The Expression of *novH* in Adrenocortical Cells Is Down-regulated by TGFβ1 through c-Jun in a Smad-independent Manner*

Jérôme Lafont†, Maryvonne Laurent‡, Hélène Thibout‡, François Lallemand§, Yves Le Bouc‡, Azeddine Attif§, and Cécile Martinec‡

From †INSERM U515 and ‡INSERM U482, Hôpital Saint-Antoine, 75571 Paris Cedex 12, France

The human NOV secreted glycoprotein (NOVH) is abundant in the fetal and adult adrenal cortex. The amount of NOVH increases in benign adrenocortical tumors and decreases in malignant adrenocortical tumors, suggesting that NOVH plays a role in tumorigenesis in the adrenal cortex. Transforming growth factor β1 (TGFβ1), fibroblast growth factor 2 (FGF2), and insulin growth factors (IGFs) play crucial roles in the physiology of the adrenal cortex. We investigated the effects of these factors on the expression of *novH* in the NCI H295R adrenocortical cell line. The amounts of NOVH protein and *novH* transcripts were down-regulated by TGFβ1 and up-regulated by FGF2, whereas IGFs had no effect. Furthermore, the TGFβ1-dependent inhibition of *novH* promoter activity was completely abrogated following site-directed mutation of two activating protein (AP-1) sequences (positions −473 and −447), whereas the stimulatory effect of FGF2 was not affected. Co-transfection of dominant negative forms of c-Jun and MEKK1 also abrogated *novH*-targeted regulation by TGFβ1, whereas the overproduction of Smad proteins or dominant negative forms of Smad had no effect. Taken together, these results suggest that c-Jun and MEKK1 signaling but not Smad signaling are involved in the TGFβ1-dependent decrease in NOVH in NCI H295R cells. In conclusion, our data provide evidence that *novH* is a new target of TGFβ1; unlike other members of the CCN (*cyr61, ctgf, nov*) family, however, its expression is repressed rather than induced.

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‡ To whom correspondence should be addressed: INSERM U515, Hôpital Saint-Antoine, 184 rue du Faubourg Saint-Antoine, 75571 Paris Cedex 12, France. Tel.: 33-1-4928-4664 or 4631; Fax: 33-1-4343-1065; Email: martinec@st-antoine.insERM.fr.

§ The abbreviations used are: nov (NOV), nephroblastoma overexpressed gene (protein); *novH* (NOVH), human *nov* gene (protein); *novM*, mouse *nov* gene; CCN, *cyr61, ctgf, nov* family; TGFβ1, transforming growth factor β1; IGF, insulin-like growth factor; FGF2, fibroblast growth factor 2; AP-1, activator protein 1; MLP, major late promoter; CAT, chloramphenicol acetyl transferase; MEKK1, mitogen-activated/extra cellular response kinase kinase 1; MKK7, mitogen-activated kinase kinase 7; JNK, c-Jun NH2-terminal protein kinase; SAPK, stress activated protein kinase; GST, glutathione S-transferase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase (gene); DN Smad4, dominant negative Smad4 protein.

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* The expression of *novH* is altered in several human tumors, including Wilms’ tumors (4, 10) and adrenocortical tumors (19). Adrenocortical tumors, which have a very poor prognosis (20, 21), quantitative and qualitative changes in *novH* expression are correlated with the acquisition of the tumor phenotype by adrenocortical tissue (19). Significant differences have been detected in the concentrations of NOVH and *novH* mRNA in benign and malignant tumors. Furthermore, the NOV protein profiles are different in the two types of tumor, suggesting that *novH* plays a role in the early stages of tumorigenesis. The enhanced expression of *novH* in benign tumors may contribute to the benign phenotype by decreasing cell adhesion, whereas the lower expression of *novH* in malignant tumors could be involved in cell invasiveness (19). Alternatively, the down-regulation of *novH* in malignant adrenocortical tumors suggests that *novH* could act as a tumor suppressor. This hypothesis is supported by the inverse cor-

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‡ M. Laurent, unpublished results.
lation between tumorigenicity and novH expression in glioma cells (22) and by the fact that the ectopic expression of novH in glioma cells reduces their tumorigenicity in xenografts (23).

Therefore, we decided to investigate the molecular mechanisms responsible for the alterations in novH expression in tumoral adrenocortical cells. For this purpose, in the present study we used the human NCI H295R cell line, which is derived from a human adrenocortical carcinoma that produces steroids (24, 25). A number of growth factors and cytokines such as epidermal growth factor (EGF), transforming growth factor-\(\alpha\) (TGF-\(\alpha\)), tumor necrosis growth factor (TNF-\(\alpha\)), interleukins (26–28), insulin-like growth factors (IGFs) (for a review see Refs. 26 and 29), fibroblast growth factor 2 (FGF2) (26), and transforming growth factor \(\beta\) (TGF-\(\beta\)) (for a review see Refs. 26 and 29) regulate adrenal growth and functions in normal and fetal adrenal glands. The concentration of IGF-II, which also plays a role in adrenocortical tumorigenesis (24, 30, 31), is inversely correlated to novH expression in several malignant adrenocortical tumors, suggesting that IGF-II regulates the expression of novH or vice versa (19). Moreover, FGF2 and TGF-\(\beta\) can induce the production of other members of the CCN family such as CTGF and CYR61 in fibroblasts and in some epithelial cells (32–36).

Thus, we examined whether novH expression is affected by IGFs, FGF2, and TGF-\(\beta\) in NCI H295R cells. We showed that the expression of novH is not modulated by IGFs but is upregulated by FGF2 and down-regulated by TGF-\(\beta\). These regulations occur at the transcriptional level. Further studies indicated that two AP-1 consensus binding sites (−473 and −447) within the novH promoter play a crucial role in TGF-\(\beta\) regulation but not in the stimulatory effect of FGF2. Finally, we provide evidence that c-Jun and MEKK1, but not Smad, can mediate the TGF-\(\beta\)-dependent decrease of novH expression.

MATERIALS AND METHODS

Plasmids

Reporter Constructs and Expression Vectors—The novH promoter constructs p625NH-Luc and p492NH-Luc were derived from p625NH-CAT and p492NH-CAT, respectively (37). Following digestion with HindIII and BglII, the novH promoter fragments were subcloned into the promoterless p2KMBIT luciferase reporter vector (38). p52540NH-Luc was obtained by replacing the 493-bp HindIII-BsrI fragment from p625NH-Luc with the 1.8-kb HindIII-BsrI fragment derived from the novH pBlH7 subclone (4). The (CAGA)9-MLP-Luc reporter was a gift from Dr. J. M. Gauthier. Myc-Smad2, Smad3, Smad4, FLAG-Smad7, pCMV-TAM67, c-Jun-Ala, and MEKK1/K432A have been described previously (39–42). The constitutively activate MKK7 and the dominant negative Smad4 mutant were gifts from Dr. E. Nishida and Dr. R. Derynick, respectively. The reporter construct containing four copies of the AP-1 enhancer (pAP-1-Luc) was purchased from Clontech.

Site-directed Mutagenesis— Either one or both of the AP-1 consensus sites present at positions −473 (GGTGACA/CAT) and −447 (CATGAC/TAAC) (Fig. 7A) of p625NH-Luc were changed to TGATGA using a two-step PCR strategy. Both strands of all the constructs were fully sequenced (Genome Express, Grenoble, France) to confirm the mutations before use.

Cells

Cell Culture—NCI H295R cells (ATCC) were maintained in Dulbecco’s modified Eagle’s/F12 medium supplemented with 2% Ultrapro G (Invitrogen), 5 \(\mu\)g/ml insulin, 5 \(\mu\)g/ml transferrin, and 5 ng/ml sodium selenite (Sigma); 200 units/ml penicillin, 200 ng/ml streptomycin, and 2.5 mm l-glutamine. Primary cultures of mouse astrocytes were obtained as follows. Brains from 3–4-day-old mice were dissected and crushed in minimum Eagle’s medium. Cells were recovered by filtration through sterile filters (70-\(\mu\)m pores), resuspended in minimum Eagle’s medium containing 25 mm Hepes, nonessential amino acids, and 10% fetal calf serum and plated out. Fibroblasts were allowed to adhere for 4 h, and any non-adhering astrocytes were replated on complete medium.

To eliminate the influence of serum, NCI H295R cells were transferred into Dulbecco’s modified Eagle’s/F12 medium supplemented with transferrin (5 \(\mu\)g/ml), selenium (5 ng/ml), 200 units/ml penicillin, 200 \(\mu\)g/ml streptomycin, and 2.5 mm l-glutamine. All experiments were performed with cell lines obtained from passages 2–8 following thawing or with primary cultures from passage 2.

Cytokine Treatment of Cell Cultures—Adrenocortical cell lines (NCI H295R) plated out at a density of 5 × 10^6 cells per 100-mm dish were incubated in a serum-free medium for 24 h and then treated with TGF-\(\beta\) 1 (Sigma) or FGF2 (R&D Systems) or left untreated as indicated in the text. In some experiments, cycloheximide (10 \(\mu\)g/ml, Sigma) and actinomycin D (5 \(\mu\)g/ml, Sigma) were added to the medium 1 h before the addition of the growth factor.

RNA Extraction and Northern Blotting—Total RNA was isolated from cultured cells by use of the acid-guanidium-thiocyanate-phenol-chloroform extraction kit according to the manufacturer’s instructions (Tri-Reagent, Sigma). Total RNA samples (10 \(\mu\)g) were loaded onto a 1% agarose-2.2 mol/liter formaldehyde gel, subjected to electrophoresis, and transferred onto nylon membranes. The membranes were hybridized as previously described with the 1.9-kb EcoRI novH probe or the 2.3-kb EcoRI-XhoI mouse nov (novM) probes (4, 10, 43) labeled by random hexamer priming (Amersham Biosciences) in the presence of \([\beta^32P]dCTP\). The signal for novH or novM was normalized according to the intensity of the gapdh signal (Clontech).

Luciferase Reporter Assays—NCI H295R cells plated in 6-well plates (5 × 10^5 cells per well) were transfected using LipofectAMINE Plus (Invitrogen) as described in the user’s manual. For reporter assays, the reporter constructs (0.5 \(\mu\)g) were co-transfected with 0.1 \(\mu\)g of pCMV-\(\beta\)-galactosidase as an internal transfection control (Clontech). For assays in which the role of the transacting proteins was to be tested, 1.5 \(\mu\)g of the empty pcDNA3 (Invitrogen) vector or pcDNA3 encoding the protein of interest was used. In dose response experiments, the total amount of the expression vectors was kept constant by use of the empty

\[\text{A} \quad \text{B} \]

\[\text{NovH (48 kDa)} \quad \text{(31-32 kDa)} \]

\(\text{Conditioned medium} \)

**FIG. 1. The expression of novH increases with cell density and in the presence of serum in NCI H295R cells. A. Western blot analysis showing the amount of NOVH produced in 48 h by 4.5 × 10^6 (lane 1), 1.75 × 10^6 (lane 2), 4.5 × 10^6 (lane 3), and 10^7 (lane 4) NCI H295R cells in serum-free conditions. B. Western blot analysis showing the amount of NOVH produced by 10^7 NCI H295R cells in 48 h in the presence of serum (lane 5) and in serum-free conditions (lane 6). A fraction of each medium corresponding to 10^6 cells was incubated with heparin-Sepharose overnight at 4 °C before SDS-PAGE and immunodetection with K19M antibody at a 1:500 dilution (19). SF9/82-conditioned medium (20 \(\mu\)l) containing NOVH baculoviral recombinant protein (19) was used as a control (lane 7). The amount of NOVH increased by 1.25 ± 0.08, 2.1 ± 0.57, and 5.3 ± 0.11-fold in the presence of serum. Arrow indicates the two (48 and 31–32 kDa) NOVH isoforms produced in conditioned medium, and asterisks indicate the two (44 and 27 kDa) baculoviral recombinant isoforms of the NOVH protein.**
vector. In each assay, cell cultures were serum starved prior to treatment with TGFβ1 (4 ng/ml) or FGF2 (10 ng/ml). Luciferase and β-galactosidase activities were assayed by use of kits from Promega and PerkinElmer Life Sciences (Galacto-Star system), respectively. The data are presented as means ± S.E. of representative experiments performed in triplicate on at least two separate occasions.

Immunoblotting—For the detection of endogenous NOV, cultured NCI H295R cells were lysed in radioimmune precipitation assay buffer (50 mM Tris (pH 7.4), 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM 4-(2-aminoethyl) benzene sulfonyl fluoride, 5 mM EDTA, 20 mg/ml aprotinin, 20 mg/ml leupeptin). For the detection of secreted NOV, proteins from conditioned medium corresponding to 2 × 10⁵ cells (unless otherwise indicated) were collected after incubation overnight at 4°C with heparin-Sepharose as described previously (10).

Protein samples from the lysates (10 μg) or from the conditioned medium were subjected to electrophoresis in 12% reducing SDS-polyacrylamide gels before being transferred to polyvinylidene difluoride membranes (Hybond P, Amersham Biosciences) for immunological detection. The membrane was incubated with the K19M anti-NOV (1:500 dilution) polyclonal antibody (10) for 1 h at 37°C. Immunoreactive proteins were detected by ECL (Amersham Biosciences) according to the manufacturer’s instructions. For the detection of proteins encoded by transfected expression vectors, protein samples (40 μg) derived from the same cell lysates used for luciferase were subjected to immunoblotting. The anti-Myc (9E10; Santa Cruz Biotechnology) monoclonal antibody was used to detect c-Myc-tagged Smad2, 3, and 4. Immunoreactive proteins were visualized by ECL.

Protein Kinase Assay—JNK activity was determined as described previously (42) with minor modifications. Briefly, cells were lysed in a buffer containing 25 mM Heps (pH 7.5), 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% Triton X-100, 0.5 mM dithiothreitol, 0.5 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 20 μM sodium vanadate, and 10% glycerol. Lysates were clarified by centrifugation, and endogenous kinases were immunoprecipitated using anti-JNK (C-17; Santa Cruz Biotechnology) antibody. Immune complexes were collected by adding electrophoresis sample buffer, and proteins were separated on 12% SDS-polyacrylamide gels before being transferred to polyvinylidene difluoride membranes (Hybond P, Amersham Biosciences) for immunological detection. The membrane was incubated with the K19M anti-NOV antibody (10) for 1 h at 37°C. Immunoreactive proteins were detected by ECL (Amersham Biosciences) according to the manufacturer’s instructions.

Densitometry—Western blots were scanned with a GS700 imaging densitometer and processed with the Molecular Analyst data system (Bio-Rad). Northern blots were analyzed with a Storm PhosphorImager (Amersham Biosciences).

RESULTS

Expression of novH in NCI H295R Is Down-regulated by TGFβ1 and Up-regulated by FGF2—NCI H295R cells were consolidated to be a good cellular model for adrenocortical tumors (24). To determine whether the expression of novH is influenced by environmental conditions, we used Western blotting to examine the amount of NOVH present in a conditioned medium when these cells were plated out at different densities. In serum-free medium, the amount of NOVH detected, corresponding to the same number of NCI H295R cells (10⁵) tested, increased with cell density (Fig. 1A). The concentration of secreted NOVH also increased in the presence of serum (Fig. 1B).

Fig. 2. Down-regulation of novH expression by TGFβ1 and up-regulation of novH expression by FGF2 in NCI H295R. Western blot analysis showing the amount of NOVH in NCI H295R or secreted into the medium following 24 h in serum-free medium with IGF-I (50 ng/ml), TGFβ1 (4 ng/ml), IGF-I (50 ng/ml), or FGF2 (10 ng/ml) plus TGFβ1 (4 ng/ml). C, untreated control. Arrows indicate the two isoforms of NOVH.

Fig. 3. Dose-response and time-course expression of novH in NCI H295R cells treated with TGFβ1 and FGF2. A, Northern blot analysis of novH in NCI H295R treated with increasing concentrations of TGFβ1 or FGF2 for 24 h or not treated. B, Northern blot analysis of novH in NCI H295R cells treated with TGFβ1 (4 ng/ml) or FGF2 (10 ng/ml) for increasing periods of time. Blots were successively hybridized with novH and gapdh probes and subjected to autoradiography. Densitometric analyses of the normalized novH/gapdh concentrations are presented in the lower panels. AU, arbitrary units.
Thus, the production of NOVH in NCI H295R cells may be regulated by cell-cell contact and by growth factors present in serum.

We next tried to identify growth factors that affect the production of NOVH in these cells. We focused on TGFβ1, FGF2, and IGFs because they play important roles in adrenocortical development and physiology (see Ref. 26 for a review). As shown in Fig. 2, the amount of NOVH did not change following treatment with IGF-II or IGF-I; however, the amount of NOVH (in cell lysates or in medium) was decreased (~80%) by TGFβ1 (4 ng/ml) and increased (~3-fold) by FGF2 (10 ng/ml). The amount of NOVH increased only slightly in the presence of both factors, suggesting that TGFβ1 inhibits both basal and FGF2-induced expression of NOVH.

The amount of novH mRNA was also affected by TGFβ1 and FGF2 in a dose-dependent manner (Fig. 3A). Time-course experiments showed (Fig. 3B) that novH down-regulation by TGFβ1 (4 ng/ml) was maximal after 24 h (72 ± 20%, n = 8), whereas stimulation by FGF2 (10 ng/ml) reached a maximum after 6 h, and this level was maintained for at least 24 h (2.5 ± 0.4-fold, n = 8).

Next, we examined whether protein synthesis was required for the regulation of novH by TGFβ1 or FGF2 in NCI H295R. Pretreatment with the translation inhibitor cycloheximide 1 h before the addition of TGFβ1 or FGF2 did not block the effects of TGFβ1 but completely abolished the effects of FGF2 (Fig. 4A). Thus, TGFβ1 directly regulates the expression of novH, and FGF2 requires de novo protein synthesis, which could be rapidly induced.

To gain further insight into the molecular mechanisms by which TGFβ1 and FGF2 regulate the amount of novH mRNA, we investigated the effects of these factors on the steady-state levels of transcripts. From Fig. 4B it can be seen that when transcription was blocked with actinomycin D for 9 h, the basal level of novH mRNA decreased (~3-fold), indicating that the half-life of novH transcripts is less than 9 h in these cells. Under these conditions, TGFβ1 and FGF2 did not significantly modulate the inhibitory effect of actinomycin D. This is consistent with the hypothesis that these factors regulate the expression of novH at the transcriptional level.

**TGFβ1 and FGF2 Regulate novH Promoter Activity**—To better understand the mechanism by which TGFβ1 and FGF2 regulate the transcriptional activity of novH, we analyzed their effects on the novH promoter fused to the luciferase reporter gene in transient transfections in NCI H295R cells. We assessed the regulation of three different promoter constructs, p2540NH-Luc (~2540 to +87), p625NH-Luc (~625 to +87), and p492NH-Luc (~492 to +87) by TGFβ1 and FGF2 (Fig. 5A). Treatment of all three constructs with TGFβ1 resulted in ~50% inhibition, and treatment with FGF2 resulted in ~150% stimulation (Fig. 5, B and C). Thus, the promoter region between ~2540 and ~492 is not involved in the regulation of novH expression by these two factors. Furthermore, the stimulatory effect of FGF2 on p625NH-Luc promoter activity was reduced following the addition of TGFβ1 (Fig. 5D), which is consistent with our results for the endogenous NOVH. Therefore, we carefully examined the promoter region beyond position ~492 to try to identify any specific cis-acting elements that could be involved in the regulation of novH by TGFβ1 and FGF2.

We also investigated the ability of TGFβ1 and FGF2 to modulate the expression of nov in cells from other species. Using primary cultures of mouse astrocytes, we observed that FGF2 had no effect, whereas TGFβ1 also decreased the amount of nov RNA in these mouse cells (Fig. 6). Therefore, we compared the sequences of the human (novH) and murine (novM)
promoter sequences (Fig. 7A). We found two consensus sequences corresponding to AP-1 binding sites in the novH promoter region (at positions -473 and -447). Interestingly, these sequences were also present in the novM promoter region.

The AP-1 family of transcription factors is implicated in various regulatory activities of TGF\(\beta\) and FGF2. To determine the role of AP-1 in the regulation of nov expression by TGF\(\beta\) and FGF2, we used site-directed mutagenesis to alter the AP-1 sites (Fig. 7B). Each of the point mutations resulted in a substantial decrease in basal promoter activity (~4–5-fold) when these constructs were used to transfet NCI H295R. No further effect was observed when both AP-1 sites were mutated simultaneously (Fig. 7B and C).

As shown in Fig. 7C, none of the AP-1 mutations prevented FGF2 from stimulating novH promoter activity (~100%), indicating that this process does not involve the binding of AP-1 to these sites. However, each of these mutations completely abrogated the effects of TGF\(\beta\) on novH promoter activity in these cells (Fig. 7D) even following stimulation by FGF2 (data not shown). These data show that these AP-1 sites mediate the inhibitory effects of TGF\(\beta\) and also suggest that the mechanism by which TGF\(\beta\) inhibits FGF2 stimulation of novH expression is not a direct competition between transcription factors for binding on AP-1 sites.

**Fig. 5. Effects of TGF\(\beta\) and FGF2 on novH promoter activity.** A, schematic structures of novH-Luc reporter constructs. The potential consensus sequences of the transcription binding sites are indicated (37). B and C, NCI H295R cells were transfected with p2540NH-Luc, p625NH-Luc, or p492NH-Luc (0.5 \(\mu\)g). Cells were or were not treated with TGF\(\beta\) or FGF2 24 h prior to lysis and subjected to a luciferase assay. The results are expressed as the mean ± S.E. of a representative experiment performed in triplicate. D, NCI H295R cells were transfected with p625NH-Luc. 4 h later the cells were or were not treated with FGF2. After 20 h they were treated with TGF\(\beta\) in the presence or absence of FGF2 (10 ng/ml). Cells were subjected to the luciferase assay 24 h later. The increase in p625NH-Luc promoter activity (~370%) due to FGF2 in these experiments compared with control was probably due to longer treatment times of cells with the growth factor. The results are expressed as the mean of a representative experiment, performed in triplicate, ± S.E. AU, arbitrary units.
C-Jun and MEKK1 Are Involved in the Effects of TGFβ1 on novH Expression—Next, we were interested in determining which signaling pathways mediate the inhibitory effect of TGFβ1 on novH expression. TGFβ1 signaling is mediated by two types of serine-threonine kinase receptors (50, 51). The highly conserved Smad proteins act as downstream signal transducers (51, 52). Smad2 and Smad3 are restricted to the TGFβ/activin pathway. After phosphorylation by TGFβ1-activated type I receptors, pathway-restricted Smads form hetero-

Fig. 6. Regulation of the expression of novM by TGFβ1 and FGF2 in mouse astrocytes. Northern blot analysis of novM in primary cultures of mouse astrocytes (15 μg total RNA) treated with TGFβ1 (4 ng/ml), FGF2 (10 ng/ml), or not treated (C, control) for 24 or 48 h. Blots were successively hybridized with novM and GAPDH probes and processed for autoradiography.

Fig. 7. Regulation of the novH promoter by TGFβ1 through AP-1 sites. A, alignment of the ~700-bp region encompassing the human and mouse nov promoter. The top strand represents the human (H) nov promoter and the bottom strand represents the mouse (M) nov promoter (deposited in the GenBank®/EMBL database under the accession number AJ431713). Nucleotides of the human nov promoter are numbered relative to the transcription start site (37). Because the transcription start site of novM has not yet been determined, nucleotides are numbered relative to the first coding ATG. Several putative cis-regulatory elements for known transcription factors conserved in the two promoters are indicated in boldface. B, schematic diagram of the AP-1 mutants used in the transient transfection assays. The substitutions at positions −473 and −447 of AP-1 are indicated. C and D, NCI H295R cells were transiently transfected with p625NH-Luc or AP-1-mutated constructs and the internal CMV-β-galactosidase control. Cells were or were not treated with FGF2 (C) or TGF β1 (D) 24 h prior to lysis and subjected to the luciferase assay. The mean luciferase activity ± S.E. of a representative experiment performed in triplicate is presented. AU, arbitrary units.
We carried out further experiments to determine whether the activation of JNK contributes to the down-regulation of *novH* expression by TGFβ1. NCI H295R cells were treated for various periods of time with TGFβ1, and endogenous JNK activity was examined by an immune complex kinase assay using GST-Jun (1–79) as a substrate. Under our experimental conditions, the basal phosphorylation level of GST-Jun observed was relatively high (Fig 8B), and it remained elevated without any significant increase for all the time periods studied (up to 24 h). A weak but not reproducible increase was detected at 6 and 24 h in this representative experiment. We also checked whether JNK, was not transiently activated within the first 15 min as has been reported in some cells (59). Immunoblotting analysis of total cell lysates from NCI H295R with the anti-JNK antibody demonstrated that approximately equivalent amounts of the JNK, protein were present (Fig 8B). Thus, although c-Jun must be phosphorylated by JNK if *novH* is to be down-regulated by TGFβ1, because TGFβ1 did not significantly activate JNK, our results suggest that the basal level of JNK activity detected in these cells is sufficient for this inhibition to occur. Consistent with this, the production of increasing concentrations of a constitutively active MKK7 protein, a specific activator of JNK (60), neither decreased the basal level nor increased the TGFβ1-induced down-regulation of the *novH* promoter activity, which was still inhibited by ~50% (data not shown).

MEKK1 is an upstream activator of the JNK pathways that is also able to mediate the effects of TGFβ1 activation on AP-1-responsive promoters (59). Transient transfection of NCI H295R cells with a dominant negative interfering MEKK1 mutant (K432A) significantly blocked the TGFβ1-induced down-regulation of both p625NH-Luc and AP1-Luc promoter activities (Fig. 8C). These results suggest that additional components besides those activated by the JNK pathway are involved in the TGFβ1-mediated inhibition of these two reporter constructs.

### The Smad Pathway Is Not Required for the Down-regulation of *novH* Expression by TGFβ1—No Smad-binding elements

H295R cells were co-transfected with p625NH-Luc (0.4 μg) and increasing concentrations of TAM67 (0.2, 0.4, 0.8, or 1.2 μg) or c-Jun-Ala (1.2 μg). The total amount of transfecting DNA was kept constant (1.6 μg) by adding an empty pCDNA3 vector. Transfected cells were treated with TGFβ1 (4 ng/ml) or not treated 24 h prior to the luciferase assay. Results are presented as the ratio of the luciferase activity of TGFβ1 (10 ng/ml) to the luciferase activity of untreated control cultures for each concentration of dominant negative constructs. Mean values ± S.E. are presented. B, effect of TGFβ1 on JNK activity. NCI H295R cells were exposed to TGFβ1 (10 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with anti-JNK (Santa Cruz Biotechnology), and immunoprecipitates were subjected to an in vitro kinase assay using GST-Jun (1–79) as a substrate. The phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Immunoblotting of a whole cell extract using anti-JNK showed that similar amounts of JNK proteins were present in each sample. Results are representative of at least three experiments. C, effect of a dominant negative form of MEKK1 (K432A). NCI H295R cells were co-transfected with p625NH-Luc (0.4 μg) and increasing concentrations of a dominant form of MEKK1 (K432A) (0.2, 0.4, 0.8, or 1.2 μg) or pAP1-Luc (0.4 μg) (1.2 μg). The total amount of transfecting DNA was kept constant (1.6 μg) by adding an empty pCDNA3 vector. Transfected cells were treated as in A. Results are presented as in A, AU, arbitrary units.

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**Fig. 8.** Effect of the JNK pathway and of MEKK1 on the TGFβ1-mediated down-regulation of *novH* transcription. A, effect of dominant negative forms of c-Jun (TAM67 and c-Jun-Ala). NCI H295R cells were co-transfected with p625NH-Luc (0.4 μg) and increasing concentrations of TAM67 (0.2, 0.4, 0.8, or 1.2 μg) or c-Jun-Ala (1.2 μg). The total amount of transfecting DNA was kept constant (1.6 μg) by adding an empty pCDNA3 vector. Transfected cells were treated with TGFβ1 (4 ng/ml) or not treated 24 h prior to the luciferase assay. Results are presented as the ratio of the luciferase activity of TGFβ1 (10 ng/ml) to the luciferase activity of untreated control cultures for each concentration of dominant negative constructs. Mean values ± S.E. are presented. B, effect of TGFβ1 on JNK activity. NCI H295R cells were exposed to TGFβ1 (10 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with anti-JNK (Santa Cruz Biotechnology), and immunoprecipitates were subjected to an in vitro kinase assay using GST-Jun (1–79) as a substrate. The phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Immunoblotting of a whole cell extract using anti-JNK showed that similar amounts of JNK proteins were present in each sample. Results are representative of at least three experiments. C, effect of a dominant negative form of MEKK1 (K432A). NCI H295R cells were co-transfected with p625NH-Luc (0.4 μg) and increasing concentrations of a dominant form of MEKK1 (K432A) (0.2, 0.4, 0.8, or 1.2 μg) or pAP1-Luc (0.4 μg) (1.2 μg). The total amount of transfecting DNA was kept constant (1.6 μg) by adding an empty pCDNA3 vector. Transfected cells were treated as in A. Results are presented as in A, AU, arbitrary units.
FIG. 9. Effect of Smad proteins and of dominant forms of Smad on the TGFβ1-mediated down-regulation of novH promoter activity. A, NCI H295R cells were transfected with (CAGA)₉-MLP-Luc reporter vector. Cells were or were not treated with TGFβ1 (4 ng/ml) 24 h prior to lysis and subjected to the luciferase assay. B, NCI H295R cells were co-transfected with p625NH-Luc (0.4 μg) and increasing concentrations of Smad2, Smad3, or Smad4 (0.2, 0.4, 0.8, and 1.2 μg). Total amount of transfecting DNA was kept constant (1.6 μg) by adding the empty pCDNA3 vector. Cells were treated with TGFβ1 (4 ng/ml) or not treated 24 h prior to the luciferase assay. The mean luciferase activity ± S.E. of a representative experiment performed in triplicate is presented. Protein samples (20 μg) derived from the same Smad cell lysates used for the luciferase assay were subjected to Western blot analysis. c-Myc-tagged Smad2, Smad3, and Smad4 proteins were detected by use of a monoclonal anti-c-Myc antibody (bottom). C and D, NCI H295R cells were co-transfected with p625NH-Luc (0.4 μg) and (CAGA) have been found in the p625NH-Luc promoter sequence (61, 62); however, the Smad and JNK pathways may converge at the transcriptional levels (58). In particular, c-Jun physically interacts with Smad2, Smad3, and Smad4 (63), resulting in a synergy of activation on AP-1 site-mediated transcription (63, 64). In contrast, c-Jun was shown to repress a TGFβ1-inducible promoter containing the Smad3/4 binding element CAGA (58, 61). MEKK1 was also shown to modulate Smad2-mediated transcriptional activation selectively (65).

To determine whether the Smad pathway was functional in NCI H295R cells, we used the CAGA reporter containing nine copies of the Smad-binding site derived from the PAI-1 promoter (61). Treatment of the transfected NCI H295R cells with TGFβ1 led to a ~100-fold increase in the CAGA reporter activity (Fig. 9A), indicating that TGFβ1 can induce the Smad pathway in these cells.

We therefore co-transfected NCI H295R cells with p625NH-Luc and increasing concentrations of either the Smad2, Smad3, or Smad4 expression vector. As presented in Fig. 9B, we observed that the overexpression of Smad2, Smad3, or Smad4 in the absence of TGFβ1 did not significantly affect the basal novH promoter activity. This is consistent with previous studies of CAGA-mediated transcription (61, 66). More importantly, TGFβ1 still down-regulated novH promoter activity as efficiently as it does in the absence of co-transfected Smad proteins. In all of these experiments, the expression of the c-Myc tagged-Smad proteins was checked by immunoblotting using an anti-c-Myc monoclonal antibody (Fig. 9B). A similar conclusion could be drawn when Smad2 or Smad3 were transfected together with Smad4 (data not shown). We also investigated the effects on this process of the overexpression of a dominant negative interfering form of Smad4 (DN Smad4) and Smad7, a natural inhibitor of the Smad pathway (67, 68). The expression of both DN Smad4 and Smad7 in NCI H295R significantly decreased the TGFβ1-induced CAGA promoter activity but did not affect the TGFβ1-dependent down-regulation of novH promoter activity (Figs. 9, C and D). Thus, these results suggest that Smad signaling does not participate in the TGFβ1-dependent down-regulation of novH expression.

DISCUSSION

Because IGF-I, IGF-II, FGF2, and TGFβ1 are involved in the physiological functions of adrenocortical cells, (26, 29), we analyzed their effects on the expression of novH in the NCI H295R cell line, which is derived from a human adrenocortical carcinoma (25). This cell line allowed us to show for the first time that novH expression is up-regulated by FGF2 and down-regulated by TGFβ1, whereas IGF-I and II have no influence. These data suggested that FGF2 and TGFβ1, which are involved in the development of various tumors (69, 70) and are also produced by adrenocortical cells (71, 72), could be considered as potential candidates involved in the modulation of novH expression in adrenocortical tumors (19). Our results also show that TGFβ1 reduces the up-regulation of novH expression induced by FGF2, suggesting that the basal level of novH expression results from a balance between the actions of these two growth factors. This balance may vary during tumorigenesis, and FGF2 detected in adrenocortical tumors (72) may play increasing concentrations of either a dominant negative form of Smad4 or Smad7 (0.4, 0.8, and 1.2 μg). As a control, NCI H295R cells were also co-transfected with (CAGA)₉-MLP-Luc reporter vector and DN Smad4 or Smad7 (1.2 μg). The total amount of transfecting DNA was kept constant (1.6 μg) by adding the empty pCDNA3 vector. Cells were treated as in A and B. Results are presented as the ratio of the luciferase activity of TGFβ1-treated cultures to the luciferase activity of untreated control cultures for each concentration of DN Smad4 or Smad7 constructs. Mean values ± S.E. are presented.
a major role in the overexpression of noxH during the earlier stages of tumorigenesis. We cannot exclude the possibility that other factors also influence the levels of noxH expression during adrenocortical tumorigenesis.

FGF2 has been shown to up-regulate the expression of noxH in NCI H295R cells; however, we showed that down-regulation of the expression of noxH by TGFβ1 is not restricted to tumoral adrenocortical cells, because it was also observed in primary astrocytes and could be detected in human as well as murine cells. The regulation of noxH by TGFβ1 might, however, present some specificity, because it has not been reported in human prostatic cells (36).

noxH is the first member of the CCN family that has been shown to be down-regulated by TGFβ1. The other members of this family such as ctsf and cyr61 are induced by TGFβ1 in different cell systems (33, 36). It is noteworthy that nox is also regulated oppositely from ctsf and cyr61 in chicken embryonic fibroblasts. In these cells, ctsf and cyr61 behave as immediate-early genes induced by serum and oncogenes (73, 74), whereas the expression of nox is down-regulated by these factors and associated with quiescence (75). Primary cultures of mouse astrocytes are another cell type in which the expression of noxH has been found to be down-regulated by TGFβ1. However, the expression of nox and ctsf has been reported to be down-regulated by Wilms' tumor suppressor gene 1 (WT1) in renal cells (37, 76), indicating that these two molecules may also cooperate in some cells.

Under our experimental conditions, the expression of ctsf and cyr61 was barely detectable in NCI H295R cells, and the expression of ctsf was only slightly stimulated by TGFβ1. In fibroblasts, CTGF can function as a downstream mediator of TGFβ1 activity. For example, it can stimulate cell proliferation and extracellular matrix protein synthesis (77, 78). In vivo, CTGF plays a role in TGFβ1-mediated formation of granulation tissue and cooperates with TGFβ1 to induce persistent fibrosis (79). Whether NOV mediates a function of TGFβ1 in adrenocortical cells remains to be determined. However, no correlation could be found between the levels of noxH expression in NCI H295R cells treated with TGFβ1 or FGF2 and their proliferative state as assessed by [3H]thymidine incorporation. 

TGFβ1 has been reported to be a strong inhibitor of steroidogenesis in adrenocortical cells (26); therefore, noxH may antagonize the effect of TGFβ1 in this function. This hypothesis is currently under investigation. TGFβ1 can potentiate tumorigenesis by down-regulating the genes involved in cell-cell adhesion and by up-regulating the expression of genes involved in cell-extracellular matrix association, ultimately improving the migration and invasiveness of the cell (69). These properties are more consistent with NOVH having a role as an adhesive protein (13) that is able to regulate the expression of genes involved in extracellular matrix remodeling.

Because the expression of noxH is directly regulated by TGFβ1 and different elements of the TGFβ1 signaling pathway can also be altered in cancer (69), we analyzed the signaling pathway involved in the TGFβ1-mediated down-regulation of noxH. Comparison of the human nox promoter region, which is targeted by TGFβ1, with the corresponding mouse sequences revealed a high degree of sequence homology (69%). These conserved regions included several consensus sequences involved in the binding of transcription factors (such as USF, NFκB, NPY, and AP-1), suggesting that noxH and noxM could be subjected to common regulations. Our data provide an example of one of those, as the expression of noxH and noxM can be down-regulated by TGFβ1. We further demonstrated that down-regulation of noxH expression is mediated by AP-1 sites, which are found in the same region of the two promoters. Our results suggest that the TGFβ1 signaling pathway targets AP-1 sites in the noxH promoter to inhibit the expression of noxH. However, we consistently observed that the mutation of AP-1 sites also results in a decrease in the basal activity of the noxH promoter. The molecular mechanisms involved in maintaining the expression of noxH in unstimulated NCI H295R cells are currently unknown. The identification of environmental cues that regulate the expression of noxH will help to clarify this point.

Whereas both ctsf and noxH can be regulated by TGFβ1, quite different promoter sequences are involved in this regulation, because the Smad pathway is responsible for the up-regulation of ctsf expression by TGFβ1 in fibroblasts (80). The induction of gene expression by TGFβ1 involving Smad or AP-1 binding sequences and c-Jun has been well documented (81, 82), but there are only a few reports of the down-regulation of gene expression by TGFβ1 involving c-Jun (41, 58). For example, TGFβ1 down-regulates the expression of the gene that encodes the metalloproteinase MMP12 (83) through AP-1 sites, but this inhibitory effect is dependent on signaling through Smad3. Our results showed that the mechanism by which noxH is negatively regulated by TGFβ1 in NCI H295R cells is different. We demonstrated that although the Smad pathway in these cells was induced by TGFβ1, which is in agreement with a previous report (84), this pathway is not involved in the TGFβ1-mediated inhibition of noxH expression. Our data concerning the noxH promoter mutated in the AP-1 sites and the dominant forms of c-Jun mutated in the JNK binding domain or in JNK-specific phosphorylation indicate that the SAPK/JNK pathway is required in this regulation. Two other studies (59, 85) showed that the activation of JNK independently of Smads leads to the regulation of fibronectin and insulin-like growth factor binding protein 5 (IGFBP5) by TGFβ1. In contrast, our data suggest that the down-regulation of noxH expression by TGFβ1 requires a basal level of JNK activity to phosphorylate c-Jun and an additional TGFβ1-dependent mechanism. We provide evidence that MEKK1 could play a crucial role in this regulation in a manner that does not involve the activation of JNK. It has recently been shown that MEKK1 is able to directly activate, independently of JNK, other proteins such as p300/cAMP-response element-binding protein-binding protein (86). It has also been reported that by reinforcing the association between c-Jun and TGFβ1, TGFβ1 leads to the repression of AP1-mediated transcriptional activity (41). It is therefore tempting to speculate that when NCI H295R are treated with TGFβ1, a factor specifically regulated by MEKK1 could participate in an interaction between c-Jun and TGFβ1, resulting in the down-regulation of noxH expression. However, the molecular mechanism by which MEKK1 could participate in the TGFβ1-negative regulation of noxH expression awaits further investigation. The study of noxH regulation in NCI H295R cells, in which the Smad pathway is functional, may therefore represent a good model for a better understanding of the molecular mechanisms involved in the TGFβ1-mediated inhibition of gene expression.

In summary, the data presented here demonstrate that noxH is a new target for TGFβ1. Further studies aimed at determining which of the functions of TGFβ1 are mediated by noxH might be useful to the development of therapeutic agents for the treatment of diseases involving also other members of the CCN family such as fibrosis or cancer.

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4 C. Martinerie and M. Laurent, unpublished results.
