

Activin A-Induced HepG2 Liver Cell Apoptosis: Involvement of Activin Receptors and Smad Proteins*

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ABSTRACT

A balance between cell proliferation and apoptosis is important for regulating normal liver function. Proteins of the transforming growth factor- β superfamily are known to be important mediators of apoptosis in the liver. In this study we demonstrate that activin A potently induces apoptotic cell death in a hepatoma cell line, HepG2 cells. To determine the roles of activin receptors and downstream signaling proteins in activin A-induced apoptosis in these cells, the activin signaling pathway was analyzed using the transcription of an activin-responsive reporter gene, p3TP-Lux, as an assay. Although individual activin receptors had little effect on transcriptional activity, coexpression of an activin type I receptor and a type II receptor significantly increased both basal and activin-induced transcriptional activation, with the combination of receptors IB and IIB being the most potent. Similarly, expression of individual Smad proteins had only a

modest effect on reporter gene activity, but the combination of Smad2 and Smad4 strongly stimulated transcription. Activin signaling induced a rapid relocation of Smad2 to the nucleus, as determined using a green fluorescence protein-Smad2 fusion protein. In contrast, green fluorescence protein-Smad4 remained localized to the cytoplasm unless it was coexpressed with Smad2. In agreement with the transcriptional response assays, overexpression or suppression of activin signaling components in HepG2 cells altered apoptosis. Overexpression of receptors IB and IIB or Smad proteins 2 and 4 stimulated apoptosis, whereas dominant negative mutant forms of the activin type IIB receptor or Smad2 blocked activin-stimulated apoptosis. These studies suggest that signaling from the cell surface to the nucleus through Smad proteins is a required component of the activin A-induced cell death process in liver cells. (*Endocrinology* 141: 1263–1272, 2000)

APOPTOSIS IS A controlled form of cell death involving the activation of an intracellular protease cascade, eventually leading to membrane blebbing, nuclear condensation, and DNA fragmentation (1). Apoptosis is essential for normal development, host defense, and suppression of oncogenesis. In liver, apoptosis is known to occur in hepatocytes and is an important part of the tightly controlled homeostatic mechanisms regulating liver function (2). One of the distinguishing features of liver is that it has a tremendous regenerative capacity in response to cell loss through physical, infectious, or hepatotoxic injury. This process requires apoptosis to fine tune the extent to which the organ regenerates (2). Abnormalities of liver regeneration caused by an imbalance between cell growth and apoptosis may contribute to chronic hepatitis, cirrhosis, and liver cancer (3). Furthermore, failure of apoptosis to delete genetically altered cells appears to contribute to the process of hepatocarcinogenesis (4, 5). In contrast to the large number of hepatotropic factors that are known, very few negative regulators, such as growth inhibitors and apoptotic inducers, have been identified and characterized in the liver (2). Activin, a member of the transforming growth factor- β (TGF β) superfamily, is one of the few identified negative regulators of liver cell mass, and it appears to act by induction of apoptosis (6). However,

the molecular mechanisms that mediate activin-induced apoptosis remain poorly defined.

Activins are formed by the combinatorial assembly of two closely related subunits, β_A and β_B , generating three dimeric isoforms, activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$), and activin AB ($\beta_A\beta_B$). Although originally identified as a gonadal peptide hormone capable of stimulating the release of FSH from the pituitary, activin has a wide variety of biological functions, including the regulation of cell proliferation and differentiation, and induction of mesoderm tissues in amphibian development (7). Several lines of evidence suggest an important role for activin in the liver. Injection of [125 I]activin A into rats has identified the liver as a major target of activin binding or clearance (8). In inhibin α -subunit-deficient mice, activin levels are elevated, leading to a severe cancer cachexia-like wasting syndrome that includes hepatocellular death in the liver (9). Infusion of recombinant activin A in mice and rats causes a marked reduction in liver mass resulting from extensive cell death (6, 10). Activin also causes cell death in primary hepatocyte cultures, and this effect can be blocked by follistatin, an activin-binding protein, indicating that it is a specific biological response to activin (6). The hepatocyte death induced by activin exhibits characteristic nuclear and cytoplasmic features of apoptosis (6). Similarly, activin has been shown to induce apoptosis in other cell types, including mouse B cell hybridomas and mouse and human myeloma cells (11, 12). The activin-related protein TGF β also causes apoptosis in normal and transformed hepatocytes (13, 14). Transgenic mice overexpressing TGF β 1 selectively develop hepatic fibrosis and exhibit apoptosis of hepatocytes (15).

Members of the TGF β family of proteins, including TGF β s,

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activins and bone morphogenic proteins (BMPs), exert their biological actions through two types of cell surface receptors, designated type I and type II receptors, both of which are serine/threonine protein kinases (16, 17). Ligand binding to a type II receptor, which is a constitutively active kinase, recruits a type I receptor into the complex (18). The type I receptor is phosphorylated and activated by the type II receptor and propagates the signal to downstream proteins. Two type II receptors, ActRII (19) and ActRIIB (20), bind activin with high affinity and selectivity. The type I receptor ActRIB (also known as ALK-4) (21), selectively mediates activin signaling (22, 23). An additional type I receptor, ActRI (also known as ALK-2), associates with activin type II receptors to form heteromeric complexes after activin binding to the type II receptors (24, 25). However, ActRI has recently been shown to coimmunoprecipitate with a BMP type II receptor (26) and mediate the effects of BMP (27), and thus may be a common type I receptor shared by the activin and BMP signaling pathways.

The Smad family of proteins plays key roles in transducing signals from cell surface serine/threonine kinase receptors for TGF β superfamily proteins to nuclear target genes (28–30). To date, eight vertebrate Smad proteins have been identified, and these can be grouped into three classes. The first class is the receptor-regulated Smads. Among them, Smad1, Smad5, and Smad8 mediate BMP signaling, whereas Smad2 and Smad3 transduce activin and TGF β signals (28). These receptor-regulated Smads transiently interact with and become phosphorylated by specific activated type I receptors (31–33). Once phosphorylated, they associate with a common partner, Smad4 (also known as DPC4), which is the only known member of the second class of Smad proteins in vertebrates (34). The Smad complex then translocates to the nucleus and regulates transcription of target genes through interaction with specific DNA sequences and other DNA-binding proteins. For example, the Smad2/Smad4 complex has been shown to associate with the winged helix transcription factor FAST (35–37) and thereby interact with activin-responsive elements. Smad6 and Smad7 form the third class of Smad proteins, the inhibitory Smads. They act as inhibitors of BMP, activin, and TGF β signaling by competing with the receptor-regulated Smads for binding to the type I receptors or by competing with Smad4 for binding to the receptor-regulated Smads (38–40).

In the present study we observed that activin A induces apoptosis in a human hepatoma cell line, HepG2 cells. We use this model system to investigate the involvement of known activin signaling components, including the four activin receptors and two of the Smad proteins, in the activin A-induced apoptotic pathway. We demonstrate by blocking receptor action using a dominant negative approach that activin receptors are essential for mediating the apoptotic response. Furthermore, we show that overexpression of activin receptors and Smad proteins can mimic activin action and induce apoptosis in HepG2 cells. Our data suggest that these signaling molecules play a critical role in mediating apoptotic cell death induced by activin A in HepG2 cells.

Materials and Methods

Complementary DNA (cDNA) cloning and expression constructs

Full-length cDNAs for rat activin receptors ActRI, ActRIIB, ActRII, and ActRIIB were isolated by a combination of cDNA library screening and RT-PCR procedures. All sequences were verified by the dideoxynucleotide chain termination method (Sequenase 2.0 kit, U.S. Biochemical Corp., Cleveland, OH). There are four alternative splicing isoforms for ActRIIB, and the form used in this study corresponds to mouse ActRIIB₂ (20). All receptor constructs were cloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA). To generate the ActRIIB mutant (ActRIIB-DN), a fragment from 10 bp upstream of the start codon to 590 bp downstream of the start codon was cloned into pcDNA3. An in-frame stop codon was provided by the vector. The resulting ActRIIB-DN mutant contains the extracellular domain and the transmembrane domain of the receptor, but is truncated at the fifth amino acid of the cytoplasmic kinase domain.

The cDNA clones for pCMV5-Flag-Smad2 and pCMV5-Flag-Smad4 were provided by Dr. Jeffrey Wrana (Hospital for Sick Children, Toronto, Canada) (32, 41). For luciferase assays, Flag-Smad2 and Flag-Smad4 were subcloned into the pcDNA3 vector. For fluorescence analysis, Smad2 and Smad4 were subcloned into the C-terminus of green fluorescence protein (GFP) in the pEGFP vector (CLONTECH Laboratories, Inc., Palo Alto, CA). To generate the Smad2 mutant (Smad2-DN), Smad2 was truncated 30 bp before the stop codon, deleting the last 10 amino acids, including the SSMS motif at the C-terminus of the protein.

Cell culture and transfection

HepG2 cells were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 2 mM sodium pyruvate and 10% FCS (Life Technologies, Inc./BRL, Grand Island, NY) at 5% CO₂ in a 37°C incubator. For luciferase assays and fluorescence microscopy, cells were transfected with cationic liposomes prepared as previously described (42). The plasmid DNAs were preincubated with liposomes for 30 min in Opti-MEM I medium (Life Technologies, Inc./BRL) before the DNA/liposome mixture was added to the cells. To generate stable cell lines, constructs encoding ActRIIB-DN and a neomycin-resistant selectable marker were transfected into HepG2 cells using lipofectin (Life Technologies, Inc./BRL). Stable transfectants were selected in 450 μ g/ml geneticin (Mediatech, Herndon, VA) and identified by RNA blot analysis. Positive clones were expanded and maintained in geneticin.

Luciferase assay

HepG2 cells cultured in 12-well plates were transiently cotransfected with p3TP-Lux (provided by Dr. Joan Massague, Memorial Sloan-Kettering Cancer Center, New York, NY) and the indicated expression constructs for 6 h. pcDNA3 vector DNA was used to keep the total amount of DNA in all samples constant. After 6 h, the cells were allowed to recover in fresh growth medium for 18 h and were treated with or without 1 nM activin A in DMEM containing 0.2% FBS for 20 h. Cells were washed with PBS twice and lysed in 150 μ l lysis buffer [25 mM HEPES (pH 7.8), 15 mM MgSO₄, 0.5 mM EGTA, 1 mM dithiothreitol, and 0.2% Triton X-100]. Four hundred microliters of assay buffer (lysis buffer minus Triton X-100 and with 2.5 mM ATP and 1 μ g/ml BSA) and 100 μ l 1 mM luciferin (sodium salt, Analytical Bioluminescence, San Diego, CA) were added to 100 μ l cell lysate, and emitted luminescence over 10 sec was measured using a Monolight 2010 Luminometer (Analytical Bioluminescence). Luciferase activity was normalized to the amount of protein in each extract, determined using a Bio-Rad Laboratories, Inc., protein assay (Richmond, CA). Each experiment was repeated at least three times.

Messenger RNA (mRNA) measurements

For RT-PCR, 5 μ g total RNA were reverse transcribed into cDNA using random hexameric oligonucleotides. Aliquots of the cDNA were then amplified by PCR using an annealing temperature of 62°C with the incorporation of [³²P]deoxy-CTP (Amersham Pharmacia Biotech, Arlington Heights, IL). The PCR products were separated by electrophoresis on a 6.5% polyacrylamide gel and visualized by autoradiography.

The sequence-specific primers used and the expected sizes of the fragments amplified are shown in Table 1.

For Northern blots, 20 µg total RNA were separated by denaturing agarose gel electrophoresis and transferred to a Biotrans Nylon membrane (ICN Biomedicals, Inc., Irvine, CA). RNA was immobilized on the membrane and hybridized to the ³²P-labeled probes in hybridization solution (50% formamide, 10% dextran sulfate, 1 × Denhardt's, 0.6 M NaCl, 1 mM EDTA, 2.5 mM HEPES (pH 6.5), 50 mM sodium phosphate (pH 6.5), 0.1% SDS, and 50 µg/ml yeast transfer RNA) overnight at 42 C. The blot was washed in 0.1 × SSC-0.1% SDS at 65 C.

Fluorescence microscopy

HepG2 cells cultured on 15-mm square glass coverslips were transfected with the indicated constructs. After recovery in fresh medium for 24 h, cells were fixed with 4% paraformaldehyde and mounted in Mowiol 4-88 (Calbiochem, La Jolla, CA) mounting medium. Fluorescence was observed using a ×63 oil immersion objective on a Carl Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

In situ detection of apoptotic cells

Apoptosis was examined *in situ* by terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling (TUNEL) using an ApopTag detection kit (Oncor, Gaithersburg, MD). Briefly, HepG2 cells cultured on glass coverslips were fixed in 4% formalin (Sigma) at room temperature for 10 min, permeabilized with 0.5% Triton X-100 for 3 min, and stored in 70% ethanol at -20 C for up to 3 days. Cells were then rehydrated in PBS, transferred to a humidified chamber, and covered with equilibration buffer for 5 min, which was replaced with the working strength terminal deoxynucleotide transferase enzyme solution. After incubation at 37 C for 1.5 h, the coverslips were submerged in the working strength stop/wash buffer for 10 min and washed in PBS. dRhodamine-conjugated antidigoxigenin antibody was applied to the cells for 1 h. After the PBS wash, cells were mounted and visualized using a ×40 or ×63 oil immersion objective on a Carl Zeiss LSM410 confocal microscope (fitted with an argon laser with a band at 488 nm for fluorescein isothiocyanate (FITC) and an He-Ne laser with a band at 543 nm for rhodamine). GFP fusion proteins were detected using an FITC filter set. Positive TUNEL staining was detected using a rhodamine filter set. Fields in each of the four quadrants of each slide were randomly chosen before visual inspection, and at least 300 cells/slide were counted. Apoptotic cells were expressed as a percentage of the total cells. Each experiment was repeated at least three times.

Statistical analysis

All values are expressed as the mean ± SD. Student's *t* test was used to evaluate differences between the control samples and activin-treated samples or between samples transfected with vector alone and samples transfected with the different receptor and Smad constructs. *P* < 0.05 was considered statistically significant.

Results

Activin induces apoptosis in HepG2 cells

To determine the effect of activin on apoptosis in HepG2 cells, cells were incubated with increasing concentrations of recombinant human activin A for 2.5 days. Compared with control cells, activin-treated cells showed the classical morphological features of apoptosis, including chromosomal condensation and nuclear fragmentation. These cells were TUNEL positive, as shown in Fig. 1A. Quantification of the TUNEL assays revealed that activin A induced a dose-dependent increase in the number of apoptotic cells, with a maximal effect observed between 1–10 nM activin A (Fig. 1B). The stimulatory effect of activin A on apoptosis was antagonized by cotreatment with the activin-binding protein follistatin at a 20-fold excess molar ratio (Fig. 1C). Inhibin, which antagonizes the effects of activin in many systems, only partially reduced activin A-induced apoptosis in HepG2 cells. Finally, the related ligand TGFβ induced apoptosis to a similar extent as did activin A in HepG2 cells.

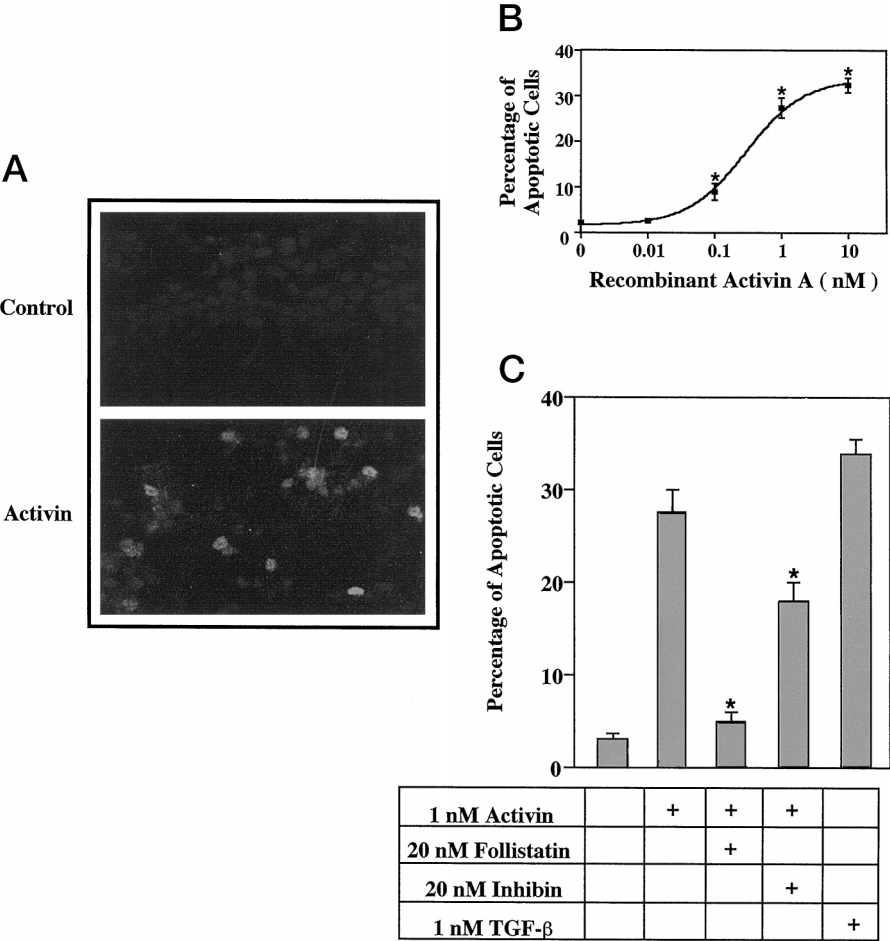
Expression of activin, activin receptor, and Smad protein mRNAs in HepG2 cells

HepG2 cells were studied to establish the profile of expression of activin or several components of the activin signaling pathway at the mRNA level. This was accomplished using RT-PCR with sequence-specific primers, which are shown in Table 1. Neither the activin β_A-subunit nor the activin β_B-subunit mRNAs are expressed in HepG2 cells, although they are readily detected in a control tissue known to express activin, the ovary (Fig. 2A). In contrast, mRNA for

TABLE 1. Primer sequences

Gene	Primer sequence				Size of PCR fragment (bp)
β _A	5' Primer	5'-CTCAT	GGAGC	AGACC TCGGA G	565
	3' Primer	5'-GTGAG	GATGG	TCTTC AGACT G	
β _B	5' Primer	5'-TATGT	CCTGG	AGAAG GGCAG C	310
	3' Primer	5'-GCGAT	GTCTG	CTATC GCCCA G	
Follistatin	5' Primer	5'-AATTG	CTGGC	TCCGC CAAG	900
	3' Primer	5'-AGCTG	TAGTC	CTGGT CTTC	
ActRI	5' Primer	5'-CTGGC	CAAGC	TGTGG AGTGC TGCCA A	570
	3' Primer	5'-GTACT	GGAGT	GTCTA GAGGT CATGT	
ActRIB	5' Primer	5'-GGAAA	GCTTA	TGGCG GAGTC GGCCG GA	545
	3' Primer	5'-GATCG	TAGAC	GAGAT CCTGG AGCGT	
ActRII	5' Primer	5'-CGACG	ACATT	GTTT GCTAC C	285
	3' Primer	5'-CCCCG	CAATT	AACAT AAGTG G	
ActRIIB	5' Primer	5'-GAGAA	TTCGG	AACAT GACGG CGCCC TG	571
	3' Primer	5'-CCTCG	AGCCT	TGATC TCCAG	
Smad2	5' Primer	5'-GCCAT	CACCA	CTCAG AATTG CAATA C	265
	3' Primer	5'-AGCAT	ATTCG	CAGTT TTCCA TTGCC	
Smad4	5' Primer	5'-AC(A/T)AC	(A/T)AATG	GNGCN CATCC TAGTA A	305
	3' Primer	5'-TGNAC	ATATT	CGTCT TTNAC CATCA T	

FIG. 1. The effect of activin on apoptotic cell death of HepG2 cells. A, *In situ* detection of apoptotic cells in HepG2 cells treated with 1 nM activin A. HepG2 cells were cultured in DMEM-0.2% FBS in the presence or absence of 1 nM activin A for 2.5 days. Apoptotic cells were detected by DNA fragmentation analysis using TUNEL staining as described in *Materials and Methods*. Magnification, $\times 40$. B, Dose-response curve for activin induction of HepG2 cell apoptosis. HepG2 cells were treated with concentrations of activin A ranging between 10 pM and 10 nM. Apoptotic cells were quantified as described in *Materials and Methods*. Results shown are the mean \pm SD of triplicate samples from a representative experiment. *, Significant stimulation compared with untreated cells ($P < 0.01$). C, Follistatin and inhibin antagonize activin A-induced apoptosis in HepG2 cells. HepG2 cells were treated with the indicated proteins. Apoptotic cells were quantified as described in *Materials and Methods*. Results shown are the mean \pm SD of triplicate samples from a representative experiment. *, Significant repression compared with the activin-treated cells ($P < 0.01$).



the activin-binding protein follistatin was detected at a low level in HepG2 cells (Fig. 2A). Activin signaling is mediated by at least four receptors (ActRI, -IB, -II, and -IIB) and several Smad proteins, including Smad2 and Smad4 (17, 28–30). Figure 2B shows that all of these signaling components are expressed in HepG2 cells, although their relative abundance at the mRNA level varies quite substantially.

Activin receptors and Smad2 and Smad4 synergize to mediate transcriptional activation of an activin-responsive promoter in HepG2 cells

To understand the role of these signaling molecules in mediating activin A action in HepG2 cells, we used a transcriptional activation assay as a model system. The reporter gene used in these studies is p3TP-Lux, which contains a luciferase gene under the control of a synthetic promoter that has been widely used in studies of the TGF β and activin signaling pathways (18, 27). In HepG2 cells, the reporter gene had very low basal activity, but activity was stimulated by more than 30-fold after treatment with 1 nM activin A (Fig. 3A). Because there are two isoforms of each type of activin receptor, and all isoforms are expressed in HepG2 cells, we determined the abilities of these various isoforms to mediate transcriptional activation of the p3TP-Lux reporter gene. Neither ActRI, ActRIB, nor ActRII alone had an effect on basal reporter gene activity (Fig. 3A). Expression of ActRIIB

alone increased basal activity 5-fold compared with that in control cells. In activin-treated cells, neither ActRI, ActRII, nor ActRIIB alone had an effect on reporter gene activity, whereas ActRIB increased luciferase activity 2.5-fold compared with that in activin-treated control cells. Strikingly, coexpression of one of the type I receptors together with one of the type II receptors resulted in a synergistic activation of the p3TP-Lux reporter gene. Even in the absence of activin, basal activity in these cells reached levels as high as those in activin-treated control cells. Activin treatment further increased reporter gene activity in these cells. Coexpression of ActRIB and ActRIIB gave the strongest ligand-independent activation of the reporter gene, implying constitutive activation of the activin signaling pathway by the combination of these two overexpressed receptors. To investigate further the involvement of activin type II receptors in activin signaling, we overexpressed a truncated form of ActRIIB (ActRIIB-DN) to interrupt activin signaling. Similar mutants have been successfully used by others to inhibit activin signaling through a dominant negative action (43, 44). Overexpression of ActRIIB-DN blocked activin-induced transcriptional activation of the reporter gene in HepG2 cells. Transfected wild-type ActRIIB was able to overcome the inhibitory effect of the ActRIIB-DN mutant and restore the transcriptional activation of the reporter gene in a dose-dependent manner (Fig. 3B).

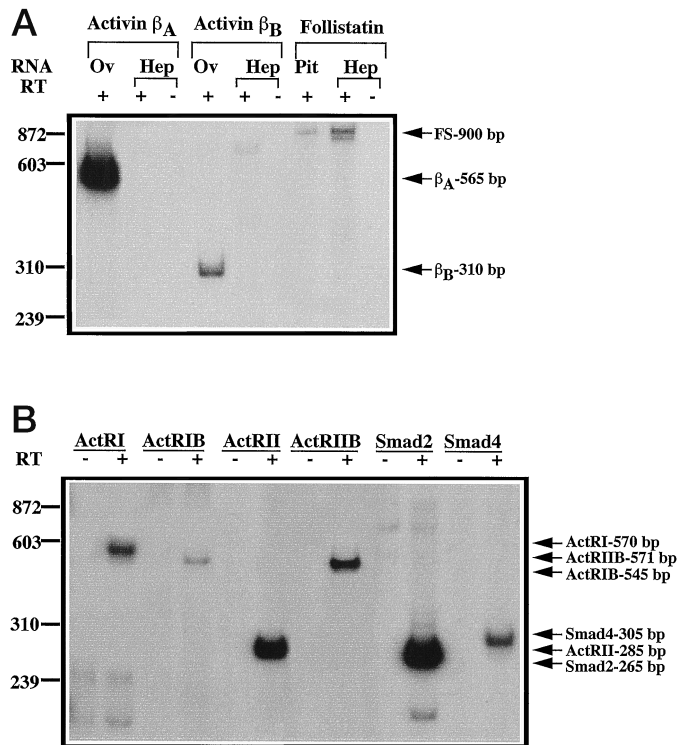


FIG. 2. Expression of activin, follistatin, activin receptor, and Smad protein mRNAs in HepG2 cells. A, RT-PCR of activin β_A and β_B and follistatin mRNAs in HepG2 cells or control tissues. B, RT-PCR of mRNA for activin receptors ActRI, -IB, -II, and -IIB and Smad proteins, Smad2 and Smad4, in HepG2 cells. Five micrograms of total RNA from HepG2 cells or control tissues were reverse transcribed and amplified by PCR with incorporation of [32 P]deoxy-CTP into the PCR product. PCR amplification without RT was used as a negative control. Products were resolved by PAGE and visualized using autoradiography. The PCR primers used and the expected sizes of the PCR products are indicated in Table 1.

The involvement of Smad2 and Smad4 in activin signaling in HepG2 cells was also investigated by examining transcriptional activation of the p3TP-Lux reporter gene. Expression of either Smad2 or Smad4 alone had little effect on reporter gene activity (Fig. 4A). However, coexpression of Smad2 along with Smad4 resulted in a 70-fold increase in basal activity compared with that in control cells. Reporter gene activity was further increased 3-fold after activin treatment of the cells. Consistent with our previous results, transfection of ActRIIB and ActRIB led to a ligand-independent activation of the reporter gene (Fig. 4B). This activity could be further increased when Smad2 or Smad4 was coexpressed with the activin receptors. Maximal activation of the 3TP-Lux reporter gene was obtained when all four proteins (ActRIB, ActRIIB, Smad2, and Smad4) were coexpressed in the cells.

To test further the role of the Smad proteins in this pathway, we constructed a Smad2 mutant that lacks the C-terminal 10 amino acids of the protein. This removes the 2 serine residues at the C-terminus that are phosphorylated in response to activin and required for Smad2 biological activity (45, 46). A similar Smad3 truncation mutant has been shown to exert a dominant interfering activity (33). Expression of Smad2-DN completely blocked transcriptional activation of the reporter gene mediated by both endogenous Smads as

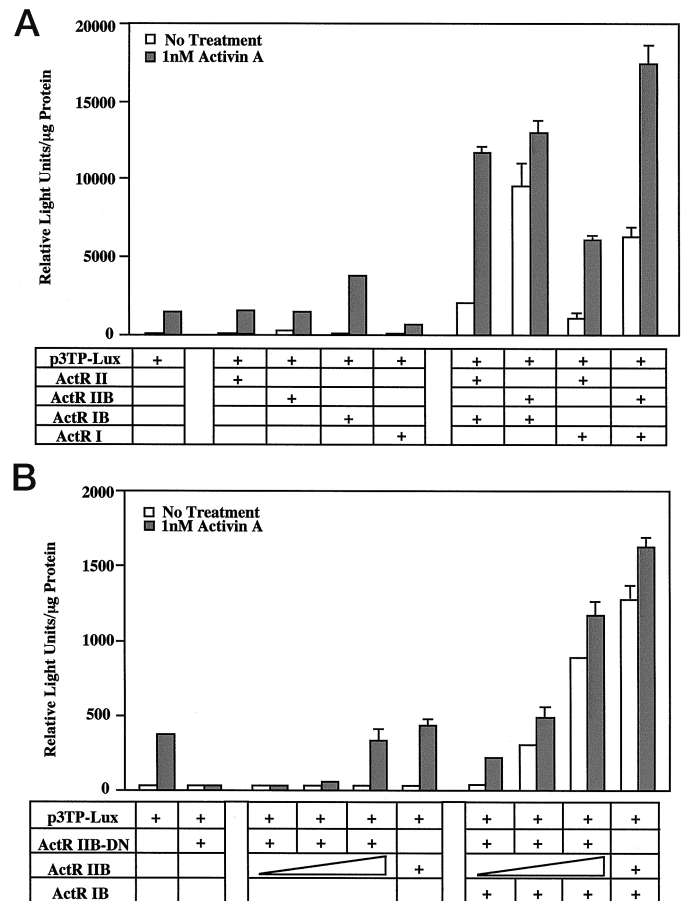


FIG. 3. Activin and activin receptor stimulation of reporter gene transcription in HepG2 cells. A, Activin receptors synergize to mediate transcriptional activation of the p3TP-Lux reporter gene. HepG2 cells were transfected with 1 μ g p3TP-Lux along with 0.75 μ g of the indicated receptor constructs. B, ActRIIB-DN blocks activin-stimulated transcriptional activation. Cells were transfected with 0.5 μ g p3TP-Lux, 2 μ g ActRIIB-DN, and 0.5 μ g each of ActRIB and ActRIIB. For the dose-response experiment, the amounts of wild-type ActRIIB DNA transfected were 0.1, 0.5, and 4 μ g. Vector DNA was used to keep the total amount of transfected DNA for each treatment group constant. Transfected cells were cultured with or without 1 nM activin A for 20 h, and the relative luciferase activity was measured as described in *Materials and Methods*. The data were normalized to the total amount of protein and are expressed as the mean \pm SD of triplicate samples from a representative experiment. Some of the error bars are too small to be visualized. Note the difference in scales for A and B.

well as transfected Smad2 and Smad4 (Fig. 4A). More importantly, Smad2-DN abolished the constitutive activation of the reporter gene by activin receptors ActRIB and ActRIIB (Fig. 4B).

Nuclear translocation of Smad2 and Smad4 proteins

The subcellular localization of Smad2 and Smad4 was examined after activation of the activin signaling pathway in HepG2 cells. The Smad2 and Smad4 proteins were fused to the C-terminus of GFP, allowing direct visualization of the fusion proteins by fluorescence microscopy. Addition of the GFP did not disrupt the functional properties of the Smad proteins, as determined by their ability to activate the p3TP-

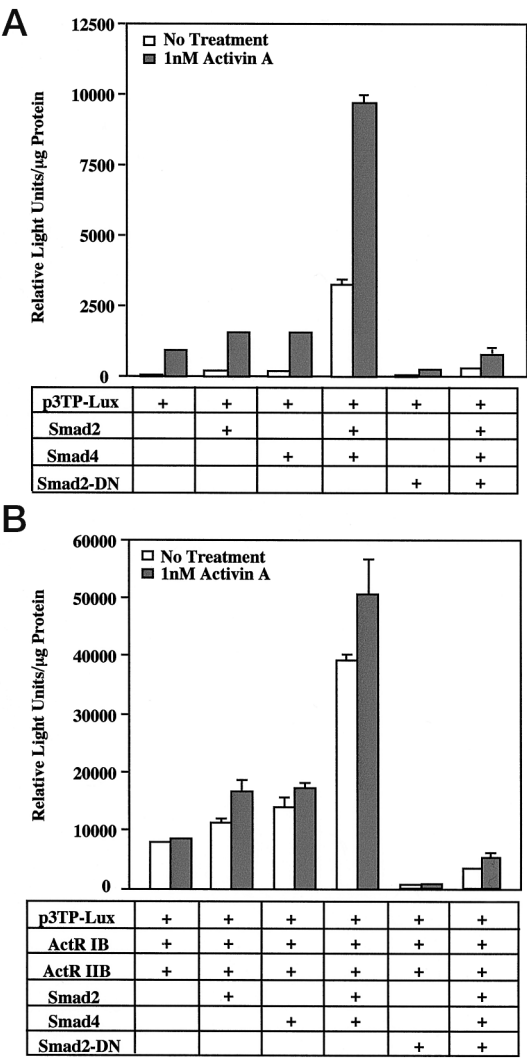


FIG. 4. Smad protein stimulation of reporter gene transcription in HepG2 cells. **A**, Smad2 and Smad4 synergize to mediate transcriptional activation of the p3TP-Lux reporter gene. **B**, Coexpression of activin receptors enhances transcriptional activation by the Smad proteins. HepG2 cells were transfected with 1 μ g p3TP-Lux plasmid and 0.75 μ g of the indicated Smad DNA constructs. For Smad2-DN, 2 μ g DNA were used. For ActRIB and ActRIIB, 0.25 μ g of each construct was used. Cells were cultured with or without 1 nM activin A for 20 h, and the relative luciferase activity was measured. The data were normalized to the total amount of protein and are expressed as the mean \pm SD of triplicate samples from a representative experiment. Some of the error bars are too small to be visualized. Note the difference in scales for A and B.

Lux reporter gene in transfected HepG2 cells treated with activin (data not shown). When expressed alone, GFP-Smad2 was expressed diffusely throughout the cells. Activation of the activin signaling pathway by coexpression of activin receptors ActRIB and ActRIIB caused Smad2 to accumulate in the nucleus of 95% of the transfected cells (Fig. 5). Similar nuclear translocation of GFP-Smad2 was also observed in cells cotransfected with combinations of receptors ActRIB and ActRII, ActRI and ActRIIB, and ActRI and ActRII, although the effect was not as robust (data not shown), consistent with the transcriptional response data. In contrast, GFP-Smad4 was detected predominantly in the cytoplasm

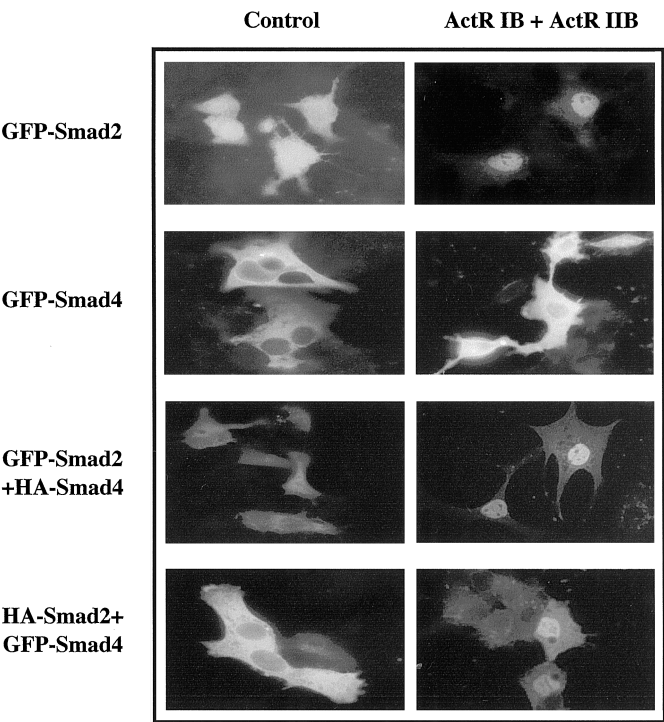


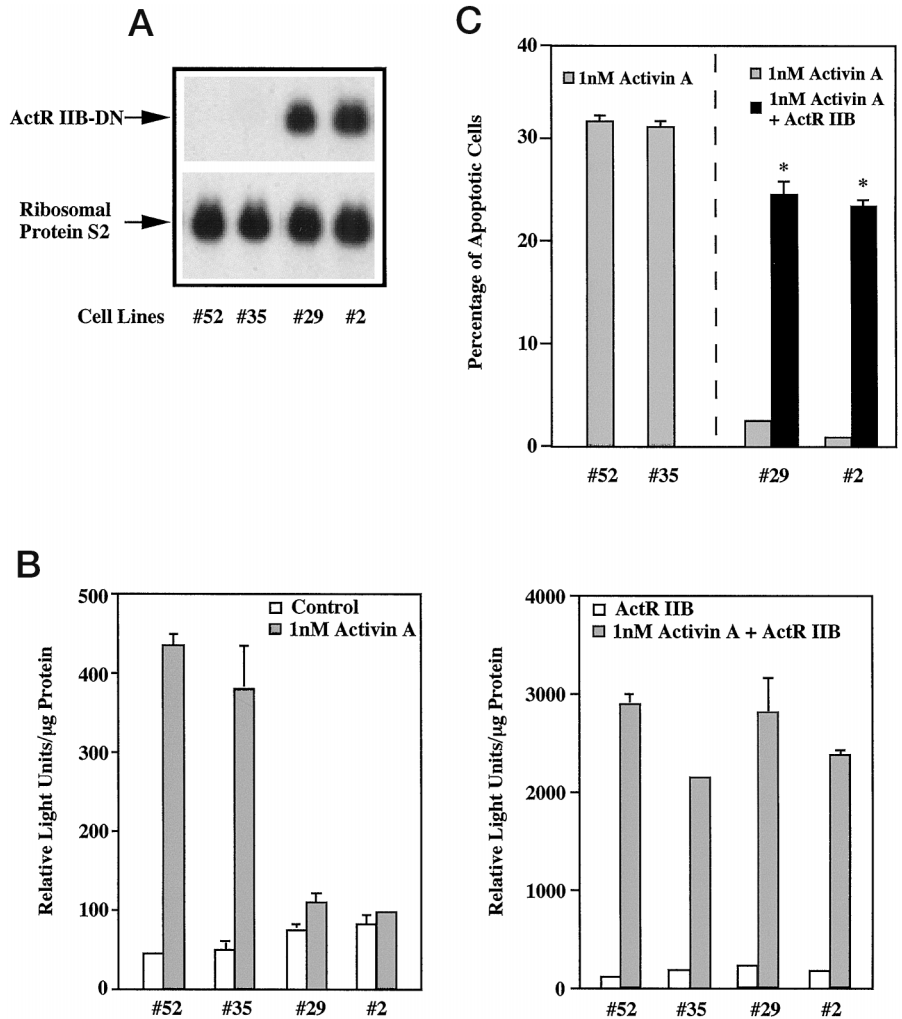
FIG. 5. Activin signaling induces translocation of Smad proteins to the nucleus in HepG2 cells. HepG2 cells were transfected with the indicated DNA constructs without (*left panels*) or with (*right panels*) cotransfection of the activin receptors ActRIB and ActRIIB. Cells were fixed and visualized directly using a $\times 63$ objective on a Carl Zeiss Axiophot microscope.

regardless of receptor coexpression. In cells cotransfected with Smad2 and the activin receptors, GFP-Smad4 translocated into the nucleus, although only in a subset of transfected cells ($\sim 20\%$). Coexpression of activin receptors did not have any effect on localization of the transfected GFP protein, which was found throughout the cell (data not shown).

Activin-induced apoptosis is blocked in HepG2 cells expressing a dominant negative IIB activin receptor

Expression of the dominant negative ActRIIB mutant, ActRIIB-DN, blocked activin-induced transcriptional activation of the p3TP-Lux reporter gene in HepG2 cells (see Fig. 3B). To determine whether this mutant could also disrupt the activin-induced apoptotic response, stable cell lines expressing ActRIIB-DN were established (Fig. 6A). Cell lines 2 and 29 express ActRIIB-DN RNA at high levels, whereas lines 35 and 52 do not express detectable levels of ActRIIB-DN RNA. Because of its low abundance, endogenous ActRIIB was below the level of detection in this RNA blot analysis. Cell lines 35 and 52 maintained normal responsiveness to activin, as shown by the transcriptional activation and apoptotic cell death assays (Fig. 6, B and C). In ActRIIB-DN high expression cell lines 2 and 29, activin-induced activation of the reporter gene was abolished (Fig. 6B), consistent with our earlier results using transient assays. TUNEL staining indicated that these cell lines no longer exhibited an apoptotic response to activin (Fig. 6C). Activation of the reporter gene could be rescued, and the apoptotic response could be partially re-

FIG. 6. A dominant negative ActRIIB mutant blocks activin-induced transcriptional activation and cell death in HepG2 cells. **A**, Expression of ActRIIB-DN RNA in stable HepG2 cell lines. Twenty micrograms of total RNA from cell lines were hybridized to a 32 P-labeled ActRIIB probe. The same filter was then stripped and rehybridized with a ribosomal protein S2 probe to compare the amount of RNA loaded. **B**, Activin-induced transcriptional activation in ActRIIB-DN cell lines. Stable cell lines were transfected with 1 μ g p3TP-Lux without (*left panel*) or with (*right panel*) 3 μ g ActRIIB and cultured in the presence or absence of 1 nM activin A. Luciferase activities were determined as described previously and are expressed as the mean \pm SD of triplicate samples. **C**, Quantification of apoptotic cells detected by TUNEL staining in cell lines expressing ActRIIB-DN. Cells were incubated with activin A for 3 days and apoptotic cells were detected and quantified as described in *Materials and Methods*. For cell lines 2 and 29, cells transiently transfected with the wild-type ActRIIB were also included. Results shown are the mean \pm SD of triplicate samples from a representative experiment. *, $P < 0.01$ compared with the corresponding cell lines without ActRIIB transfection.



stored in these cell lines by overexpressing the wild-type ActRIIB receptor (Fig. 6, B and C) or the ActRII receptor (data not shown).

As a complementary approach to suppressing activin signaling using the dominant negative mutant, we assessed the ability of wild-type activin receptors ActRII and ActRIIB to induce apoptosis when they were transiently overexpressed in HepG2 cells. In cells cotransfected with ActRII and ActRIIB, about 40% of the cells were apoptotic, as determined by TUNEL staining, compared with 2% apoptotic cells in control cells transfected with vector DNA alone (Fig. 7).

Overexpression of Smad2 and Smad4 induces apoptosis in HepG2 cells

The effect of transient overexpression of the Smad proteins on apoptosis was similarly determined in HepG2 cells. Transfection of either Smad2 or Smad4 into HepG2 cells caused a 20-fold increase in the number of apoptotic cells compared with that in vector-transfected cells (Fig. 7). Cotransfection of the Smad2-DN construct blocked the stimulatory effect of cotransfected Smad2 and Smad4 on apoptosis. Consistent with the function of the Smad proteins being downstream of the activin receptor, the Smad2-DN construct

also blocked HepG2 cell apoptosis induced by either activin or cotransfected activin receptors IB and IIB (Fig. 7).

To establish at a cellular level the relationship between Smad protein expression and apoptosis, GFP-Smad fusion proteins were used to visualize directly cells that were overexpressing the Smad proteins. In cells transfected with GFP-Smad2 alone, GFP was colocalized with positive TUNEL staining (Fig. 8). Similar colocalization was observed in cells transfected with GFP-Smad4 or GFP-Smad2 plus Smad4. Smad2-DN was able to suppress apoptotic cell death induced by Smad2 or Smad4, indicating that apoptosis in Smad2- or Smad4-overexpressing cells is a direct consequence of Smad2 or Smad4 activity in these cells.

Discussion

Although activin has been reported to negatively regulate liver mass and stimulate liver cell apoptosis, little is known about the molecular mechanisms by which this occurs. In the present study we demonstrate that activin A stimulates dose-dependent apoptotic cell death in a human hepatoma cell line, HepG2 cells. Using HepG2 cells as a model system, we establish the involvement of several activin signaling components, including the activin type I and type II receptors

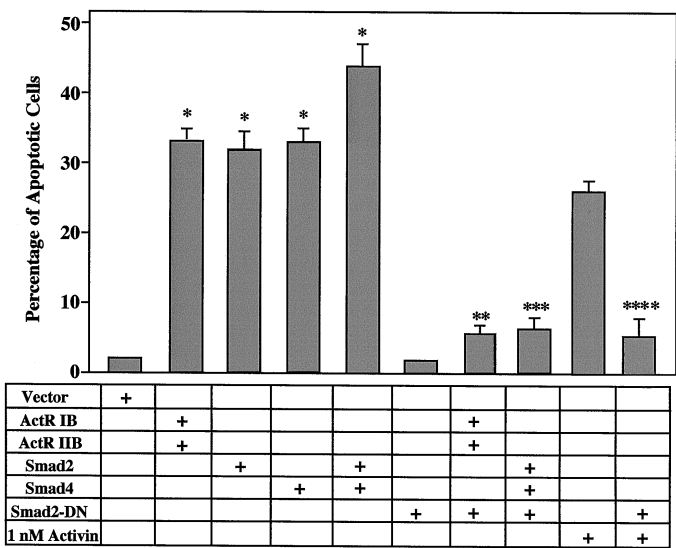


FIG. 7. Overexpression of activin receptors and Smad proteins induces apoptosis in HepG2 cells. HepG2 cells were transfected with 5 μ g of the indicated DNA constructs, allowed to recover in fresh medium for 48 h, and processed for TUNEL analysis. Apoptotic cells were detected and quantified as described in *Materials and Methods*. Results shown are the mean \pm SD of triplicate samples from a representative experiment. *, $P < 0.01$ compared with cells transfected with the vector alone; **, $P < 0.01$ compared with cells transfected with ActRIB and ActRIIB; ***, $P < 0.01$ compared with cells transfected with Smad2 and Smad4; ****, $P < 0.01$ compared with cells treated with 1 nM activin A.

and Smad2 and Smad4, in activin A-stimulated liver cell apoptosis.

Binding of activin to a type II receptor is the first step in the activin signaling pathway. In our studies, expression of the ActRIIB-DN mutant receptor that lacks the cytoplasmic domain resulted in a loss of activin A-stimulated transcriptional activation and cellular apoptosis. This is presumably because ActRIIB-DN is still competent for ligand binding and interaction with a type I receptor, but is unable to phosphorylate and activate the type I receptor (47, 48), and therefore prevents signal transduction. Activin responsiveness could be restored by the coexpression of exogenous wild-type Act II or ActRIIB, suggesting that both isoforms of the activin type II receptor are capable of binding the type I receptor and forming a functional receptor complex that transduces activin signals in HepG2 cells. Interestingly, ActRIIB-DN is less efficient at suppressing signaling in the presence of coexpressed ActRIB. This raises the possibility that endogenous ActRIB is being trapped by the mutant type II receptor, in agreement with studies showing an interaction between an analogous deletion mutant of the type II TGF β receptor and its type I receptor (48). However, in other studies a kinase-deficient ActRIIB receptor failed to block the activity of a constitutively active ActRIB, arguing against trapping of the type I receptor in this system (23).

Consistent with earlier overexpression studies on TGF β and activin receptors (18, 23), coexpression of both a type I and a type II receptor activated the activin signaling pathway even in the absence of the ligand, indicating there is a ligand-independent interaction between the two receptors. Biochemical analysis has confirmed that in the absence of ac-

tivin, overexpressed ActRIB and ActRIIB are able to form a stable heteromeric complex (our unpublished results) (27).

Overexpression of either Smad2 or Smad4 caused HepG2 cells to undergo apoptosis. Although the transfected GFP-Smad4 fusion protein showed predominantly a cytoplasmic localization, even when cotransfected with receptors ActRIB and ActRIIB, it is likely that some fraction of the Smad4 does translocate to the nucleus and is sufficient to mediate an apoptotic signal. Using an inducible nuclear translocation expression system, it was reported that overexpression of Smad4 in the nucleus was able to induce cellular apoptosis, and a tumor-derived mutation affecting the DNA binding of Smad4 caused a significant decrease in apoptotic cell numbers (49). Taken together, these data suggest that nuclear Smad4 is able to induce apoptosis, an activity that may contribute to its tumor-suppressive role.

A critical role for Smad proteins in the pathway leading to apoptosis is further indicated by the results with the Smad2-DN mutant, which acts as a dominant negative regulator of activin A-dependent gene transcription and cellular apoptosis. This protein lacks the 10 amino acids at the C-terminus of the wild-type protein and hence is unable to be phosphorylated by the activated type I receptors. Phosphorylation of Smad2 is important for its association with Smad4 and subsequent nuclear translocation (45, 46). The ability of Smad2-DN to block activin, activin receptor, and Smad protein-mediated apoptosis suggests that Smad-dependent signaling to the nucleus is important for activin A-stimulated apoptosis. The Smad proteins are also important mediators of TGF β signaling (28–30). TGF β has been shown to induce apoptotic cell death in liver cells (13, 14), and we observed an apoptotic response to TGF β in HepG2 cells. It is likely that the Smad pathway is also involved in TGF β -induced liver cell apoptosis, providing for cross-talk between the activin- and TGF β pathways in regulating liver cell function.

Our identification of Smad proteins as intracellular mediators of activin-induced apoptosis provides a potential point for cross-talk between activin signaling and other signaling pathways involved in the regulation of apoptosis. One such pathway might involve Ca²⁺ signaling. Recently, calmodulin (CaM) has been shown to physically interact with Smad proteins in a calcium-dependent manner (50). CaM is an important intracellular receptor for calcium ions (51), and alterations in intracellular calcium appear to be essential for apoptosis. Calcium is required for activation of the latent Ca²⁺-dependent endonuclease that degrades internucleosomal DNA, a hallmark of apoptosis (51). Ca²⁺-bound CaM also activates a serine-threonine phosphatase, calcineurin, which has been demonstrated to induce apoptosis in the presence of calcium and the absence of growth factors (52).

The final stage of the apoptotic process, called execution, occurs through the activation and proteolytic function of a family of cysteine proteases, the caspases (53). Studies on the FaO rat and Hep3B human hepatoma cell lines provide evidence that the activity of the caspase-3 subfamily is stimulated in TGF β 1-induced apoptosis (54, 55). In future studies it will be important to determine whether these same caspases are involved in activin-induced apoptosis and to establish how they are activated by TGF β family proteins. The inhibitory Smads, Smad6 and Smad7, have been shown

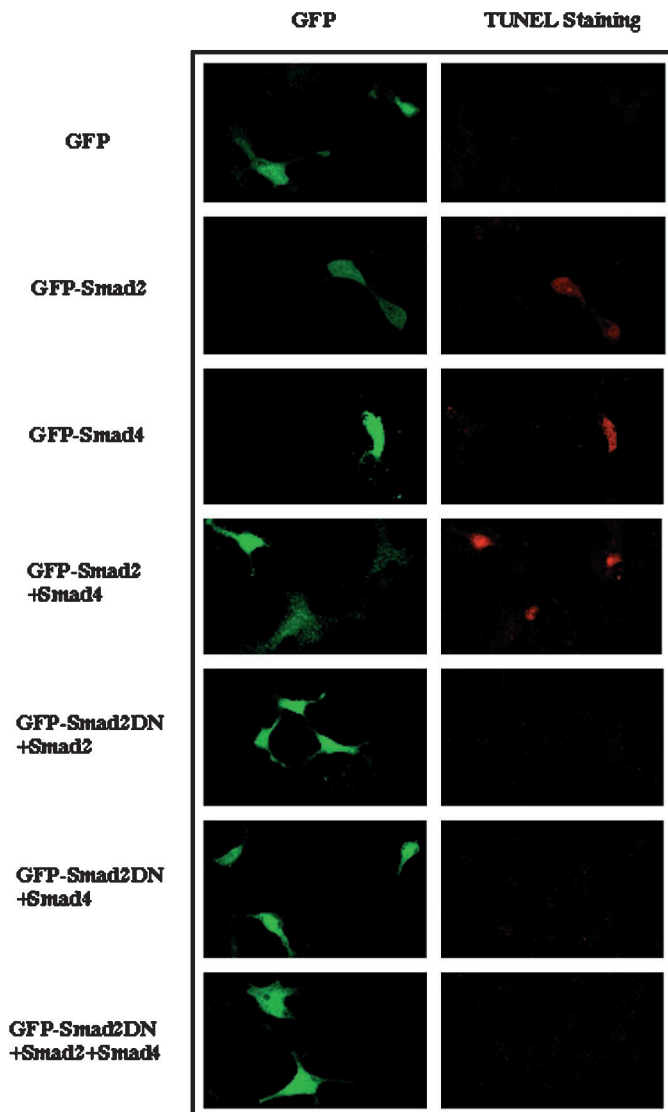


FIG. 8. HepG2 cells expressing transfected Smad proteins undergo apoptosis. HepG2 cells were transfected with 5 μ g of the indicated DNA constructs. After being allowed to recover in fresh medium for 48 h, cells were fixed and processed for TUNEL analysis. The GFP fusion proteins were visualized directly using the FITC filter (left panels), and TUNEL staining was visualized using a rhodamine filter (right panels) in the same focal plane and the same field. Magnification, $\times 63$.

to negatively regulate TGF β /activin signaling (38–40). In mouse B hybridoma HS-72 cells, Smad7 was induced by activin A and the ectopic expression of Smad7 suppressed activin A-induced apoptosis (56). It will also be important to determine whether Smad7 is induced by activin A and antagonizes activin A-induced apoptosis in HepG2 cells.

In summary, our data show that activin receptors and Smad proteins are expressed in HepG2 cells and are functional in an activin signaling pathway, leading to the activation of gene expression and cellular apoptosis. Furthermore, we show that activin receptors and Smad proteins are essential for mediating the apoptotic response induced by activin and that overexpression of activin receptors or Smad proteins can mimic activin action to induce apoptosis in

HepG2 cells. These studies provide a framework for understanding interactions between the Smad protein signaling pathway and the pathways leading to cellular apoptosis and for understanding the complex roles of activin in modulating liver cell function.

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