Identification of Smad2, a Human Mad-related Protein in the Transforming Growth Factor β Signaling Pathway*

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Transforming growth factor-β (TGF-β) superfamily members are multifunctional cytokines that exert their effects via heteromeric complexes of two distinct serine and threonine kinase receptors. Drosophila mothers against dpp and related genes in Caenorhabditis elegans, Xenopus, and mammals were shown to function downstream in the intracellular signaling pathways of TGF-β superfamily members. Here we report the cloning of a Mad-related protein, termed Smad2 and Mad-related protein 2 (Smad2). TGF-β stimulated the phosphorylation and nuclear translocation of Smad2 in nontransfected Mv1Lu cells. In addition, we demonstrated that TGF-β and activin mediated phosphorylation of Smad2 after its overexpression with appropriate type I and II receptors in COS cells. Smad2 and Smad1 were found to be broadly expressed in human tissues. Smad2 is closely linked to DPC4 on chromosome 18q21.1, a region often deleted in human cancers. Cells that lack Smad2 may escape from TGF-β-mediated growth inhibition and promote cancer progression.

Transforming growth factor β (TGF-β)1 is the prototype of a family of structurally related cytokines, which also includes activins and bone morphogenetic proteins (BMPs). TGF-β superfamily members are multifunctional and have been shown to control proliferation, differentiation, migration, and apoptosis of many different cell types (1). Signaling of these proteins occurs via ligand-mediated hetero-oligomerization of type I and II receptors, which are both endowed with intrinsic serine and threonine kinase activities (2). The TGF-β and activin type II receptors are constitutively active kinases that transphosphorylate their specific type I receptors on association. The type I receptors thereby become activated, which is necessary and sufficient for most signaling responses (3–5). The functionally active receptor complex may be heterotetrameric, consisting of two molecules of each receptor type (6).

Targets downstream of serine and threonine kinase receptors that provide the link with the transcriptionally induced effects of TGF-β superfamily members are poorly understood. TGF-β-like signaling pathways are present in Drosophila and Caenorhabditis elegans. Genetic analysis of signaling mediated by decapentaplegic, a member of the TGF-β superfamily in Drosophila, has led to the identification of mothers against dpp (Mad) (7), a gene the activity of which is controlled by Dpp. Genetic evidence suggests that Mad is an essential downstream component for all Dpp-dependent signaling events (7–10). In addition, Mad homologous genes have recently been identified in C. elegans (smn genes) (11) and Xenopus (12) and mammals (Smad genes) (10, 13–15). A putative tumor suppressor gene function in pancreatic cancer was ascribed to DPC4 (14). The Mad family of proteins lacks known sequence motifs but has conserved N-terminal (MH1) and C-terminal (MH2) domains, which are linked by a sequence rich in serine, threonine and proline residues. Smad1 is phosphorylated and translocated to the nucleus after stimulation of BMP-2 (10). In addition, the C-terminal domain of Smad1 was found to have transcriptional activity when fused to a heterologous DNA binding domain. The whole Smad1 protein was found to be transcriptionally latent and became activated on BMP receptor-mediated phosphorylation (13). In Xenopus, Smad1 induced ventral mesoderm, a BMP-like response, and Smad2 induced dorsal mesoderm, a Vg1- and nodal-like response (12). These results suggest that Mad family members may encode transcription factors that mediate distinct responses to TGF-β family members.

Here we demonstrate that the TGF-β-induced phosphorylation and nuclear translocation of a ubiquitously expressed human Mad-related protein, Smad2. The chromosomal location of Smad2 is close to DPC4 on chromosome 18q21.1, a region often deleted in cancer, suggesting a putative tumor suppressor function for Smad2.

EXPERIMENTAL PROCEDURES

cDNA Cloning—Smad1 was cloned by reverse transcription-polymerase chain reaction (RT-PCR) from placenta tissue using the primers 5′-TATAACTAGTCTGTGCTATTAG-3′ and 5′-TCGAAGGCTCAATACTTTCAT-3′ that were based on the expression sequence tags (ESTs) R19015 and R10104 from the Washington University-Merck EST Project. Smad2 was identified by RT-PCR from human erythroleukemia cells using a degenerated sense primer, 5′-CCGAATTCTGGATCCATCTACTAAC/CTCCACCTAC-3′, and an antisense primer, 5′-CCGAGATCCGAT/RACTCA/CTAGT/TTAGCTA/CAAG-3′, that were based on regions of sequence similarity between the Drosophila Mad and C. elegans Smn genes. The 0.5-kb insert of the Smad2 PCR recombinant was used as a probe in the screening of a human brain cDNA library, and the sequence of a 2-kb Smad2 cDNA clone was determined.

Northern Blot Analysis—Northern blot filters with mRNAs from different tissues were obtained from Clontech. cDNA restriction fragments encoding complete coding regions of Smad1 and Smad2 were used as probes. The filters were hybridized and washed as described previously (16).

Constructs—Expression constructs for TβR-I, TβR-II, activin type IB receptor (ActR-IB), and activin type II receptor (ActR-II) were previously described (16). Smad1-Flag in pCMV5 was obtained from Dr. J.
FIG. 1. Smad1 and Smad2 are ubiquitously expressed. Northern blots with mRNA prepared from different tissues were hybridized with specific probes for Smad1 and Smad2. Lane 1, pancreas; lane 2, kidney; lane 3, skeletal muscle; lane 4, liver; lane 5, lung; lane 6, placenta; lane 7, brain; lane 8, heart; lane 9, spleen; lane 10, thymus; lane 11, prostate; lane 12, testis; lane 13, ovary; lane 14, small intestine; lane 15, colon; lane 16, peripheral blood leukocyte. Each lane contained 2 μg of mRNA from the indicated tissues. Smad1 and Smad2 transcripts are indicated by arrows. Autoradiograms from the hybridized blots are shown.

FIG. 2. Smad2 is a 58-kDa protein, which is specifically recognized by anti-Smad2 antiserum. A, COS cells transfected with expression plasmid containing Smad2-His/Flag were metabolically labeled and immunoprecipitated by SED or Flag antiserum or preimmune serum. Blocking of the SED antiserum was performed by addition of 10 μg of peptide to the immunoprecipitate reaction. B, cell lysates from metabolically labeled Mv1Lu cells were subjected to precipitation using preimmune serum (pre), or SED antiserum. Blocking of the immune serum was performed with 10 μg of peptide. The migration of Smad2 is indicated by arrows. Precipitates were analyzed by SDS-PAGE and visualized using a Fuji-X BioImager (A) or autoradiography (B).

Smad2 cDNA probe containing the complete coding region. The mapping of Smad2 was also confirmed and refined by FISH. The yeast artificial chromosome (YAC) Y739A3 was cohybridized with a chromosome 18 α satellite probe (D18Z1; Oncor) as described previously (20). PCR Analysis of YAC DNA—Three Centre d’Etude du Polyomavirus Human YACs, Y747A6, Y945B11, and Y739A3 (obtained from Genome Systems, Inc.), known to contain DPC4 and the Mad-related gene JV18–1 (15, 21), were analyzed by PCR for the presence of Smad2 sequences using two primer pairs (sense primer1, 5’-TCGAAAAGGAT-TGCCACAT-3; antisense primer 1, 5’-AGGGTTTACACATACTGCAATCT-3; antisense primer 2, 5’-GCTTGAGCAACGCACTGA-3) corresponding to exons 2 and 11 of Smad2.

RESULTS
cDNA Cloning and mRNA Expression of Smad1 and Smad2—A data base search for mammalian sequences related to the Mad gene revealed the existence of multiple human and mouse ESTs corresponding to different genes. PCR using placenta cDNA with two Mad-related EST primers resulted in the isolation of Smad1 (10, 13). The closely related Smad2 was identified by RT-PCR using human erythroleukemia cell mRNA with two degenerate primers that were based on short stretches of sequence identity between Drosophila Mad (7) and C. elegans Smn gene (11). A cDNA encoding the complete Smad2 protein was obtained from a human brain cDNA library. As reported previously by others [10, 13, 15, 22], the cDNA sequences predict that Smad1 and Smad2 are proteins of 465 and 467 amino acid residues, respectively.

The distribution of Smad1 and Smad2 in various human tissues was determined by Northern blot analysis of mRNA (Fig. 1). One Smad1 transcript of approximately 3.2 kb was
were labeled with \([^{32}\text{P}]\)orthophosphate and treated with 10 ng/ml TGF-\(\beta\)-1 or 30 ng/ml activin. For kinetics of Smad2 phosphorylation, Mv1Lu cells were stimulated with 10 ng/ml TGF-\(\beta\)-1 for the indicated times. A representative experiment of three experiments is shown. Cell lysates were subjected to immune precipitation with SED antiserum and analyzed by SDS-PAGE. C, phosphorylated Smad2 was subjected to phosphoamino acid analysis. The migration of phosphorylated serine (S), threonine (T), and tyrosine (Y) that were used as standards are shown. D, COS cells transfected with Smad1-Flag, Smad2 alone, or the indicated type I and II receptors were labeled with \([^{32}\text{P}]\)orthophosphate, immunoprecipitated with anti-Flag antibodies or SED antiserum, and analyzed by SDS-PAGE. COS cells were treated with 10 ng/ml TGF-\(\beta\)-1 or 100 ng/ml activin (A) where indicated. In parallel, we confirmed the expression of type I and II receptors and Smads by affinity labeling with iodinated ligands and immunoprecipitation of metabolically labeled cells, respectively (data not shown). Autoradiographs are shown.

Smad2 Encodes a 58-kDa Protein in Mv1Lu Cells—After transfection of Smad2-Flag/His in COS cells and metabolic labeling with \([^{35}\text{S}]\)methionine and \([^{35}\text{S}]\)cysteine, a cell extract was prepared and subjected to immunoprecipitation using Flag antiserum or a Smad2 antiserum, termed SED, which was raised against a peptide corresponding to amino acid residues 227–244 in the proline-rich region of Smad2. This region is divergent in sequence among the Smads. With both Flag and SED antiserum a component of 62 kDa was specifically immunoprecipitated, which was not seen when preimmune serum was used or when excess cognate SED peptide was added together with the SED antiserum (Fig. 2A). Moreover, it was not detectable in samples derived from untransfected COS cells. When overexpressed in COS cells, Smad1 and DPC4 were not immunoprecipitated with SED antiserum (data not shown), indicating that SED antiserum does not cross-react with these proteins. The SED antiserum precipitated a 58-kDa component from Mv1Lu cells. This component was not observed when preimmune serum was used or when excess cognate SED peptide was added together with the SED antiserum (Fig. 2A). Moreover, it was not detectable in samples derived from untransfected COS cells. When overexpressed in COS cells, Smad1 and DPC4 were not immunoprecipitated with SED antiserum (data not shown), indicating that SED antiserum does not cross-react with these proteins. The SED antiserum precipitated a 58-kDa component from Mv1Lu cells. This component was not observed when preimmune serum was used or when excess cognate SED peptide was added together with the SED antiserum (Fig. 2A). Moreover, it was not detectable in samples derived from untransfected COS cells. When overexpressed in COS cells, Smad1 and DPC4 were not immunoprecipitated with SED antiserum (data not shown), indicating that SED antiserum does not cross-react with these proteins. The SED antiserum precipitated a 58-kDa component from Mv1Lu cells. This component was not observed when preimmune serum was used or when excess cognate SED peptide was added together with the SED antiserum (Fig. 2A). Moreover, it was not detectable in samples derived from untransfected COS cells. When overexpressed in COS cells, Smad1 and DPC4 were not immunoprecipitated with SED antiserum (data not shown), indicating that SED antiserum does not cross-react with these proteins. The SED antiserum precipitated a 58-kDa component from Mv1Lu cells. This component was not observed when preimmune serum was used or when excess cognate SED peptide was added together with the SED antiserum. The observed mass of 58 kDa for Smad2 is somewhat higher than the predicted size of 52.5 kDa predicted from the cDNA sequence.

**Ligand-induced Phosphorylation of Smad2**—The effect of TGF-\(\beta\) on the phosphorylation of Smad2 was analyzed using \([^{32}\text{P}]\)orthophosphate-labeled Mv1Lu cells. Smad2 was not appreciably phosphorylated in the absence of ligand in Mv1Lu cells. The phosphorylation of Smad2 was strongly induced after stimulation with TGF-\(\beta\)-1 (Fig. 3A). Significant phosphorylation was observed 15–30 min after addition of TGF-\(\beta\)-1 and plateaued after about 2 h of incubation at 37 °C (Fig. 3B). Activin A stimulated no (or in some experiments weak) increase in Smad2 phosphorylation in Mv1Lu cells (Fig. 3A). BMP-2 and BMP-7 did not stimulate the phosphorylation of Smad2 in Mv1Lu cells (data not shown). Phosphoamino acid analysis of phosphorylated Smad2 after TGF-\(\beta\)-stimulation revealed that phosphorylation was mainly on serine, little on threonine, and not on tyrosine residue(s) (Fig. 3C).

Phosphorylation of Smad2 was also investigated using \([^{32}\text{P}]\)orthophosphate-labeled COS cells, transfected with Smad2 alone or cotransfected with T\(\beta\)R-II and T\(\beta\)R-I, in the absence or presence of TGF-\(\beta\). Analysis of the Smad2 immunoprecipitates showed that Smad2 in the absence of overexpressed receptor and ligand was not (or weakly) phosphorylated. The observed increase in Smad2 phosphorylation after cotransfection with receptors is most likely due to ligand-independent complex formation of a T\(\beta\)R-I and T\(\beta\)R-II complex induced by their high overexpression. Further receptor activation by TGF-\(\beta\)-1 addition led to a strong increase in the Smad2 phosphorylation (Fig. 3D). The phosphoamino acid analysis pattern and two-dimensional trypic phosphopeptide maps of phosphorylated Smad2 from transfected COS cells and Mv1Lu

**FIG. 3. Ligand-induced phosphorylation of Smad2.** A and B, Mv1Lu cells were labeled with \([^{32}\text{P}]\)orthophosphate and treated with 10 ng/ml TGF-\(\beta\)-1 or 30 ng/ml activin. For kinetics of Smad2 phosphorylation, Mv1Lu cells were stimulated with 10 ng/ml TGF-\(\beta\)-1 for the indicated times. A representative experiment of three experiments is shown. Cell lysates were subjected to immune precipitation with SED antiserum and analyzed by SDS-PAGE. C, phosphorylated Smad2 was subjected to phosphoamino acid analysis. The migration of phosphorylated serine (S), threonine (T), and tyrosine (Y) that were used as standards are shown. D, COS cells transfected with Smad1-Flag, Smad2 alone, or the indicated type I and II receptors were labeled with \([^{32}\text{P}]\)orthophosphate, immunoprecipitated with anti-Flag antibodies or SED antiserum, and analyzed by SDS-PAGE. COS cells were treated with 10 ng/ml TGF-\(\beta\)-1 or 100 ng/ml activin (A) where indicated. In parallel, we confirmed the expression of type I and II receptors and Smads by affinity labeling with iodinated ligands and immunoprecipitation of metabolically labeled cells, respectively (data not shown). Autoradiographs are shown.

**FIG. 4. Nuclear accumulation of Smad2 is induced by TGF-\(\beta\).** Mv1Lu cells were incubated in the absence or presence of 10 ng/ml TGF-\(\beta\)-1 for 1 h. Smad2 was localized in the cells by immunofluorescence using SED antiserum. Staining was predominant in the cytoplasm in the absence of TGF-\(\beta\), whereas nuclear staining was observed with TGF-\(\beta\) stimulation.
of the somatic cell hybrid panel used for mapping of Smad2. The breakpoints in hybrids GM12088 and GM12083 are only approximate (19). This panel allows assignment of genes to at least six different intervals on chromosome 18. Southern blot analysis showed that the presence of Smad2-specific sequences correlated with the presence of the region 18cen → q21.3. The presence (+) or absence (−) of Smad2 sequences in the hybrid lines are indicated. B, chromosomal sublocalization of Smad2 by FISH using YAC Y793A3 as probe. YAC DNA (green signal) was co-hybridized with a chromosome 18-specific α satellite probe (red signal). Note the presence of Smad2-specific hybridization signals on both chromosomes 18 at q21.1. Chromosomes are counterstained in blue with 4,6-diamino-2-phenylindole.

**FIG. 5. Chromosomal location of Smad2 suggests a tumor suppressor gene function.** A, ideogram showing the chromosome 18 content of the somatic cell hybrid panel used for mapping of Smad2. The breakpoints in hybrids GM12088 and GM12083 are only approximate (19). This panel allows assignment of genes to at least six different intervals on chromosome 18. Southern blot analysis showed that the presence of Smad2-specific sequences correlated with the presence of the region 18cen → q21.3. The presence (+) or absence (−) of Smad2 sequences in the hybrid lines are indicated. B, chromosomal sublocalization of Smad2 by FISH using YAC Y793A3 as probe. YAC DNA (green signal) was co-hybridized with a chromosome 18-specific α satellite probe (red signal). Note the presence of Smad2-specific hybridization signals on both chromosomes 18 at q21.1. Chromosomes are counterstained in blue with 4,6-diamino-2-phenylindole.

**DISCUSSION**

Recently, a conserved cytoplasmic family of Mad-like proteins, termed Smads, was identified and shown to play a pivotal role in the downstream signal transduction pathway of various TGF-β superfamily members (24). In the present study we report the identification of a human Mad-related protein, Smad2, which acts in the TGF-β signaling pathway.

Smad1 and Smad2 have 66% overall sequence identity, with the highest level of similarity in the MH1 and MH2 domains. The proline-rich intervening region is divergent, and a SED satellite probe (22) recently demonstrated Smad2 regulation by TGF-β.

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mously induced by Smad1 (ventral mesoderm) and Smad2 (dorsal mesoderm) in Xenopus (12) resemble the ligand-specific activation of Smad1 and Smad2, as detected by biochemical means. However, Lechleider et al. (27) and Yingling et al. (28) reported the phosphorylation of endogenous Smad1 (or immunologically related Smads) in response to TGF-β. The latter result may suggest a functional redundancy of Smads in TGF-β and BMP signal transduction. Differences may also prevail between different cell types. In addition, there may be a need for multiple Smads in TGF-β signaling. It will be interesting, therefore, to test whether TGF-β can induce an association between Smad2 and other Smads, e.g., Smad1 and DPC4. Involvement of multiple Mads in a TGF-β-like signaling pathway was proposed for the C. elegans MAD homologs, as mutant phenotypes for sma-2, sma-3, and sma-4 all closely resemble the mutant phenotype of daf-4 (11).

TGF-β stimulated the nuclear accumulation of Smad2 in Mv1Lu cells. Recently, Baker and Harland (29) reported the induction of nuclear localization of Smad2 by activin in Xenopus explants. In addition, they observed that the N-terminally truncated Smad2 is localized in the nucleus in the absence of ligand. It is unclear whether phosphorylation provides the trigger for nuclear accumulation, e.g., by unmasking a nuclear localization signal for active nuclear entry or by inducing the dissociation from a putative cytoplasmic retention protein.

Smad2 was localized to chromosome 18q21.1 by a combination of mapping techniques. Using YACs we could demonstrate that Smad2 and DPC4 are closely linked within a 3-Mb region, with Smad2 being proximal to DPC4. The 18q21 chromosomal region is frequently deleted or rearranged in a variety of human cancers. Recently, DPC4 was found to be frequently homozygously deleted or mutated in pancreatic cancers (14) but only rarely in other types of cancers with chromosome 18q alterations (25). The interesting possibility that Smad2 has a tumor suppressor function in these other cancers or in pancreatic cancer needs to be examined. In agreement with our results, Riggins et al. (15) and Eppert et al. (22) reported the chromosomal assignment of Smad2 to 18q21 by PCR analysis of YAC clones. In addition, both groups found that certain colorectal cancers contain somatic mutations in Smad2.

The elucidation of intracellular signal transduction pathways by which TGF-β executes its multifunctional effects is of great importance for a better understanding of the (patho)physiological processes in which TGF-β has been implicated and for future therapeutic interventions. In this respect the identification of Smad2 as an intracellular phosphorylation target in the TGF-β signaling pathway is an important finding. The identification of the upstream Smad2 activating serine and threonine kinase and the downstream Smad2 nuclear effectors that result in the transcriptional modulation of Smad2 target genes will be the subject of future studies.

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