosidase plasmid (Promega) and 2 µg of plasmids encoding MAN1, its N-terminal domain or its C-terminal domain (21,41). Endogenous MAN1 was detected in HepG2 cells by immunoblotting using MAN antiserum. Expression of proteins encoded by transfected plasmids was confirmed by immunofluorescence microscopy using anti-FLAG antibodies (Sigma). Immunofluorescence microscopy was performed using a Zeiss LSM 410 confocal laser scanning system attached to a Zeiss Axiovert 100TV inverted microscope (Carl Zeiss, Inc.) as described previously (21,41). Images were processed using Photoshop software (Adobe Systems) on a Macintosh G3 computer (Apple Computer). Cells were treated with TGF-β1 (Sigma) for 24 h, and the relative luciferase activities in cell extracts were measured using a luminometer (Monolight 2010) and the Luciferase Assay System (Promega). To correct for differences in transfection efficiency between experiments, luciferase activities were normalized to β-galactosidase activities.

Cell proliferation assay

Full-length MAN1 cDNA with a FLAG epitope tag coding region at the N-terminal (21) was cloned into pcDNA3.1-myc-His(-)B (Invitrogen). This plasmid was used to establish stable transfected Mv1Lu cell lines, which expressed

full-length MAN1 with myc, histidine and FLAG epitope tags. Expressed protein was detected by immunofluorescence microscopy with anti-FLAG antibodies as described earlier. CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) was used to determine the number of viable cells. Cells were seeded in 96 well plates with approximately 5000 cells in 100 µl of medium and incubated with TGF-β1 at serial 2-fold dilutions. After 48 h of incubation, 20 µl of CellTiter 96 AQueous One Solution reagent was added per well and incubated for 2 h. Absorbance at 490 nm was then measured using a 96-well plate reader.

Materials

Unless otherwise indicated, routine chemical reagents were purchased from Sigma or Fisher. Enzymes for molecular biology were obtained from New England Biolabs, Invitrogen or Fisher.

ACKNOWLEDGEMENTS

This work was supported by a grant from Muscular Dystrophy Association. The confocal microscopy facility used for part of this project was established by National Institutes of Health Grant 1S10-RR10506 and supported by National Institutes of Health Grant 5P30-CA13696 as part of the Herbert Irving Cancer Center at Columbia University. We thank Dr Joan Massagué (Memorial Sloan-Kettering Cancer Center) and Dr Micheline Paulin-Levasseur (University of Ottawa) for providing essential reagents for this project.

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