Identification of a Critical Sp1 Site within the Endoglin Promoter and Its Involvement in the Transforming Growth Factor-β Stimulation*

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Endoglin, a component of the transforming growth factor-β (TGF-β) receptor complex expressed on endothelial cells, is involved in cardiovascular morphogenesis and vascular remodeling, as exemplified by the fact that the endoglin gene is the target for the autosomal dominant disorder known as hereditary hemorrhagic telangiectasia type 1. Since haploinsufficiency is the underlying mechanism for hereditary hemorrhagic telangiectasia type 1, understanding the regulation of endoglin gene expression appears to be a crucial step to correct the disease. In this study we have identified an Sp1 site at −37 as a critical element for the basal transcription of the endoglin TATA-less promoter. Since endoglin promoter activity is stimulated by TGF-β and this stimulation is located at the Sp1-containing proximal region, we have investigated the possible involvement of Sp1 in the TGF-β-mediated induction. Mutation of the Sp1-binding sequence, or addition of the Sp1 inhibitor WP631, abolished both the basal transcription activity and the TGF-β responsiveness of the endoglin promoter. Binding of Sp1 and Smad3 to the proximal promoter region −50/−29 was evidenced by electrophoretic mobility shift assays and DNA affinity precipitation studies. Furthermore, synergistic cooperation on the promoter activity between Sp1 and TGF-β or Smad3 could be demonstrated by co-transfection experiments of reporter promoter constructs. The molecular mechanism underlying this cooperation appears to involve a direct physical interaction between Sp1 and Smad3/Smad4.

Support an important role for endoglin in cardiovascular development and vascular remodeling. First, endoglin expression is regulated during heart development in humans and chicken (6, 7). It is highly expressed at the level of endocardial cushion during valve formation and heart septation by the mesenchymal cells of the atrioventricular canal (6). The contribution of endoglin to the vascular morphogenesis has been demonstrated by its genetic inactivation in the mouse as embryos homozygous for mutant endoglin die at 10–10.5 days post coitus due to vascular and cardiac anomalies (8–10). In addition, the gene encoding endoglin is targeted for the autosomal dominant disorder known as hereditary hemorrhagic telangiectasia type 1 (HHT1) (11). HHT1 is a vascular disorder, which shows a prevalence of up to 1 in 8,000, exhibiting age-dependent penetrance and variable expressivity (Online Mendelian Inheritance in Man, OMIM 187390). The most common clinical manifestations involve the development of vascular abnormalities seen as telangiectases on skin and lesions in nasal mucosa with bleeding. The lesions are the result of direct arteriovenous connections that can lead to large arteriovenous malformations in brain, lung, and liver. Many of the mutations reported to date in endoglin include deletions, insertion, and point mutations in the coding region, leading to premature stop codons and frameshifts that result in predicted truncated proteins that are neither expressed at the cell surface nor secreted. This and the existence of null allele mutations support haploinsufficiency as the underlying mechanism for HHT1 (12–14). Thus, the study of the regulation of endoglin gene expression appears to be critical to correct the disease. As a first step to ascertain the factors involved in the regulation of endoglin expression, we initiated the functional characterization of its promoter region (15). The primary analysis of endoglin promoter reveals that it is a TATA- and CAAT-less promoter, but with GC-rich tracts, including an Sp1 consensus site at −37, in the vicinity of the transcription initiation nucleus. These features are typical of TATA-less promoters with multiple transcription start sites (16) and are also present in other components of the mammalian TGF-β system, including human TGF-β1 (17), mouse inhibin/activin βC gene (18), and TGF-β receptor type II (19, 20). We have shown previously that endoglin expression is stimulated by TGF-β1, at protein and mRNA levels (4), and that TGF-β1 is also able to induce the transcriptional activity of the endoglin promoter (15). The stimulation by TGF-β could be detected in different endoglin promoter constructs, representing successive deletions from −400 to −141 base pairs, but the putative TGF-β responsive elements within this fragment have not been identified yet.

The transcription modulatory effect of TGF-β is exerted by binding to type I and type II serine-threonine kinase receptors, which propagate intracellular signaling by phosphorylation of...
members of the Smad family of proteins (21, 22). Smads may be classified into three different subclasses: receptor-regulated or R-Smads, as Smad2, and Smad3 (activated by TGF-β); the common partner, Smad4; and finally the inhibitory Smads, I-Smads, Smad6, and Smad7. R-Smads are activated by phosphorylation at their C-terminal SS/M/V/S-motifs, whereafter they heterologomerize with the common partner, Smad4. The Smad complexes are then translocated to the nucleus, where the transcriptional activities of different genes are affected. Within the nucleus, Smads bind to their target genes at the motifs called Smad binding elements (SBE) (23–26). However, Smads bind with low affinity to their target sequences, requiring interaction with additional transcription factors in order to exert its function (27, 28). Several transcription factors and transcriptional co-activators have been identified to cooperate with Smad in transcriptional activation such as CBP/p300, FAST, TFE-3, PEBP-2/CBF, ATF-2, OAZ, and AP-1 (21, 27, 29). The mechanisms whereby different transcription factors participate cooperatively in TGF-β induced transcription involve the stabilization of protein-DNA complexes, bridging Smads with the basal transcription machinery, or stimulating the acetylation activity in promoter regions (21, 27).

In the proximal endoglin promoter, there are G/C-rich motifs, which are putative binding sites for the Sp1 transcription factor. Sp1 is an ubiquitously expressed protein with a zinc finger DNA-binding domain (16, 30, 31). Sp1 is required for early embryogenesis and regulates terminal differentiation of cells by affecting the methylation of DNA CpG islands (32). Sp1 interacts directly with basal transcription machinery factors and cooperates with several transcriptional activators (33–38). In the present work we analyze the role of Sp1 in endoglin gene transcription. We show that Sp1 is a critical factor for the basal transcription and for the TGF-β-induced transcriptional stimulation, mediated by Sp1, of the proximal promoter of endoglin.

**EXPERIMENTAL PROCEDURES**

*Cell Culture*—All the cell lines were cultured in a 5% CO₂ atmosphere at 37 °C (unless otherwise indicated) in medium containing 10% fetal calf serum and 2 mM L-glutamine and antibiotics (penicillin-streptomycin, as G418, and monkey kidney COS cells were grown in Dulbecco’s modified Eagle’s medium supplemented with penicillin-streptomycin. The human monocytic line U-937 was cultured in RPMI 1640 medium supplemented with penicillin-streptomycin. The Drosophila Schneider S-2 cells were grown at room temperature in 100-ml culture bottles containing S-2 medium (Sigma) supplemented with 100 IU/ml gentamicin.

*Reporter Constructs, Transfections, and Luciferase Assays*—The reporter vectors pCD105(−250/+350), pCD105(−150/+350), and pCD105(−50/+350) were derived from the human endoglin promoter (15). Polymerase chain reaction was carried out in the presence of sequence-specific primers surrounded by HindIII/XhoI sites for directional cloning and the resulting −250/+350, −150/+350, and −50/+350 fragments were inserted into the reporter luciferase vector pX2 by previously digested with HindIII/XhoI. Mutagenesis of Sp1 site at −37 was performed by recombinant polymerase chain reaction, using mutated primers −50/−24 D (GACA GGC GCC CTG GTG ttt AGC CCC TTTTC) and −50/−24 R (GAGG GGG GGT CAT aaa CCC AGG CCG CCT GC), where the mutated nucleotides appear in lowercase (CCC in the original sequence was changed by TTT). Using the pCD105(−50/+350) construct as template, mutant oligonucleotides −50/−24 D and −50/−24 R, together with flanking primers of the pX2 vector, allowed the amplification of the mutant −50/−24 C fragment, which was inserted into the HindIII/XhoI site of the pX2 vector leading to the pCD105(−50/+350) construct. The altered construct was confirmed by automated sequencing (ABI Prism 310 genetic analyzer). The reporter plasmid pHRL-LUC, containing the prolactin promoter with a minimal TATA box, was kindly provided by Dr. Mercedes Rincón (Yale University School of Medicine, New Haven, CT). Transfections were performed with 1 µg of reporter vector, the indicated amounts of expression vectors, and Superfect reagent (Qiagen), following the instructions of manufacturer for each type of cell line. Luciferase activity was determined with the Luciferase assay reagent (Promega, Madison, WI) using a TD-20/20 luminometer (Promega). Luciferase activity values were corrected for internal normalization by internal controls consisting of plasmids with β-galactosidase, yielding the relative luciferase units as a measurement of the promoter activity. Luciferase assays were carried out in duplicate, and each experiment of transfection was repeated at least three times with similar results. Data plotted in the figures represent the mean ± standard deviations of intra assay values from representative experiments. When required, after transfection, cells were treated for 20 h with 10 ng/ml TGF-β1 (R&D Systems). The increase of the promoter activity by different stimuli was measured by fold induction values using as a reference the activity of untreated samples whose arbitrary value is 1. For inhibition by bisanthracycline (WP361) of Sp1 binding to DNA, cells were incubated with different concentrations (0.1–1 µM) of WP361 (Tropix) 3 h after transfection and kept for 20 h.

The expression vectors pCMV5-Flag-Smad3, and pCMV5-Smad4-Flag- or hemagglutinin (HA), encoding human Smad members Flag- or hemagglutinin (HA) epitope-tagged, and pCMV5-TjRI encoding a constitutively activated form of the TGF-β receptor type I (ALK-5) have been described previously (39).

*Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)*—EMSAs were carried out as described (15, 40). To obtain nuclear extracts, subconfluent cell cultures, either untreated or treated with TGF-β1 (10 ng/ml), were washed, collected in cold phosphate-buffered saline, and resuspended in appropriate buffers to collect the nuclear fraction and nuclear extracts. Complementary oligonucleotides, constituting the different binding motifs, were annealed followed by 5' end labeling using [γ-32P]ATP and T4 polynucleotide kinase. Approximately 5 ng (100,000 cpm) of the respective probe was incubated with 5–10 µg of nuclear extract and 2 µg/reaction of poly(dI-dC) for 30 min on ice. Oligonucleotides used as probes were −50/−24 WT (GACGGCG GCCCTGGG CCCCAGCC CTTCTC), and −50/−24 Sp1-Mut (GACGGCG GCCCTGGG TTTAGCC CTTCTC). For competition experiments, an excess of 100- or 200-fold excess of unlabeled double-stranded oligonucleotide was added. When required, EMSAs were carried out in the presence of recombinant Smad or Sp1 proteins. In experiments with antibody, protein extract and 1 µg of commercial antibody were preincubated for 60 min on ice prior to the addition of the remaining components of the binding reaction. Rabbit polyclonal antibodies anti-Sp1, anti-AP-2, and anti-Smad3 were from Santa Cruz Biotechnology (Santa Cruz, CA). For specific inhibition of Sp1 binding, nuclear extracts were incubated with the indicated concentrations of WP361 (Tropix). Binding reactions were separated by nondenaturing 6% PAGE in Tris-Borate-EDTA buffer at 4 °C, dried, and visualized by autoradiography. EMSAs were repeated at least three times with similar results, and representative experiments are shown in the corresponding figures.

*PINPOINT*—Position Identification with Nuclease Tail (PINPOINT) Experiments—The PINPOINT technique is an alternative, in vitro method to the in vivo footprinting and allows the identification and positioning of a given transcription factor on the promoter (41). The sense +42/+24 (CAG CAG GGA GCT CCC GGC) and antisense −73/−56 (GTG GCA CTT CCT CTA CCC) endoglin primers, as well as the Sp1 pointer −58/−56 (GTG GCA CTT CCT CTA CCC) were used. Before the addition of Sp1 binding to DNA, the cleavage site was determined by primer extension. Two primers were used: an antisense primer, complementary to +46/+24 positions of the endoglin promoter; and the sense primer −73/−56. Total DNA from transfected cells was collected and subjected to primer extension with the above-mentioned 5'-kinased oligonucleotides. Reactions were loaded in a 6% polyacrylamide: 7.5 M urea sequencing gels, in the presence of a G+A sequencing lane. Gels were dried and autoradiographed. PINPOINT experiments were repeated at least four times with similar results, and a representative experiment is shown in the corresponding figure.

Expression of Recombinant Proteins—The glutathione S-transferase (GST) fusion proteins, GST-Smad3, and GST-Smad4, kindly provided by Dr. Lilianna Attisano (University of Toronto, Toronto, Ontario, Canada), were expressed in Escherichia coli strain DH5α and purified as described (42). In vitro transcribed and translated Sp1 protein was
obtained using a TnT Kit (TnT® T7 coupled reticulocyte lysate system, Promega) in the presence of 1 μg of pcGNeosp1 vector generously provided by Dr. Scott L. Friedman (Mount Sinai Medical Center, New York, NY), and one tenth of the reaction was used for each DNA binding experiment.

Co-immunoprecipitation Assays—Forty hours after transfection of COS-7 cells, total extracts were prepared by lysing the cells in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mM aprotinin, and 2 mM Pefabloc at 4°C. Afterward, immunocomplexes were precipitated with protein G-Sepharose, washed with lysis buffer twice, and dissolved in Laemmli-SDS-polyacrylamide gel electrophoresis loading buffer. After 8% SDS-PAGE, the resolved proteins were transferred to Hybond-C extra nitrocellulose (Amersham Pharmacia Biotech) and the antigens were detected by incubation with the specific antibodies (anti-Sp1 or anti-Smad3, Santa Cruz Biotechnology) followed by incubation with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham Pharmacia Biotech). The DNA was detected with a biotinylated DNA probe in the presence of ImmunoPure streptavidin-agarose (Pierce), washed, and developed as indicated in the previous section. Experiments were repeated at least five times with similar results, and a representative experiment is shown in the corresponding figure.

DNA Affinity Precipitation—COS-7 cells were transfected with the expression vectors pCMV5-Flag-Smad3, pCMV5-Smad4-HA, and pCMV5-T7sp1 using Superfect (Qiagen). After 48 h, cells were resuspended in a solution containing 50 mM HEPES, pH 7.4, 50 mM NaCl, 0.1% Tween 20, and 10% glycerol supplemented with protease and phosphatase inhibitors, and lysed by sonication. The lysates were pre-cleared in the presence of ImmunoPure streptavidin-agarose (Pierce), followed by incubation with 200 ng of biotinylated –50/–35 oligonucleotide and 2 μg of poly(dI-dC). Endogenous Sp1 bound to the endoglin promoter was then captured by streptavidin-agarose, washed in the same lysis buffer, fractionated on SDS-PAGE (8%), transferred onto nitrocellulose membranes, and developed as indicated in the previous section. Experiments were repeated at least five times with similar results, and a representative experiment is shown in the corresponding figure.

RESULTS

Effect of TGF-β on the Activity of the Proximal Endoglin Promoter Is Mediated by Smads—We have shown previously that TGF-β stimulates the activity of the endoglin promoter (15). Since TGF-β signaling is exerted through Smads, we assessed whether Smad proteins could reproduce the TGF-β-mediated stimulation of the endoglin promoter. Thus, HepG2 cells were transiently cotransfected with the reporter constructs pCD105 (–350/+350), pCD105 (–250/+350), and pCD105 (–50/+350), derived from the proximal endoglin promoter (Fig. 1), together with an expression vector encoding Smad3. These constructs were transactivated by Smad3, and significant induction values were obtained with the –350/+350 (3-fold), –250/+350 (2.5-fold) and –50/+350 (2.5-fold) promoter fragments, whereas the promoterless pX2 vector was unresponsive. The fact that the pCD105 (–50/+350) construct, containing only 50 base pairs upstream of the transcription start site, displayed a level of stimulation by Smad3 similar to the longer constructs, suggested that a TGF-β/Smad-responsive element was located in this proximal region of the endoglin promoter. Interestingly, within this region, three putative Smad binding elements and one Sp1 motif were identified (Fig. 1).

The Endoglin Proximal Promoter Contains DNA Binding Sites for Smads and Sp1—To determine the sequences responsible for the TGF-β/Smad responsiveness of the endoglin promoter, DNase I footprinting experiments with TGF-β treated versus untreated cells were performed. In different cell lines (U-937, L6E9, and HMEC-1), a protection area spanning the region –50/–20 was revealed (data not shown). Within this sequence, there is a Sp1 consensus site at –37/–29 (CACCAG-CCC) partially overlapping with a preceding GC-rich region at –50/–35. To test whether the protection of the Sp1 motif was meaningful in vivo, the PINPOINT technique (41) was applied. COS cells were cotransfected with pCD105 (–150/+350) as reporter, together with the expression Sp1 pointer, and the cleavage site was detected by primer extension. Specific extended bands were obtained with both sense and antisense primers (Fig. 2A). Although the antisense primer gave a fragment of around 75 nucleotides, corresponding to a cut point by the nuclease around the sequence GCT from –33 to –35, the sense primer was extended in a series of discrete bands from –35 to –46. Taking into account the flexibility of the nuclease cutting point (around 4 base pairs from the Sp1 binding site in the DNA; Ref. 41), it can be concluded that Sp1 binds the endoglin promoter at around –33/–46.

To confirm the Sp1 binding to the endoglin proximal promoter region, a synthetic oligonucleotide encompassing this region (–50/–24) was used as a probe in EMSA experiments. A slow migrating DNA-protein band, which was significantly increased after TGF-β treatment, was detected in both U937 and HepG2 cells (Fig. 2B). This band was specific because it was competed with a 100-fold excess of cold oligonucleotide. Sp1 was present in the DNA complex, as the band decreased significantly (more than 5 times) using a Sp1-specific antibody, but was not affected by an anti-AP-2 antibody (Fig. 2B). The involvement of Sp1 was also demonstrated by the absence of a retarded complex when using a probe mutated at the Sp1 site (Fig. 2C, lanes 3 and 4). These results support the participation of Sp1 in the DNA-protein complex.

Smad3 contains DNA binding activity with affinity toward the so-called SBE (GCN5), whereas Smad4 has affinity toward both the SBE and the GC-rich motifs (39, 43). In the region between –47 and –40 of the endoglin proximal promoter
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Fig. 2. Characterization of Sp1 and Smad binding to the −50°−24 region of the endoglin promoter. A, DNA PINPOINT assay in both directions using the Sp1 FokI nuclease as pointer and the endoglin promoter vector pCD105−150°+350 as reporter. The 5’ end-labeled endoglin promoter primers +42/+24 or −73−56 were extended in the presence of total DNA from COS cells previously transfected with 1 μg of the expression vector for Sp1 FokI nuclease and the endoglin promoter vector pCD105−150°+350 as reporter. The extended products are shown in lanes 2, whereas lanes 1 correspond to the same reactions in the absence of template. The size of the extended products was determined by including a G+A sequence of the endoglin promoter in the same gel. Numbers to the left of the gels indicate the size in nucleotides (nt). The 5’ end-labeled primers (input) used in each sample are shown in the lower part of the panel. B, EMSA with U-937 and HepG2 nuclear extracts. Cells were either untreated or treated with 10 ng/ml TGF-β for 20 h. Nuclear extracts were incubated with the radiolabeled −50°−24 oligonucleotide as probe in the absence or presence of 100-fold excess unlabeled oligonucleotide (wt, Competitor). Specific antibodies anti-Sp1 (S) or anti-AP2 (A) were also used. The Sp1-containing complex is indicated by an arrow. C, EMSA using as probes either the labeled wild type −50°−24 oligonucleotide (WT) or the labeled Sp1 mutated version (MUT), as indicated. A specific antibody anti-Smad3 was also used. Experimental conditions were as in B, D, DNA affinity precipitation analysis (DNA-P). COS cells were transfected with 1 μg of expression vectors encoding Smad3 and a constitutively activated form of the TGF-β receptor type I (TβRI). Cell extracts were incubated with the biotinylated −50°−24 oligonucleotide and DNA-protein complexes isolated by centrifugation with streptavidin-agarose beads. Complexed proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the presence of Sp1 or Smad3 was revealed with specific monoclonal antibodies. As control, expression of Sp1 and Smad3 in total lysates was determined. Specific binding of Sp1 and Smad3 was inhibited with an excess of 100× unlabeled oligonucleotide (Competitor). E, EMSA with recombinant Smad proteins. The labeled −50°−24 probe was incubated with 1 μg of bacterially expressed GST, GST-Smad3 (GST-S3), or GST-Smad4 (GST-S4). F, EMSA with recombinant proteins and nuclear extracts. The labeled −50°−24 probe was incubated with bacterially expressed GST, GST-Smad3 (GST-S3), and GST-Smad4 (GST-S4) (1 μg each), COS nuclear extracts (CNE), in vitro transcribed and translated Sp1 (TnT-Sp1), or a specific antibody anti-Sp1 (aSp1), as indicated. The Smad-containing complex is shown by an arrow. The asterisk indicates the position of the new complex originated in the presence of Smads and Sp1 proteins. In lane 7 containing TnT-Sp1, the broad band with an electrophoretic mobility higher than the indicated Sp1 complex is unspecific (see panel G). G, EMSA with recombinant Smad and Sp1 proteins. The labeled −50°−24 probe was incubated with GST, GST-Smad3, and GST-Smad4 (0.1 μg each), in vitro transcribed and translated Sp1 (TnT-Sp1), anti-Sp1 (aSp1), or anti-Smad3 (aSmad3), as indicated. The Smad-containing complex is indicated by an arrow. The asterisk indicates the position of the new complex originated in the presence of Smads and Sp1 proteins. In the lanes containing translated Sp1, the broad band with an electrophoretic mobility higher than the indicated Sp1 complex is unspecific, as it also appears in control TnT extracts without the Sp1 cDNA (TnT-Control, lane 7).

(Fig. 1), two putative SBE motifs were identified (GGCG-GGCC). In addition, a third putative site at −38/−35 shares 3 bases with the Sp1 consensus sequence (GGCC). On the other hand, the region between −50 and −29 is very G/C-rich. These data suggest the involvement of Smad proteins in a nucleoprotein complex containing Sp1 and this particular region of the
endoglin promoter. Supporting this view, in EMSA experiments with nuclear extracts from TGF-β-treated cells, anti-Smad3 antibodies are able to decrease the amount of the retarded complex below the level of untreated cells (Fig. 2C, compare lanes 5, 6, and 9). In addition, specific binding of Smad3 and Sp1 to the −50/−24 endoglin promoter was also observed in DNA affinity precipitation experiments (Fig. 2D). COS-7 cells were transfected to overexpress Smad3/Smad4 and their lysates incubated with a biotinylated oligonucleotide encompassing the −50/−24 region. Binding of Smad3 and Sp1 was demonstrated upon immunodecoration with specific antibodies. Overexpression of Sp1 by cotransfection of exogenous Sp1 did not alter the results of the experiment (data not shown). As a control, competition with unlabeled oligonucleotide markedly diminished the binding of both Smad3 and Sp1 to the biotinylated oligonucleotide (Fig. 2D).

Next, the interaction of recombinant Sp1 and Smad3/Smad4 proteins with the endoglin promoter in EMSA experiments was analyzed (Fig. 2, E–G). As shown in Fig. 2E, the specific binding of 1 μg of GST-Smad3 or GST-Smad4 could be detected, although the binding of GST-Smad4 was −50-fold more efficient than that of GST-Smad3. This difference is likely explained because the C terminus of R-Smads is self-inhibiting the DNA binding by the MH1 domain (N terminus), and this inhibition is not present in Smad4 (27). The formation of a ternary complex between Smads, Sp1, and the endoglin promoter is suggested by the TGF-β-dependent increase of the nucleoprotein complex (Fig. 2B), and by the inhibition of this complex in the presence of anti-Smad antibodies (Fig. 2C). To confirm the formation of this ternary complex, EMSA experiments with recombinant proteins and nuclear extracts were carried out (Fig. 2, F and G). As a source of Sp1 without interference of Smads, nuclear extracts of COS cells, or in vitro transcribed and translated Sp1 were used. As depicted in Fig. 2F (lane 3), COS nuclear extracts allowed the formation of a nucleoprotein complex which decreased in intensity upon preincubation with anti-Sp1 antibody (lane 4). Furthermore, this nuclear extract-derived complex displayed a similar electrophoretic mobility as the one formed with in vitro transcribed and translated Sp1 (lane 7). On the other hand, binding complexes formed by GST-Smad3 and GST-Smad4 (1 μg each) could be observed in lane 2. When both COS nuclear extracts and GST-Smads were coincubated with the endoglin probe, a distinct retarded complex with lower mobility than individual bands corresponding to Sp1 or GST-Smads was observed (see asterisk in lane 5). The intensity of this complex was markedly decreased by anti-Sp1 antibodies (lane 6), indicating that Sp1 is present in the complex. Further experiments were carried out using transcribed/translated Sp1 (Fig. 2G). Thus, recombinant Sp1 alone yielded the expected nucleoprotein band (lane 1), whereas no complex could be detected when using low amounts (0.1 μg) of GST-Smads (lanes 2 and 3). However, addition of GST-Smads to transcribed/translated Sp1 resulted in a clearly distinct and slower migrating band than the one observed with Sp1 alone (lane 4, asterisk). Addition of antibodies to either Sp1 or Smad proteins abolished the formation of this DNA multiprotein complex (lanes 5 and 6), confirming the presence of Sp1 and Smads in the complex.

Taken together, these results indicate that Sp1 and Smad3/Smad4 are able to bind the promoter at positions −50/−29 forming a multiprotein complex, and suggest their cooperation in the transcriptional activity of endoglin.

**Functional Role of Sp1 in the Proximal Promoter of Endoglin**—Since the endoglin promoter does not contain a TATA box (15), the presence of a Sp1 motif immediately upstream of the transcription initiation site suggests a functional role for Sp1 in the basal transcription. Thus, the functional importance of the Sp1 site at −37 in the basal activity of the proximal endoglin promoter was studied by using a specific inhibitor of Sp1 and by site-directed mutagenesis of the promoter. First, cell transfection experiments in HeLa cells were performed to determine the basal activity of the proximal endoglin promoter in the presence of different concentrations of the bisintercalating anthracycline drug, WP631, which specifically inhibits Sp1-dependent transcription (44). Concentrations of WP631 as low as 0.1 μM significantly diminished the promoter basal activity of the pCD105(−50/−350) construct (Fig. 3A). As a control for WP631 specificity, a reporter vector, pRL-LUC, containing the minimal prolactin promoter, was used in the same experiment, and its activity was not inhibited by the drug. WP631 displays a remarkably high DNA-binding affinity, by intercalating between the G/C tracts like the ones present in Sp1 sites. This fact was demonstrated by including in the binding reactions WP631 at two different doses (0.1 and 1 μM). As depicted in Fig. 3B (lower panel), the intercalating effect is shown in EMSA by the stepwise migration of the free DNA representing the endoglin promoter from positions (−50/−24) (detected after short exposures). Furthermore, the retarded band, which appears in EMSA experiments using the Sp1 probe, is either markedly reduced or completely abolished in the presence of increasing WP631 doses (Fig. 3B, upper panel). Since WP631 intercalates between the G/C tracts and the putative SBE on this promoter region are G/C-rich, it is possible that WP631 is affecting not only Sp1, but also Smad binding to DNA. Next, the functional contribution of Sp1 to the endoglin proximal promoter was assessed by transient transfections in Sp1-deficient Schneider Drosophila cells (Fig. 3C). Transfection with the pCD105(−50/+350) construct yielded background levels of promoter activity, whereas co-expression of Sp1 resulted in a marked increased activity (almost 90-fold), which was abrogated in the presence of the Sp1 specific inhibitor WP631. Additional proof for the functionality of Sp1 was obtained by transfection experiments using wild type and Sp1 mutant endoglin promoter constructs. HeLa and HepG2 cells were transfected with pCD105(−50/+350)WT and pCD105(−50/+350)Sp1Mut vectors, and the promoter activity was measured (Fig. 3D). The mutation in the Sp1 site, dramatically suppressed the basal promoter activity of endoglin in the two cell types tested. Taken together, these results demonstrate that Sp1 is an essential transcription factor for the basal activity of the endoglin promoter.

**Cooperative Effect between Smad3 and Sp1 at Positions −50/−29 of the Proximal Promoter of Endoglin**—To assess the involvement of Sp1 in the TGF-β responsiveness of the endoglin promoter, HeLa and HepG2 cells were transfected with the wild type and Sp1 mutant endoglin promoter constructs, and their basal and TGF-β-induced promoter activities were measured (Fig. 4A). Addition of TGF-β stimulated an average of 2.5 (HeLa) or 2.2 (HepG2) times the activity of the wild type promoter construct. By contrast, the Sp1 mutant vector displayed not only a markedly reduced basal activity (as shown in Fig. 3D), but also a much lower TGF-β response (1.6- and 1.4-fold induction in HeLa and HepG2 cells, respectively). Overall, the TGF-β-dependent stimulation of the endoglin promoter was clearly diminished (−70%) when the Sp1 site was mutated, suggesting that Sp1 is involved in the TGF-β-mediated TGF-β-induced activity of the proximal promoter of endoglin. Then, the putative cooperation between Sp1 and Smad3 was analyzed using the wild type and Sp1 mutant endoglin promoter constructs in Sp1-deficient Schneider Drosophila cells (Fig. 4B). Co-expression of Smad3 and Sp1, using suboptimal amounts of the corresponding plasmids, resulted in a strong transactivation (38-fold induction), whereas the indi-
FIG. 3. Sp1 site at −37 is essential for the basal activity of endoglin promoter. A, effect of WP631 on the basal promoter activity of endoglin. HeLa cells were transiently transfected with pCD105(−50/+350) or pRL-LUC (prolactin minimal promoter) constructs in the absence or in the presence of the specific Sp1 inhibitor WP631, at the indicated concentrations. This is a representative experiment of four different ones. Standard deviations are denoted by vertical lines. The endoglin promoter activity, but not that of the prolactin promoter is inhibited by WP 631. RLU, relative luciferase units. B, EMSA performed with nuclear extracts of TGF-β-treated U-937 cells in the absence or in the presence of two different doses (0.1 and 1 μM) of WP 631. The Sp1 inhibitor decreased the formation of the retarded DNA-protein complex (upper panel) and intercalated into the DNA probe, as shown by the stepwise migration of the free DNA probe (lower panel). C, the basal endoglin promoter activity dependent on Sp1 transcription factor, as shown in S-2 Schneider Drosophila Sp1-deficient cells when transfected with the Sp1 expression vector pPacSp1 (1 μg) and the pCD105(−50/+350) endoglin promoter construct. This is a representative experiment out of three different ones. Standard deviations are denoted by vertical lines. Sp1 activated 88-fold the endoglin basal promoter activity. This activation was abrogated by the Sp1 inhibitor WP631. D, effect of Sp1 mutation on endoglin promoter activity. HeLa and HepG2 cells were transiently transfected with either the wild type endoglin construct pCD105(−50/+350) (WT) or the Sp1 mutated version (MUT). This is a representative experiment of three different ones. Standard deviations are denoted by vertical lines. The mutation of Sp1 site abolished basal promoter activity of endoglin in both cell types.

Endoglin Regulation by Sp1

The cooperation between TGF-β/Smad3 pathway and Sp1 was also studied in the Sp1-expressing human cell lines HeLa and HepG2 (Fig. 5). Due to the high levels of endogenous Sp1 in these cells, the Sp1 transactivation is predicted to be lower than that in the Sp1-deficient S2 cells. Consistent with this, the transactivation obtained by overexpressing Sp1 alone ranged from 1.5- to 2-fold, whereas additional addition of TGF-β yielded a synergistic induction from 6- to 8-fold with respect to untreated samples (Fig. 5A). On the other hand, expression of Smad3/ Smad4 resulted in a 3- to 4-fold induction of the promoter activity in both cell lines (Fig. 5B). The joint action of Sp1 and Smad3/ Smad4 triggered a synergistic stimulation reaching up to 8- or 12-fold induction in HeLa and HepG2 cells, respectively.

Physical Interaction between Sp1 and Smad3—The previous results show a strong functional interaction between TGF-β/ Smads and Sp1, which may be defined as synergistic, because the result of the joint Smad/Sp1 cooperation is above the additive effects due to either Smads or Sp1 alone. To determine whether the functional synergy between Sp1 and Smad3 involves physical interactions, COS-7 cells were transiently transfected with Smad3 and the constitutively activated TβRI receptor. As Sp1 is abundant in COS cells, it is not necessary to overexpress Sp1 by transfection. Immunoprecipitation with anti-Sp1 antibodies followed by the immunodetection with anti-Smad3, demonstrated the association between Sp1 and Smad3 (Fig. 6A). Conversely, immunoprecipitation with anti-Smad3 antibodies, followed by the immunodetection with anti-Sp1 (Fig. 6B), confirmed this association. As a control, total lysates were analyzed by Western blot to demonstrate expression of endogenous Sp1 and transfected Smad3.

DISCUSSION

Endoglin is the locus affected in the HHT1, an inherited vascular disease whose clinical manifestations are the result of an haploinsufficiency leading to decreased levels of endoglin at the surface of endothelial cells. Therefore, the understanding of endoglin transcription and, eventually, the assessment of ways to increase its rate may be crucial in relation to strategies for the HHT1 treatment. Endoglin has been characterized previously as a component of the TGF-β receptor system (1, 4, 46), and its expression has been shown to be regulated transcriptionally by TGF-β (4, 15), although the responsible elements for the TGF-β responsiveness were not mapped. Here, we have elucidated the molecular mechanisms of TGF-β activation on endoglin promoter. The region involved in the Smad3-dependent TGF-β activation of the promoter has been mapped within 50 base pairs upstream of the transcription start (Fig. 1). Smad proteins can bind to the Smad box (GTCT), also termed CAGA box, as in the PAI-1 promoter where three CAGA motifs are present (23). However, Smad proteins may also bind to more relaxed consensus motifs (47). Thus, the change of T by other base in the second position of the GTCT box is tolerated with-
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Fig. 4. Sp1 site at −37 position in the proximal promoter of endoglin is involved in the TGF-β/Smad3-induced promoter activity. A, HeLa and HepG2 cells were transiently transfected with either the wild type or the Sp1 mutated versions of endoglin proximal construct pCD105(−50/+350). After transfection, cells were treated or not with 10 ng/ml TGF-β for 20 h and the relative promoter activity of the wild type (WT) and the Sp1 mutant (MUT) constructs was determined. This is a representative experiment out of three different ones. Standard deviations are denoted by vertical lines. The TGF-β stimulation (-fold induction) of each construct is indicated by the number at top of the corresponding bar, and it is referred to the activity of their respective untreated samples. Mutation of Sp1 site decreased TGF-β responsiveness of the proximal endoglin promoter. RLU, relative luciferase units. B, Sp1-deficient, Schneider S-2 Drosophila cells were transfected with pCD105(−50/+350) (WT) or pCD105(−50/+350) Sp1-MUT, in the absence or presence of Sp1 and/or Smad3 as indicated. Sp1 and Smad3 proteins were provided by cotransfection of the expression vectors pPacSp1 for Sp1 and pCMV5-FlagSmad3 for Smad3 (125 ng each), together with the endoglin reporter construct. This is a representative experiment out of 10 different ones. Standard deviations are denoted by vertical lines. This is a representative experiment out of three different ones. Standard deviations are denoted by vertical lines. The fold induction values are referred to the activity of the corresponding untreated samples whose arbitrary value is 1. A, HeLa or HepG2 cells were either TGF-β-treated (10 ng/ml) or untreated for 20 h after cotransfection with pCD105(−50/+350) endoglin promoter in the presence or in the absence of Smad expression vector (0.5 μg), as indicated. The endoglin proximal promoter showed a marked increase in activity as a result of a cooperation between TGF-β treatment and Sp1. B, HeLa or HepG2 cells were transfected with pCD105(−50/+350) endoglin proximal promoter in the presence or absence of Sp1 (0.5 μg) and/or Smad3/4 (125 ng) expression vectors, as shown. The endoglin promoter activity was increased as a result of the cooperation between Sp1 and Smad3/4.

out significant decrease of Smad binding (43). Additionally, Smad4 binds to G/C-rich sequences, although with relatively low affinity, in the goosecoid promoter (39). It is possible that the loss of affinity by single changes in the Smad box, might be compensated by physical interactions of Smads with spatially adjacent transcription factors. Accordingly, a more general GNCN consensus repeat sufficient for Smad-DNA binding has been identified at position 37/(−36) (GGCG GCCT GGCC) of endoglin promoter contains three GNCN tandem repeats close to the Sp1 site. Moreover, the second of these motifs fits with the GTTC box, except for a minor substitution of the second T by C, a permissive change according to Shi et al. (43). This and the ability of GST-Smad3 and GST-Smad4 to bind the −50/−24 oligonucleotide support the idea of a direct binding of Smads to the SBE sequences in this region of the promoter (Fig. 2E). Close to this region, a Sp1 consensus site has been identified at −37/−29 and found to play a critical role in the promoter because mutation of the Sp1-binding sequence, or addition of Sp1 inhibitor WP631, abolished the basal activity of the promoter (Fig. 3). This finding supports the crucial role of the Sp1 site at −37/−29 in the transcriptional activity of the TATA-less endoglin promoter. Hence, it can be speculated that similar heterozygous mutations at this site of the promoter would lead to HHT1, because it would render a non-functional endoglin promoter allele. So far, all the mutations detected in HHT1 are located downstream the ATG initiation codon for the endoglin protein. It remains to be determined whether a systematic search for mutations in the endoglin promoter region renders any disabling mutation.

Furthermore, the Sp1 site −50/−24 appears to be necessary for the TGF-β/Smad3 responsiveness of the endoglin promoter (Figs. 4 and 5). This site has been shown to be differentially protected by TGF-β treatment in experiments of DNA footprinting (data not shown), and in vivo positioning of Sp1 using the PINPOINT technique maps the Sp1 binding site over the predicted consensus sequence on endoglin promoter (Fig. 2A). From the above observations, a functional cooperation at the transcriptional level between Smads and Sp1 should be demonstrated. In Figs. 4 and 5, the synergy between both factors is shown. The transcription levels of endoglin after cotransfection with both Smad and Sp1 factors are much higher than the simple addition of individual inductions triggered by each factor. The effect is dramatic in Schneider cells, which do not express Sp1. In these cells, the effect of Sp1 increases the proximal promoter of endoglin between 10- and 100-fold, dem-
Smads are necessary, but not sufficient, to support TGF-β sequence in the DNA. This is in agreement with the view that factor sustaining the TGF-β-Sp1 could be considered in this respect as the transcription promoter. The transactivating activity of Smad in response to passing promoter (53).

b) The cooperation between Smad/Sp1 could be also observed in Sp1-containing cells, like HeLa and HepG2 cells, where the overexpression of Sp1 slightly transactivates transcription, but the cooperation with TGF-β/Smad3, significantly increases the levels of endoglin promoter activity between 5- and 8-fold (Fig. 5). We therefore propose a putative Smad/Sp1 interaction site at −50/−29 G/C-rich sequence, which could explain the ability of endoglin promoter to be stimulated by TGF-β through Sp1. This effect is not due to an increased production of Sp1 by TGF-β, because the amount of Sp1 present was not significantly affected by TGF-β treatment, as determined by Western blot analysis (data not shown).

It is well established that Smad proteins are transcriptional activators that bind DNA, and cooperate with other transcription factors in regulating target gene expression (21, 27). It is now clear that Sp1 can be included in the growing list of transcription factors cooperating with Smad proteins. Supporting this view, while this manuscript was in preparation, a burst of reports described the Sp1-dependent cooperation with Smads in a number of gene promoters (48–52). Similarly to endoglin promoter, integrity of the Sp1 was also found to be critical for the TGF-β induced activity in the Smad7 promoter, where mutation or deletion of binding site for Sp1 reduced drastically the ability of the promoter to respond to TGF-β (49).

Sp1 could be considered in this respect as the transcription factor sustaining the TGF-β response of the endoglin proximal promoter. The transactivating activity of Smad in response to TGF-β could not be exerted unless Sp1 binds its target sequence in the DNA. This is in agreement with the view that Smads are necessary, but not sufficient, to support TGF-β signaling, thus requiring the presence of other transcription factors to cooperate with. Requirements of this type have been described for example between OAZ and Smad1 in the Xvent2 promoter (53).

DNA binding experiments with an oligonucleotide encompassing −50/−24 endoglin promoter showed that treatment with TGF-β enhanced a nucleoprotein complex that contained Sp1, as evidenced by anti-Sp1 antibodies and mutation of Sp1 site (Fig. 2, B and C). Interestingly, the decrease of the TGF-β enhanced protein-DNA complex containing Sp1, after preincubation with anti-Smad antibodies (Fig. 2C), would imply that TGF-β-responsive promoters that contain both SBE sequences and G/C-rich Sp1 binding motifs provide optimal substrates for a cooperative interaction between Smads and Sp1 in transcription regulation. This observation was also made in p21 promoter, where Smad3 and Smad4 proteins increased the formation of the Sp1/DNA complex (48).

Other putative examples of this type of promoters include those of p15 (54), Smad7 (55), β3 integrin (50), TGF-β, and TGF-β I-I and II-I (56). Further proof for Sp1 and Smad3 protein interaction with the endoglin promoter was obtained by using a biotinylated oligonucleotide representing (−50/−24), which allowed the detection of the specific binding, as revealed by Western blot analysis (Fig. 2D).

These experiments support the direct binding of both Smad3 and Sp1 to the endoglin promoter, but they do not demonstrate the presence of Sp1 and Smad3 within the same nucleoprotein complex. Although the demonstration of similar complexes of Smads with other transcription factors such as c-Jun/c-Fos (57) or Fast-1 (58) has remained elusive, a distinct nucleoprotein complex migrating slower than Sp1-DNA and containing Sp1 and Smad proteins could be detected in the endoglin promoter (Fig. 2, F and G). This is in agreement with the presence of a complex, due to Smad3, with a lower mobility than Sp1-DNA in p21 (48) and p15 (51) promoters.

Smad proteins have been shown to cooperate with Sp1 in the up-regulation by TGF-β of p21 (48, 59), p15 (51), Smad7 (49), plasminogen activator inhibitor 1 (52), and β3 integrin (50) gene promoters. Together with endoglin, all these genes have in common that they are Sp1-dependent because the Sp1 site involved participates in transcription initiation regardless whether they are TATA or TATA-less promoters. However, a link with the function of each of those genes encoded proteins, which could justify a common regulated expression, is missing. Interestingly, two of these examples, endoglin and Smad7 proteins, have been reported to be negative modulators of TGF-β signaling (4, 5, 60), suggesting their involvement in a negative feedback regulation.

From the results presented in this paper, it is concluded that a direct physical interaction between Smads and Sp1 exists, as demonstrated by direct co-immunoprecipitation experiments (Fig. 6). This conclusion agrees with previous reports showing co-immunoprecipitation of Gal4-Sp1 and Smads (48, 51, 50). Furthermore, Smad3 probably interacts through its MH1 domain with two duplicated glutamine-rich domains of Sp1 (48). At the molecular level, the role of Smads in their cooperation with Sp1 could be: (a) induction of oligomerization, which results in higher binding affinity of Sp1 for the endoglin promoter; (b) recruitment of other cooperating factors as p300 or c-Jun, which could possibly stabilize or enhance transcriptional activity of Sp1; or (c) modulation of phosphorylation of Sp1, which could affect DNA-binding properties and transactivation abilities. Whatever is the mechanism involved in the synergistic cooperation between Sp1 and TGF-β/Smad3 at endoglin proximal promoter, the outcome is an important increase in the endoglin transcriptional activity. Exogenous treatments conducted to achieve this synergy could be envisaged to counteract the haploinsufficiency of patients with HHT1.

Acknowledgments—We thank Drs. Liliana Attisano and Scott Friedman for reagents and Carmen Langa for excellent technical assistance.
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Identification of a Critical Sp1 Site within the Endoglin Promoter and Its Involvement in the Transforming Growth Factor-β Stimulation

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J. Biol. Chem. 2001, 276:34486-34494.
doi: 10.1074/jbc.M011611200 originally published online June 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011611200

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