Smad Proteins Suppress CCAAT/Enhancer-binding Protein (C/EBP) β- and STAT3-mediated Transcriptional Activation of the Haptoglobin Promoter*

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The execution of activin A biological functions depends upon association of the cytokine with type I and type II serine/threonine kinase membrane receptors (1, 2). Activin A first binds to activin receptor (ActR)I type II. This is followed by recruitment into the complex and phosphorylation of activin receptor type IB (actRIB), which is responsible for downstream signal transduction. Smad proteins are effectors of intracellular signal transduction of members of the TGFβ superfamily (2–5). The mediators of activin signaling are receptor-activated Smad2 and Smad3 (6), which are present in the cytoplasm in a monomeric form. Following activation, they undergo homodimerization and then heterodimerize with Smad4 (Co-Smad) that lacks the C-terminal phosphorylation motif and is therefore not activated by type I receptors (6, 7). The complex of Smad4 and receptor-activated Smads translocates to the nucleus and binds to Smad binding elements in specific promoters (8–10). Smad3 and Smad4 bind through their conserved MH1 domain to the palindromic sequence GTCTAGAC (11, 12) and to CAGA-like sequences in the human plasminogen activator inhibitor-1 (PAI-1) and junB genes (13–15). Smads cooperate with various transcription factors such as c-Fos and c-Jun in binding to AP-1 sites (16). Smads also complex with Forkhead activin signal transducer (FAST) proteins for efficient binding to specific promoters (17) and associate with the closely related transcriptional co-activators p300 and CBP. The latter interact with a variety of transcription factors and thereby link these factors to the basal transcription machinery (18–20). A hallmark in Smad signaling is therefore their interaction with other transcription regulators. Engagement of the activin A receptors is further followed by increased expression of inhibitory Smad7, which binds to type I receptors and antagonizes further signal transduction (21–23).

This general scheme of signal transduction is common to many activin A-induced systems. One major question raised therefore is how does activin A elicit, in different cells, distinct biological responses. The ActR-triggered signaling cascade was found to interact with mitogen-activated protein kinase pathways (24), and such cross-talk among cascades may contribute to the diversity of responses. We have shown that activin A kills tumor plasmacytomans and hybridomas (25, 26), by competitively overcoming the survival and proliferation-promoting effects of IL-6 (27). This competition did not occur on the level of interactions of IL-6 with its receptor complex (27), suggesting that activin A interferes with intracellular signaling. In this hybridoma model, cell functions, survival and proliferation, all depends on IL-6. On the other hand, we found that activin A slows down HepG2 hepatoma cell growth through hypophosphorylation of retinoblastoma, without causing cell death (28). It further blocked, in these hepatoma cells, the growth of tumor plasmacytomas and hybridomas (25, 26), by competitively overcoming the survival and proliferation-promoting effects of IL-6 (27). This competition did not occur on the level of interactions of IL-6 with its receptor complex (27), suggesting that activin A interferes with intracellular signaling. In this hybridoma model, cell functions, survival and proliferation, all depends on IL-6. On the other hand, we found that activin A slows down HepG2 hepatoma cell growth through hypophosphorylation of retinoblastoma, without causing cell death (28). It further blocked, in these hepatoma cells, the growth of tumor plasmacytomas and hybridomas (25, 26), by competitively overcoming the survival and proliferation-promoting effects of IL-6 (27).

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from the NIDDK, National Institutes of Health, National Hormone and Pituitary Program and was used at 300 units/ml unless indicated otherwise.

**Western Blotting**—The acute phase protein haptoglobin was monitored in conditioned medium under nonreducing conditions (2% SDS, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.01% bromphenol blue). Extracts were subjected to 8% SDS-polyacrylamide gel electrophoresis, blotted, and probed with specific monoclonal antibodies (HG-36, Sigma). Chemiluminescent signals were generated by incubation with the ECL reagent. The gels were exposed to x-ray film.

**DNA Constructs**—pHp (190-bp CT plasmid, containing a 190-bp fragment from the Hp promoter, was a gift from Dr. Heinz Baumann (Roswell Park Cancer Institute, Buffalo, NY) (30). The 190-bp fragment was subcloned upstream of a luciferase gene into pGL3-basic plasmid (Promega, Madison, WI). The cDNA of C/EBP β was subcloned from pBlue610, a gift from Dr. Shizuo Akira (Hyogo College of Medicine, Hyogo, Japan) into pcDNA3 expression plasmid (Invitrogen). STAT3 was expressed in pCMV/STAT3 (Invitrogen). Drs. Y. Zhang and R. Derynck (University of California, San Francisco) provided the plasmids harboring Flagged Smad3, Flagged Smad4, and Flagged Smad2 (8). The Smad genes were subcloned into pcDNA3. The PAI-1 promoter-reporter construct was a gift of Dr. D. J. Loskutoff (The Scripps Research Institute, La Jolla, CA). Dr. Peter ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden) provided Flagged Smad7 and CAcActRIB (ALK4-QD) constructs. pVP22 constructs, full-length Flagged Smad genes, were constructed in-frame, into pVP22/myc-His Vector (Invitrogen) resulting in fusion proteins. The CMV promoter construct was provided by Dr. Steven Grossman (Dana-Farber Cancer Institute, Boston).

**Luciferase Assay**—Transient transfections with the appropriate vectors were carried out using the calcium phosphate method. Transfection efficiency was normalized to β-galactosidase or Renilla activities by cotransfection of 0.5 μg of CMV-β-galactosidase or 2 ng of pRL-CMV expression vectors, respectively. Cells were plated in 6-well plates (Falcon) and transfected with 0.5 μg of the reporter plasmid and different DNA constructs in a final volume of 1.5 ml. The total amount of DNA was normalized using empty vector. Six hours after transfection, cells undergoing serum shock and 24 h later were serum starved and incubated in the presence or absence of IL-6 as indicated. Cells were then harvested, and the luciferase activity in cell lysates was determined according to standard procedure with the aid of a Turner Designs luminometer.

**Electrophoretic Mobility Shift Assay**—Cells were plated in 10-cm plates and transfected with the indicated vectors using FuGENE 6 reagent (Roche Molecular Biochemicals). Nuclear cell extracts were prepared as described previously (31), mixed with 1–3 μg of poly(dI-dC) (Amersham Pharmacia Biotech) in 20 μl of binding buffer containing 110 mM KCl, 4 mM MgCl2, 4 mM Tris-HCl, pH 7.6, 10% glycerol, 0.05 mM dithiothreitol, 0.25% bromphenol blue. The mixture was kept on ice for 10 min, and then the labeled double-stranded oligonucleotide probe was added, and incubation was continued for 20 min longer on ice. The reaction product was then electrophoresed in a 5% nondenaturing polyacrylamide gel and run in a Tris-glycine running buffer (25 mM Tris-HCl, pH 8.3, 190 mM glycine, and 10 mM EDTA). For supershift analysis, rabbit polyclonal anti-C/EBP β antibodies were obtained from Santa Cruz Biotechnology. After proteins were allowed to bind the probe, samples were incubated with the antibodies for another 15 min at room temperature. The oligonucleotide probe used was a double-stranded DNA element from the haptoglobin promoter containing the C/EBP β site (top strand) as follows: CCGACATTGTGCAAACACAGAAATGGAAG. The oligonucleotide for competition was a double-stranded non-relevant DNA element from the haptoglobin promoter (top strand) as follows: TTGTGTGGTACTCGGAAAGCAGTCACTTGGCCT.

**RESULTS**

**Activin A Antagonizes the IL-6-induced secretion of the Acute Phase Protein Hp through Inhibition of Transcription Activation**—The acute phase protein gene promoter C/EBP β is a competitive antagonist of IL-6-induced functions (27). The present study was undertaken with the aim of elucidating the molecular basis for the ability of activin A to block IL-6 signaling using the HepG2 hepatoma cell model. These cells have a low basal level of secretion of APPs that is induced by IL-6. Activin A interfered with IL-6-induced secretion of the acute phase protein, Hp, by the HepG2 hepatoma cell line (Fig. 1A). Hp is known to be expressed in several isotypes, which accounts for the differences in expression bands observed. Similar results were obtained when we examined the secretion of an additional acute phase protein, α-acid glycoprotein (27). The effect of activin A was dependent on the time of its application relative to induction with IL-6. Thus, when added prior to IL-6, it caused a more pronounced reduction in Hp production than observed when activin A was added concomitantly with IL-6. By contrast, activin A had virtually no effect when added 17 h post-IL-6 induction (Fig. 1B). The effect of activin A is thus to block Hp production rather than to increase its degradation. To test the possibility that the effect of activin A is mediated by interference with IL-6-induced transcription activation of the H6 gene, we transfected HepG2 cells with a 190-bp fragment of the haptoglobin promoter (30) upstream of a luciferase reporter and measured the response to activin A following activation with IL-6. As shown in Fig. 2A, IL-6 triggered transcription from the 190-bp Hp promoter fragment, whereas addition of activin A markedly reduced this activation.

Constitutively active ActRIB mimics activin A in inhibiting IL-6-induced transcription and suppresses C/EBP β- and STAT3-induced transcriptional activation of the Hp promoter. The addition of exogenous activin A to the cells could be replaced by transfection with a constitutively active mutant form of actRIB (CAcActRIB), which almost completely abolished IL-6-induced transcription from the haptoglobin promoter (Fig. 2B). In contrast, and as expected, CAcActRIB caused marked transcriptional activation of the control luciferase reporter construct that contain the PAI promoter, which has a binding site for Smads (Fig. 2D). These experiments show that the signaling part of the activin receptor complex is sufficient to execute the biological function.

Hp promoter contains DNA binding sites to several IL-6-inducible transcription factors. It has been shown that a variety of IL-6 activities are mediated by C/EBP β and STAT proteins (32–35). Whereas C/EBP β can be tested by direct overexpression in HepG2 cells, STAT3 must first be activated by IL-6 to enable its translocation into the nucleus. CAcActRIB reduced C/EBP β-mediated transcriptional activation of Hp promoter and completely abolished STAT3-induced activation (Fig. 2C) indicating that activin A signaling affects IL-6-induced transcription either on the level of these transcription factors or downstream in the IL-6 signaling cascade. Further
transfection efficiency was normalized to 

$B_D$ induced activation of this promoter (Fig. 3, induced with IL-6 itself or with C/EBP $b$ transcription was completely restored (Fig. 3 respectively). However, the IL-6-dependent STAT3-induced 

bars 

ments—

CAactR and were treated with IL-6. 

BARs in a dose-dependent manner. $B$, similar inhibition was observed when HepG2 cells were transfected with plasmids containing cDNA encoding CAactR and were treated with IL-6. $C$, Hp promoter activity induced by C/EBP$\beta$ or STAT3 in combination with IL-6 was also reduced by CAactR.

$D$, a control experiment for the activity of the CAactR in which the activin A-inducible PAI promoter was used. In all of the luciferase assays, the transfection efficiency was normalized to $B_g$-galactosidase activity. Results of one experiment of three performed are shown. Here and in the following figures, the bars indicate the average luciferase activity ± standard deviation of triplicate determinations.

experiments were aimed at identifying the molecules within the activin A signaling pathway that mediates its effect on IL-6 signal transduction. This information could then lead to identification of the target molecule for activin A interference within the IL-6 pathway.

Smad7 Restores IL-6 Transcriptional Activation of the Hp Promoter Following Suppression with CAAcRIB—Signaling by actRIB recruits the cytoplasmic Smad proteins that upon activation and phosphorylation migrate to the nucleus and attach to specific sites in activin A-inducible promoters. Smad7 interacts with activin A type I receptors and blocks further downstream activation of receptor activated Smads. It follows that if Smad7 overrides the effects of activin A or of CAAcRIB. We found that Smad7 reverted the CAAcRIB inhibition of IL-6-induced Hp promoter transcription (Fig. 3A). Similar results were obtained following independent activation by C/EBP$\beta$ or by IL-6-dependent STAT3-induced activation of this promoter (Fig. 3, B and C, respectively). However, the IL-6-dependent STAT3-induced transcription was completely restored (Fig. 3C), whereas that induced with IL-6 itself or with C/EBP$\beta$ did not return to control values. Fig. 3D shows that PAI promoter activation by CAAcRIB is completely abolished by Smad7, verifying the ability of this protein to negatively regulate activin A signaling.

We thus showed that Hp promoter activation, which is mediated by C/EBP$\beta$ or STAT3, is suppressed by activin A or by a constitutively active form of its signaling receptor. Furthermore, the transcription activation is restored by Smad7, suggesting the involvement of Smad proteins in the suppression of IL-6-induced transcription.

Smad3 and Smad4 Suppress Transcription Activation through Hp and $\beta$-Fibrinogen Promoter Response Elements—To directly examine the role of Smad proteins in activin A-mediated suppression of IL-6-induced transcription, we transfected HepG2 cells with Smad3, Smad4, or both. Co-transfection with Smad3 and Smad4 resulted in the most pronounced suppression of IL-6-induced Hp promoter activity (Fig. 4A). Similar experiments performed with a combination of Smad2 and Smad4 yielded essentially identical results (not shown). It is noteworthy that Smad3 alone also caused significant reduction in the promoter activity, whereas Smad4 alone was inactive. These results are strongly supported by additional experiments in which Hp promoter activation was mediated by either C/EBP$\beta$ (Fig. 4B) or STAT3 (Fig. 4C). The lack of effect of Smad4 when transfected on its own could be because of its inability to enter the nucleus. To allow for borderline activation of the activin A pathway, we transfected the cells with CAAcRIB at a concentration that does not inhibit C/EBP$\beta$-mediated transcription (25–100 ng as compared with 1.5 $m$ required for complete suppression). Under these conditions Smad4 was sufficient to cause significant suppression of C/EBP$\beta$-induced transcription from the Hp promoter (Fig. 4D). It is noteworthy that the relationship between the effects of Smad3 alone, as compared with Smad3 and Smad4 together, were maintained when studied on the PAI promoter, although in the case of this activin A-inducible gene, Smads caused augmentation of transcription (Fig. 4E). We performed further experiments with promoters from several other genes, which were either constitutively active (CMV, GLN-LTR) or specifically activated (IRF, $\alpha M$ activated by C/EBP$\beta$ or STAT3 and IL-6). None of these promoters was suppressed in a manner comparable with APP promoters (data not shown). Taken together these results indicate that activin A operates by activating Smad proteins that in turn suppress IL-6-mediated transcriptional activation of specific promoters. Smad proteins also suppressed transcriptional activation by the IL-6 downstream transcription activators, C/EBP$\beta$ and STAT3. This phe-
nomenon was not restricted to the *Hp* gene because experiments conducted with an IL-6 response element of the β-fibrinogen promoter, which was reported to respond to C/EBPβ and STAT3 (36, 37), yielded similar results. As shown in Fig. 5, *A* and *B*, C/EBPβ- or STAT3-induced transcription from the β-fibrinogen IL-6 response element was suppressed by a combination of Smad3 and Smad4, whereas only Smad3 on its own had a partial suppressive effect. Thus, response elements in two independent APP gene promoters are negatively regulated in a similar manner by Smad proteins.

To examine the effect of Smad proteins on the endogenous *Hp* gene in HepG2 hepatoma cells, we used the pVP22 vector.
The VP22 protein has the ability to translocate between cells. After translation in the cytoplasm, the expressed VP22 protein is exported from transfected cells and translocates into the nucleus of adjacent non-transfected cells; this subsequently leads to the nuclear expression of the transfected plasmids in a large proportion of the cells. Full-length Flagged Smad genes were constructed in-frame into pVP22 vector as an N-terminal fusion to VP22. The cells were transfected with these constructs and were treated with IL-6 48 h after transfection for a period of 24 h. The end point of these experiments was secretion of the haptoglobin protein following induction with IL-6. Fig. 6 shows that Smad3 together with Smad4 reduced the accumulation of haptoglobin protein in the cultures.

The Transcription Co-activator p300 Overcomes Smad-mediated suppression of Hp Promoter Transcription Activation—p300 has a histone acetyltransferase domain. By acetylation of core histones, p300 loosens the nucleosomeal structure and allows access of transcription factors to the general transcription machinery. The study of interactions between Smads and partner proteins that form transcription complexes revealed the functional significance of p300 as a bridge-former that allows the execution of Smad-mediated transcriptional activation. Smad3 interacts with p300 with high affinity (18–20). p300 also binds to various transcription factors including STAT3 and C/EBPβ through different segments thereby linking them to the basal transcription machinery (38–40). Indeed, we find that p300 by itself increased the transactivation capacity of C/EBP β and STAT3 (Fig. 7, A and B, respectively). The formation of functional transcription activation complexes depends upon the correct stoichiometric concentrations of the various components. We reasoned that if Smad competes with STAT3 and C/EBPβ on binding to p300, then an excess amount of the latter would prevent the effect of Smad. We therefore co-transfected HepG2 cells with plasmids expressing the transcription factors and CAactRIB along with p300. It was found that p300 reduces, in a dose-dependent manner, the ability of CAactRIB to interfere with IL-6 induction of transcription through either C/EBPβ or STAT3 (Fig. 7, A and B, respectively). In addition, transfection of cDNA encoding E1A, which is known to bind p300, reduced completely the transactivation of the haptoglobin promoter by STAT3 and C/EBPβ (data not shown) indicating that Smads compete with the transcription factors for binding to p300.

Smad4 Reduces the Binding of C/EBPβ to Hp Promoter DNA—One possible interpretation of the suppressive effect of Smad proteins on activation of the Hp promoter is that Smads interfere, directly or indirectly, with the binding of the relevant transcription factors to DNA. To test this possibility, we performed EMSA assays by using nuclear extracts obtained from...
cells transfected with expression vectors. Differences between signals obtained in such EMSA assays may result from the loading of variable amount of protein. To exclude this possibility we verified by Western blotting that equal amounts of C/EBPβ were loaded per lane. Fig. 8 details the factors that were examined for their corresponding ability to affect the binding of C/EBPβ to a 20-bp DNA oligonucleotide probe from the Hp promoter. C/EBPβ bound effectively to this fragment as revealed by gel shift and supershift examination (Fig. 8A). This binding was highly specific because it was competed out by an excess of cold promoter fragment, whereas an irrelevant control fragment had no effect. This DNA binding was abolished by overexpression of CAactRIB (Fig. 8B) presumably through activation of Smads. To further examine this point, we transfected cells with lower amount of the constitutively active receptor to minimize the suppressive effect. Under these conditions transfection with Smad4 leads to further inhibition of DNA binding (Fig. 8C).

**DISCUSSION**

Cytokine members of the TGFβ superfamily are pleiotropic molecules exhibiting a variety of biological functions (2). The present study was focused on the mechanism by which activin A blocks the secretion of the IL-6-induced APP, Hp. Our previous studies have implied that activin A interferes with the signaling cascade induced by IL-6 downstream to the receptor (27). We therefore focused on the possible interactions of these intracellular transduction pathways. It is shown here that Smad proteins are sufficient by themselves to mediate an activin A-like suppressive effect on Hp promoter activation. Furthermore, the suppressive effect of Smads was reproduced when the Hp promoter was activated by overexpression with the IL-6 pathway transcription factor, C/EBPβ, alone. Smads operate therefore on the level of C/EBPβ and/or downstream in the cascade.

A constitutively active form of activin A receptor was effective in suppressing Hp expression by the HepG2 hepatoma cell line. Whereas activin A only partially suppressed Hp promoter activation, CAactRIB almost abolished transcription, possibly because of the sustained signal that a high number of constitutively active receptor molecules per cell elicits, followed by maximal activation of downstream targets. CAactRIB blocked transcriptional activation by C/EBPβ without IL-6 stimulation, indicating that any effect of activin on IL-6 signaling could not occur upstream to C/EBPβ. We could not perform similar experiments with STAT3 protein overexpression because this protein acts synergistically with IL-6 to activate transcription and is insufficient by itself. The 190-bp promoter region used in our experiments was activated by C/EBPβ and STAT3 proteins, which were very effectively blocked by the constitutively active form of the activin type I receptor. It therefore appears that a significant portion of the effect of activin A is due to suppression of C/EBPβ and STAT3 transcription activation.

The inhibitory Smad7 abolished the effect of CAactRIB on IL-6 or on the transcription activators C/EBPβ or STAT3. Because Smad7 has been shown to be an inhibitor of receptor-activated Smad2 and Smad3, the results implied the involvement of these intracellular mediators. Direct examination by overexpression of these proteins indicated that Smad3 on its own, and more effectively, a combination of Smad3 and Smad4, suppressed transcriptional activation of the Hp promoter fragment. Overexpression of Smad3 alone was sufficient to cause partial suppression of Hp promoter, probably because of complexing with endogenous Smad4. The use of the pVP22 vector allowed us to examine the endogenous Hp gene. In this study a combination of Smad3 and Smad4 transfection was needed to obtain an effect on Hp secretion. This may be because the endogenous promoter contains sites for binding factors that are additional to those attaching to the 190-bp fragment, and effective suppression of these requires both Smads.

The suppressive effect of CAactRIB on C/EBPβ- and STAT3-induced transcription activation was reduced by overexpression of p300. In other experimental systems relating to TGFβ-induced functions, p300 has been shown to physically bind Smad proteins and serve as a co-activator of transcription (18, 19). p300 might be expected to potentiate the Smad effect rather than reduce it. However, p300 participates in transcription activation complex formation by many other factors including C/EBPβ (38) and STAT3 (39, 40). Therefore, under conditions of limiting the amount of p300, the recruitment of this protein by Smad3 would lead to inhibition of C/EBPβ- or STAT3-dependent transcription. By analogy to the effect of Ski oncoprotein in attenuation of TGFβ signaling, wherein this protein reduces the recruitment of p300 into DNA binding complexes and thus suppresses transcription, it is possible that Smad causes dissociation of p300 from C/EBPβ and STAT3 transcription activator complexes. These data are consistent with a model in which Smad proteins sequester p300, inhibiting C/EBPβ and STAT3 functions. Activation of β-fibrinogen by IL-6 or by C/EBPβ can be similarly suppressed by p53 (41), which physically binds to p300 (42), providing further support for the above interpretation of our data.

It is well established that Smad proteins are transcription activators for genes inducible by TGFβ superfamily members (3, 43). However, Smad proteins are also involved in the suppression of transcription in specific circumstances. TGFβ (TG-interacting factor) and c-Ski repress TGFβ-mediated transcription by recruiting histone deacetylase and by directly binding to Smad proteins and reducing the recruitment of the co-activator p300 into the DNA binding complex (44–48). Thus, in the context of TGFβ-inducible genes, Smad proteins are transcrip-
tion activators but may also suppress transcription. It has not been shown, to the best of our knowledge, that Smad proteins mediate inhibition of transcription of promoters unrelated to the TGFβ-regulated genes. We show in this study that transcriptional activation of response elements in the APP genes, Hsp and β-fibrinogen, which is mediated by IL-6, is inhibited by Smads. These results thus point to a novel cross-talk channel that links the TGFβ serine/threonine receptor-initiated pathway with the IL-6 family tyrosine kinase receptor pathway. Several other studies have implied that TGFβ controls C/EBP- or STAT3-mediated pathways. In intestinal rat epithelial cells, TGFβ caused attenuation of a glucocorticoid-dependent increase in expression of C/EBPβ, as well as reduced binding of this transcription factor to specific sites in the haptoglobin promoter (49) and to α-acid glycoprotein promoter sites (50). In human T cells, TGFβ antagonizes IL-12-induced activation. Whether the effect of TGFβ in this system is mediated by blocking the phosphorylation, and hence activation of Jak or STAT proteins, remains uncertain (51, 52).

EMSA experiments using cellular proteins showed that over-expression of Smad4 interferes with the binding of C/EBPβ to DNA. It is noteworthy that Smad4 did not by itself interfere with transcriptional activation of the Hp promoter (Fig. 4, A–C) unless the cells were co-transfected with CAactRIB at a concentration that does not suppress transcription (Fig. 4D). The EMSA experiments were performed under these latter conditions. We predicted that this would assure that the signaling machinery in the cells would be triggered and would permit efficient translocation of Smad4 into the nucleus. This may not occur in the transcription activation experiments in which Smad4 is transfected into the cells on its own without the support of CAactRIB. Thus, the particular conditions used for the DNA binding experiment allowed us to observe a possible new function of Smad4, i.e. a negative effect on C/EBPβ binding to DNA.

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