Induction of Platelet-derived Growth Factor B-chain Expression by Transforming Growth Factor-β Involves Transactivation by Smads

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Transforming growth factor-β (TGF-β) regulates a diverse array of biological processes, such as proliferation, differentiation, extracellular matrix production, and apoptosis. In cultured vascular endothelial cells, TGF-β induces the expression of platelet-derived growth factor (PDGF) B-chain, a mitogen and chemotactant, at the level of transcription. The molecular mechanisms underlying this process are not presently understood. In this study, we performed serial 5′ deletion and transient transfection analysis to define a region in the PDGF-B promoter mediating inducible responsiveness to TGF-β. This region contains an atypical nucleotide recognition element for the Smad family of transcriptional regulators. Electrophoretic mobility shift analysis revealed that nuclear proteins bound to this site in a transient and specific manner. Supershift analysis demonstrated the physical association of Smad4 with the promoter. Overexpression of Smad4 activated other transcriptional regulators. Electrophoretic mobility shift analysis revealed that nuclear proteins bound to this site in a transient and specific manner. Supershift analysis; PDGF, platelet-derived growth factor; PDGF-B, platelet-derived growth factor B-chain; EMSA, electrophoretic mobility shift analysis; PDGF, platelet-derived growth factor; PDGF-B, platelet-derived growth factor B-chain; PBS, phosphate-buffered saline; DTT, dithiothreitol; CMV, cytomegalovirus.

I and type II. Upon ligand binding, the type II receptor phosphorylates the type I receptor activating a specific set of recently discovered positive and negative regulatory transcription factors known as Smads. The Smad family, mammalian homologues of the Drosophila gene Mad (2, 3), translocate to the nucleus after activation and modulate gene expression. Smad1, Smad2, Smad3, Smad5, and Smad8, the receptor-associated Smads, are direct substrates of the type I receptor. Smad4, a common mediator Smad, is not directly phosphorylated by the receptor. Rather, it physiologically associates with other Smads in the cytoplasm prior to nuclear translocation as a complex. Smad6 and Smad7 are “anti-Smads” that have been found to inhibit TGF-β-induced Smad-dependent activation. A role for Smad family members in TGF-β signal transduction has been defined in a large number of promoters, including the human plasminogen activator inhibitor-1 (3–5), JunB (6), and p21/WAF1/Cip1 (7), by interactions directly with DNA and/or other transcriptional regulators.

Platelet-derived growth factor (PDGF) is a potent mitogen and chemotactant for cells of mesenchymal origin. It consists of two chains, A and B, held together in homodimeric or heterodimeric configuration by disulfide bonds. The three isoforms of PDGF (AA, BB, and AB) modulate biological responsiveness by interacting specifically with two (α and β) cell surface tyrosine kinase receptors; α binds the A-chain and the B-chain, whereas β binds only to the B-chain. The PDGF ligand receptor system has strongly been implicated to play roles in a large number of normal and pathologic settings, such as embryonic development, wound healing, tumorigenesis, and atherosclerosis. For example, PDGF-A and PDGF-B mRNA and protein are present in human atherosclerotic tissue (8–10) and in human coronary arteries after percutaneous transluminal coronary angioplasty (11). In rat arteries, PDGF-A, PDGF-B, and the α-receptor are activated in endothelium following mechanical injury to the vessel wall (12). Ischemia of recombinant PDGF-BB stimulates migration and intimal thickening (13). Moreover, PDGF-B gene transfer into pig arteries stimulates intimal hyperplasia (14, 15).

The PDGF-B chain gene (or c-sis proto-oncogene) resides on chromosome 22 (22q12.3–q13.1) (16, 17) and spans approximately 24 kilobases of genomic DNA. The PDGF-B promoter is transcriptionally activated in a wide variety of normal and transformed cell types, including vascular endothelial cells (18–21), megakaryocytes (22, 23), fibroblasts (22), Jurkat T-(24), glioblastoma cells (22), fibrosarcoma cells (22), and osteosarcoma cells (25, 26). Since the structure of the PDGF-B transcriptional unit was first defined (27), a number of transcription factors with the ability to modulate PDGF-B promoter-dependent expression have been identified. Sp1 (19, 24, 26), Sp3 (24, 26), and early growth response factor-1 (Egr-1) (21) bind to overlapping nucleotide recognition elements in the PDGF-B promoter near the TATA box. In vivo footprinting

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The abbreviations used are: TGF-β, transforming growth factor-β; Egr-1, early growth response factor-1; EMSA, electrophoretic mobility shift analysis; PDGF, platelet-derived growth factor; PDGF-B, platelet-derived growth factor B-chain; EMSA, electrophoretic mobility shift analysis; PDGF, platelet-derived growth factor; PDGF-B, platelet-derived growth factor B-chain; PBS, phosphate-buffered saline; DTT, dithiothreitol; CMV, cytomegalovirus.
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EXPERIMENTAL PROCEDURES

Transient Transfection and Reporter Gene Expression—Bovine aortic endothelial cells were used in experiments between passages 4 and 8. The cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum supplemented with 50 μg/ml streptomycin and 50 IU/ml penicillin, at 37 °C in a 5% CO₂ incubator. At 50–60% confluency, the cells were transfected with the indicated amount of plasmid DNA and 2 μg of the Renilla luciferase reporter plasmid, pRL-TK (Promega) using the modified calcium phosphate precipitation procedure (32). After incubation overnight at 37 °C at 3% CO₂, the cells were washed twice with PBS, pH 7.4, and incubated with 0.5% fetal bovine serum/Dulbecco’s modified Eagle’s medium for 24 h at 37 °C and 5% CO₂ before exposure to 10 ng/ml of TGF-β (Promega). The cells were incubated for a further 24 h before harvesting by washing twice with PBS, pH 7.4, and scraping in 1 ml of passive lysis buffer (Promega). The suspension was freeze/thawed and spun, and the supernatant used to determine luciferase activity using the Dual Luciferase reporter assay (Promega). The suspension was freeze/thawed and spun, and the supernatant used to determine luciferase activity using the Dual Luciferase Kit (Promega) according to the manufacturer’s directions.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from 100-mm dishes. Growth-arrested endothelial cells were incubated with 10 ng/ml of TGF-β for the times indicated and harvested. Cells were washed twice with PBS, pH 7.4, and centrifuged at 1300 rpm for 15 min at 4 °C. The cell pellet was resuspended in 100 μl of ice-cold Solution A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5% Nonidet P-40, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, and 10 μg/ml leupeptin) and incubated on ice for 5 min. The suspension was microfuged at 13,000 rpm, and the cytosolic fraction was removed. The nuclei were resuspended and lysed in 20 μl of ice-cold Solution C (20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, and 10 μg/ml leupeptin) by gentle shaking for 20 min at 4 °C. Cell debris was removed by centrifugation at 13,000 rpm for 1 min, and the nuclear extract was combined and diluted 1:1 with ice-cold Solution D (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, and 10 μg/ml leupeptin). Nuclear extracts were snap frozen on dry ice and stored at −80 °C until use.

Electrophoretic Mobility Shift Assay—Binding conditions were essentially as described (33). In general, 4–6 μg of nuclear extract and 150,000 cpm of 32P-labeled double-stranded oligonucleotide were incubated in 20 μl containing 10 mM Hepes-KOH, pH 7.9, 40 mM KCl, 0.4 mM DTT, 0.4 mM EDTA, 4% glycerol, and 1 μg of poly(dI-dC) for 30 min on ice. In supershift studies, 4 μg of affinity-purified antibody (Santa Cruz Biotechnology) was incubated with the binding mixture at 4 °C overnight prior to the addition of probe. Bound complexes were separated from free probe by nondenaturing (5%) polyacrylamide gel electrophoresis in 0.5× TBE running buffer at 200 V (constant voltage). The gels were vacuum dried and exposed to Hyperfilm-MP (Amersham Pharmacia Biotech) overnight at −80 °C.

Western Blot Analysis—Transfected cells were washed twice with ice-cold PBS, pH 7.4, before being lysed on ice in 1× RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 1% sodium pyrophosphate, 0.5 mM EDTA, 4 mM DTT, 200 mM NaCl, 1 μg/ml leupeptin, 1 mM aprotinin, and 1% SDS). Lysates were collected after centrifugation at 14,000 rpm for 20 min at 4 °C, and the protein concentration was determined by BCA protein assay (Pierce). Lysates containing 10 μg of protein were prepared in SDS sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, and 30 mM DTT) and boiled for 3 min with 0.5 μl of 0.5% SDS-polyacrylamide gel with a 4% stacking gel. Gels were blotted onto Immobilon-P (polyvinylidene difluoride) membranes (Millipore, Bedford, MA) for 1 h at 100 V, dried, and blocked overnight at 4 °C in 5% skim milk powder, 0.05% Tween 20 and PBS, pH 7.4. Three 15-min washes with 0.05% Tween-PBS were made prior to 1 h of incubation with the primary antibody (1:1000 in 1% bovine serum albumin, 0.05% Tween-PBS) at 22 °C. The membrane blots were washed again before incubation at 22 °C with rabbit immunoglobulin secondary antibody (1:1000 in 1% bovine serum albumin, 0.05% Tween-PBS) conjugated with horseradish peroxidase (DAKO). The blots were washed again before chemiluminescence detection according to the manufacturer’s protocol (NEL Life Science Products).

RESULTS

TGF-β Induces PDGF-B Promoter-dependent Expression—Previous investigations have determined that TGF-β can induce PDGF-B expression in vascular endothelial cells at the level of transcription (31, 34). To understand the molecular basis for this effect, we performed transient transfection analysis using a nested series of Firefly luciferase-based reporter constructs bearing various sized fragments of the PDGF-B promoter. The endothelial cells were cotransfected with a Renilla luciferase-based expression vector to correct for transfection efficiency. PDGF-B promoter-dependent reporter activity increased in cells transfected with constructs RA-UC, NA-luc, and SDA-luc (26), whose 5′ end points extend 998, 251, and 103 base pairs upstream of the transcriptional start site (27) (Fig. 1A). However, TGF-β-inducible reporter activity was no longer observed in transientants harboring construct SPA-luc, whose expression is driven by 64 base pairs of the PDGF-B promoter (Fig. 1A) and retains binding sites for Sp1, Sp3, and Egr-1 (19, 21, 26). These findings suggested that these transcription factors may not play a direct regulatory role in TGF-β-inducible PDGF-B promoter-dependent expression. Rather, TGF-β-activation is mediated by the region located between nucleotides −103 and −64 in the PDGF-B promoter, which, interestingly, bears a putative atypical nucleotide recognition element for the Smad family of transcription factors, whose core motif is 5′-CAGA-3′ (5) (Table I). TGF-β activation of the PDGF-B promoter-reporter construct SDA-luc (Fig. 1A), which contains a single CAGA motif (−81/−78), compares favorably with TGF-β activation of construct (CAGA)_3-TK-luc (Fig. 1B), which contains six copies of the AGCCAGACA element located at position −730 in the plasminogen activator inhibitor-1 promoter (5).

Nuclear Proteins Interact with the PDGF-B Promoter in Endothelial Cells Exposed to TGF-β—To explore the mechanisms underlying PDGF-B promoter activation by TGF-β, we performed electrophoretic mobility shift analysis (EMSA) with 32P-Oligo-93/-63, a double-stranded oligonucleotide spanning this putative Smad-binding site in the PDGF-B promoter. Using nuclear extracts of growth-quiescent cells, several distinct nucleoprotein complexes were detected (Fig. 2, lane 2). Within 30 min of incubation with TGF-β, a single inducible complex was observed (Fig. 2, lane 3, indicated by arrow) that remained elevated even after exposure to TGF-β for 3 h, but not 4 h (Fig. 2).
To demonstrate that nuclear protein(s) interact with this region in a specific fashion, we performed oligonucleotide competition experiments in EMSA. A 75-fold molar excess of unlabeled Oligo-93/-63 virtually abrogated the appearance of the inducible complex (Fig. 3A, indicated by arrow). In contrast, the same fold molar excess of an irrelevant oligonucleotide LMSSREm3 (20) had no effect on the formation of this complex (Fig. 3A). Similarly, when the CAGA sequence in Oligo-93/-63 was deleted to produce Oligo-93/-63Δ2, a 75-fold molar excess of the latter oligonucleotide failed to interfere with nucleoprotein complex formation (Fig. 3A), nor could it directly support formation of the TGF-β-inducible complex (Fig. 3B, indicated by arrow).

We performed supershift analysis to shed light on the identity of the nuclear proteins interacting with 32P-Oligo-93/-63. Inclusion of polyclonal antibodies specifically targeting Smad4 in EMSA produced a distinct supershift (Fig. 4, right panel, lane 3, indicated by S), which was not observed when an identical amount of polyclonal antibodies raised against Egr-1 were used (Fig. 4, right panel, lane 4). To ensure that the Smad4 shift was not the product of a nonspecific interaction between the antibody and the radiolabeled probe, nuclear extract was omitted from the EMSA reaction. This strategy did not produce a supershift with either antibody (Fig. 4, left panel, lanes 2 and 3). The inability of Smad4 antibodies to affect the intensity of nucleoprotein complex or effect a complete supershift has previously been observed by other investigators in EMSA (4–6) and may be a reflection of the low affinity or conformation of Smad4. These findings demonstrate the presence of Smad4 in endothelial nuclei and its physical association with the PDGF-B promoter.

**TGF-β Activation of the PDGF-B Promoter Is Smad4-dependent**—To determine whether Smad4 plays a role in TGF-β-inducible PDGF-B promoter-dependent gene expression, we performed transient transfection analysis using cytomegalovirus (CMV) promoter-driven expression vectors generating wild type and dominant negative forms of Smad4. TGF-β-induced PDGF-B promoter-dependent luciferase activity in cells transfected with the empty expression vector (Fig. 5A, compare first and second columns). Overexpression of wild type Smad4 (CMV-Smad4) activated the PDGF-B promoter (Fig. 5A, third column, and Fig. 7, third column), which further increased upon exposure of the transfectants to TGF-β (Fig. 5A, sixth column). In contrast, in cells transfected with the dominant negative Smad4 expression vector, DPC4(1–514), the capacity of TGF-β to augment Smad4-inducible PDGF-B promoter ex-

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**FIG. 1.** Definition of a TGF-β response element in the PDGF-B promoter by 5′ deletion and transient transfection analysis. Endothelial cells were transfected with 10 ng of Firefly luciferase reporter constructs driven by various sized fragments (−398, −251, −103, or −64) of the PDGF-B promoter (A) and construct (CAGA)$_6$-TK-luc (B). Each construct was cotransfected with 2 μg of pRL-TK to normalize Firefly data with Renilla data for transfection efficiency. The cells were rendered growth-quiescent and exposed to 10 ng/ml of TGF-β for 24 h prior to harvest and determination of luciferase activity by 32P-luminometry.

**FIG. 2.** Nuclear proteins interact with the PDGF-B promoter in response to TGF-β in an inducible and transient manner. Nuclear extracts of growth-quiescent vascular endothelial cells exposed to 10 ng/ml of TGF-β for various times (indicated in figure) were incubated with 32P-labeled PDGF-B promoter oligonucleotide (Oligo-93/-63), and bound complexes were resolved on a 5% nondenaturing polyacrylamide gel. The nucleotide sequence in Oligo-93/-63 is 5′-CTG ACT CCG GCC CAG AAG AGG AAA GGC TGT-3′. The first lane from the left indicates the result of EMSA when probe alone without nuclear extract was used; the arrow denotes the TGF-β-inducible nucleoprotein complex.

**TABLE I**

| Gene   | Species | Sequence       | Motifs in various promoters activated by TGF-β |
|--------|---------|----------------|-----------------------------------------------|
| PAI-1  | Human   | AGCCAGAGCA     | 9'-CAGA-3'                                   |
| JunB   | Murine  | AGACAGAAGC     |                                               |
| α2(I)  | Collagen| AGACAGACAG     |                                               |
| PDGF-B | Human   | −8GTCGCCAGAG −76|                                               |

**FIG. 3.**
pression was inhibited (Fig. 5A, seventh column). These findings demonstrate the capacity of Smad4 to transactivate the PDGF-B promoter, alone and in concert with exposure to TGF-β.

Renilla luciferase activity generated by the normalizing plasmid pRL-TK was independent of Smad cotransfection or exposure to TGF-β (data not shown), indicating specificity. The comparable increase in TGF-β- and Smad-inducible PDGF-B promoter-dependent expression (Fig. 5A, second and third columns) is consistent with previous findings by different groups in other promoters (6, 35, 36). That Smad protein was generated in endothelial cells following transfection was confirmed by Western immunoblot analysis (Fig. 5B).

Integrity of the 281CAGA278 Element Is Critical for Smad Activation of the PDGF-B Promoter—To demonstrate the functional significance of the CAGA-binding site in the proximal PDGF-B promoter, we mutated the PDGF-B 281CAGA278 in Oligo-93/-63 to generate Oligo-93/-63TTTT. A 100-fold molar excess of Oligo-93/-63TTTT failed to block the formation of the TGF-β-inducible nucleoprotein complex using the 32P-labeled wild type PDGF-B promoter fragment (compare Fig. 6A with Fig. 3A). Introduction of this mutation into SDA-luc generated SDAm7-luc. Cotransfection experiments revealed that the intact 281CAGA278 element is crucial for Smad3-inducible PDGF-B promoter-dependent expression (Fig. 6B).

We next mobilized six copies of the 283GCCAGAAG276 motif into the poly linker region of pGL3-prom, driven by the SV40 promoter, in efforts to establish portability of the element and demonstrate greater transactivation upon cotransfection with Smad3. However, the heterologous promoter bearing this sequence in either the forward or the reverse orientation failed to generate any additional reporter activity (data not shown). These findings, which are not entirely unexpected, suggest that additional cis-acting elements in the PDGF-B promoter are required; these may be absent, disrupted, or mis-spaced in the
heterologous construct.

Smads Together Activate the PDGF-B Promoter—Previous studies have found that Smad proteins functionally cooperate over nucleotide elements in the promoters of a number of different genes (2, 3, 37). Having demonstrated a functional role for Smad4 in the regulation of PDGF-B promoter activity, we next determined whether this Smad member collaborates with other Smads to modulate PDGF-B promoter-dependent expression. Transient transfection studies revealed that Smad3, like Smad4, activates the PDGF-B promoter in endothelial cells (Fig. 7, second column). Simultaneous transfection of Smad3 and Smad4 further activated the PDGF-B promoter (Fig. 7, sixth column). This effect was markedly attenuated if Smad4 was substituted by its dominant negative counterpart (Fig. 7, seventh column).

Smad2 and Smad3 Activate the PDGF-B Promoter—We explored whether Smad2, like Smad3 and Smad4, activates the PDGF-B promoter. Overexpression of Smad2 alone induced PDGF-B promoter-dependent reporter gene expression (Fig. 8A, second column). Coexpression of both Smad2 and Smad3 resulted in synergistic activation (Fig. 8A, fifth column). In contrast, Smad2, Smad3, and Smad4 were not able to activate the shorter PDGF-B promoter-reporter construct, SPA-luc (Fig. 8B), thus demonstrating that Smad activation of the PDGF-B promoter is sequence-specific. Physical and cooperative interactions between these Smad proteins have previously been demonstrated by other investigators (3, 38).

DISCUSSION

In this paper, we demonstrate that TGF-β induction of the PDGF-B promoter in vascular endothelial cells is mediated by the Smad family of transcriptional regulators. Serial 5′ deletion and transient transfection analysis defined a region in the PDGF-B promoter mediating inducible responsiveness to TGF-β. This region contains an atypical nucleotide recognition element for the Smad family that preserves the 5′-CAGA-3′ motif. EMSA revealed that nuclear proteins bound to this site in an inducible, transient, and specific manner. This nucleoprotein complex was detected within 30 min of exposure to TGF-β, consistent with rapid Smad translocation from the cytoplasm to the nucleus and its interaction with the PAI-1 promoter (4, 5). Formation of the TGF-β-inducible complex was blocked by a molar excess of unlabeled Oligo-93/-63 but was unaffected by an oligonucleotide bearing a deletion of the −81 CAGA−78 motif (Oligo-93/-63Δ2) or one in which the −81 CAGA−78 motif was mutated to −81 TTGT−78 (Oligo-93/-63TTTT). Overexpression of Smad4 activated the PDGF-B promoter and superinduced PDGF-B promoter-dependent expression in endothelial cells exposed to TGF-β. Simultaneous expression of Smad3 and Smad4, or Smad2 and Smad3, activated the PDGF-B promoter. Mutation of the −81 CAGA−78 motif in the PDGF-B promoter (SDAm7-Luc) abrogated Smad-inducible reporter gene expression. These findings demonstrate the existence of a novel Smad-binding element in the PDGF-B promoter.
TGF-β activation of the PDGF-B promoter was blocked by overexpression of cDNA encoding a truncated, dominant negative form of Smad4 (amino acids 1–514). Conversely, exogenous wild type Smad4 augmented TGF-β-inducible PDGF-B expression, testing the fidelity of Smad4 activation of PDGF-B in a manner consistent with TGF-β induction of the PAI-1 promoter (35, 38). The dominant negative approach has been used previously by other groups to define the involvement of Smad4 in signal transduction stimulated by TGF-β (49). Smads, in turn, may induce the expression of PDGF-B and perhaps other growth regulatory molecules that modulate cell movement and proliferation and the eventual formation of a neointima. The response of the artery wall to injury may involve Smad activation by endogenous TGF-β, which converts the latent form to the active form (49). Smads, in turn, may induce the expression of PDGF-B and perhaps other growth regulatory molecules that modulate cell movement and replication. In developing human atherosclerotic lesions, TGF-β and PDGF-B are both overexpressed (50, 51). TGF-β and PDGF-B are also present in restenotic lesions following percutaneous transluminal coronary angioplasty and bypass...
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