

Vascular Biology, Atherosclerosis and Endothelium Biology

Peroxide-Inducible Ets-1 Mediates Platelet-Derived Growth Factor Receptor- α Gene Transcription in Vascular Smooth Muscle Cells

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Platelet-derived growth factor (PDGF) has been implicated in the pathogenesis of vascular occlusive disorders such as atherosclerosis and restenosis in part due to its regulation of smooth muscle cell phenotype. The molecular mechanisms regulating the expression of PDGF-R α , which binds all known dimeric forms of PDGF except PDGF-DD, are poorly understood. Here we demonstrate that the winged helix-turn-helix proto-oncogene Ets-1 controls PDGF-R α transcription and mRNA expression in smooth muscle cells. Mutational analysis, electrophoretic mobility shift assay, and chromatin immunoprecipitation revealed the existence of a reverse Ets binding motif ($^{-45}\text{TTCC}^{-42}$) in the proximal region of the PDGF-R α promoter, which bound both recombinant and endogenous Ets-1. Ets-1-inducible PDGF-R α expression depended on the integrity of both the $^{-45}\text{TTCC}^{-42}$ motif and the $^{-61}\text{G}_{10}^{-52}$ element, which resides upstream of $^{-45}\text{TTCC}^{-42}$ and mediates Sp1 induction. Hydrogen peroxide (H_2O_2) at nanomolar concentrations stimulated levels of Ets-1 and increased PDGF-R α transcription and mRNA expression without affecting Sp1 expression. H_2O_2 activation of the PDGF-R α promoter was abolished by disrupting $^{-45}\text{TTCC}^{-42}$ or $^{-61}\text{G}_{10}^{-52}$. These studies identify a functional Ets motif in the PDGF-R α promoter that plays a pivotal role in agonist-inducible PDGF-R α transcription. (*Am J Pathol* 2005, 167:1149–1159)

Platelet-derived growth factor (PDGF) is a family of potent mitogenic and chemoattractant proteins for cells of mesenchymal origin such as vascular smooth muscle cells (SMCs) and fibroblasts.^{1,2} PDGF proteins consist of four ligands, PDGF-A, -B, -C, and -D that form disulfide-linked

homodimers or heterodimers and bind to the transmembrane receptor tyrosine kinases, PDGF receptor- α and - β (PDGF-R α and PDGF-R β).³ PDGF-R α can bind PDGF-A, -B, or -C.^{4,5} whereas PDGF-R β binds PDGF-B and -D.^{3,6} The PDGF-Rs have distinct and overlapping biological functions. For example, PDGF-R α is involved in cell hyperplasia and hypertrophy whereas PDGF-R β is involved in both mitogenesis and migration.⁷ Cells that express both PDGF-Rs can exploit receptor differences by activating PDGF-R α that inhibits β -receptor-induced migration.^{2,8} PDGF-Rs activate signaling cascades including extracellular signal-regulated kinases (ERKs) and stress-activated protein kinase-1/c-Jun NH₂-terminal kinase-1 (JNK-1).⁹

Numerous lines of evidence indicate that the PDGF/PDGF-R system plays an important role in SMC growth and the development of vascular occlusive disorders. For example, PDGF-R α is expressed in advanced atherosclerotic lesions.¹⁰ Cardiac allograft arteriosclerosis in rats is inhibited by CGP 53716, a protein tyrosine kinase inhibitor selective for PDGF-R.¹¹ PDGF-R α levels increase after balloon injury to rat carotid arteries.^{12,13} Balloon injury also increases PDGF-R α tyrosyl phosphorylation.¹⁴ Anti-sense oligonucleotides targeting PDGF-R β inhibit neointima formation in balloon-injured rat carotid arteries.¹⁵ Chimeric antibodies raised against PDGF-R α significantly reduced SMC density in the newly formed neointima in a baboon model.⁷ Finally, blockade of PDGF signaling with antibodies against PDGF-R α and PDGF-R β in a baboon vascular graft model caused intimal atrophy.¹⁶

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The Ets family of transcription factors regulate gene expression via physical interactions with DNA (by virtue of their evolutionarily conserved DNA-binding domain and their core recognition motif, 5'GGAA/T3') and protein-protein interactions with co-factors and other transcription factors. Posttranslational modifications (such as phosphorylation) can also alter the transcriptional activity of Ets factors.¹⁷ We have recently shown that Ets-1 physically interacts with Sp1 and regulates Fas ligand gene transcription in SMCs.¹⁸ Ets-1 has also been shown to interact with nuclear factors such as nuclear factor- κ B,¹⁹ AP-1,²⁰ and GHF-1.²¹ Ets-1, like PDGF-R α , may play a permissive role in vascular remodeling. For example, Ets-1 expression is induced after arterial injury and exposure to PDGF-BB.²² Moreover, Ets-1 regulates the expression of many genes involved in degradation and remodeling of the extracellular matrix.²³

Here we demonstrate that PDGF-R α expression is under the transcriptional control of Ets-1. A functional Ets motif lies in the proximal region of the PDGF-R α promoter 5' of a recently identified Sp1-binding element.²⁴ Ets-1-inducible PDGF-R α expression is critically dependent on the Sp1 site. Surprisingly, Sp1 transactivation of the PDGF-R α promoter does not require the Ets motif. The integrity of the Ets site is critical for agonist-inducible PDGF-R α transcription in vascular SMCs. Peroxide stimulates PDGF-R α and Ets-1 expression without affecting Sp1 expression. Induction of the PDGF-R α promoter by H₂O₂ is abolished by disruption of either ⁻⁴⁵TTCC⁻⁴² or ⁻⁶¹G₁₀⁻⁵². These studies show that PDGF-R α transcription is controlled by Ets-1, which mediates peroxide-inducible PDGF-R α transcription.

Materials and Methods

Immunohistochemical Detection of PDGF-R α , Ets-1, Sp1, and α -Smooth Muscle Actin (α -SMA) in Human Carotid Arteries

Immunohistochemical analysis was performed with antibodies to PDGF-R α , Ets-1, Sp1 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or α -SMA (Novocastra, New Castle upon Tyne, UK) on sister sections of formalin-fixed, paraffin-embedded atherosclerotic carotid artery specimens obtained by endarterectomy at St. Vincent's Hospital, Sydney, Australia. Immunohistochemical analysis was performed essentially as previously described.²⁵ Briefly, before staining, deparaffinized sections were treated with 3% hydrogen peroxide and boiled in citrate buffer, pH 6.0, to retrieve antigenicity. The standard avidin-biotin complex (ABC) immunoperoxidase technique was used.²⁶ After washing in Tris-buffered saline, pH 7.6, sections were incubated in primary antibody for 60 minutes, followed by incubation with the appropriate secondary antibody for 20 minutes, and finally with ABC (Elite Vector PK-6100) for 30 minutes. Immunogenicity was visualized by treatment in 3,3'-diaminobenzidine solution for 2 minutes, which produced brown coloration. Sections were counterstained with Mayer's hematoxylin. As negative control, the primary antibody was omitted, or the sections were treated

with the immunoglobulin fraction of appropriate nonimmune serum as a substitute for the primary antibody. No positive staining was observed in any of the negative control sections.

Cell Culture

Rat aortic (pup) WKY12-22 SMCs (obtained as a generous gift from Dr. Stephen M. Schwartz, University of Washington, Seattle, WA) were cultured in Waymouth's MB752/1 medium (Life Technologies, Inc.), pH 7.4, supplemented with 10% fetal calf serum, 10 U/ml penicillin, and 10 μ g/ml streptomycin at 37°C and 5% CO₂. The cells were passaged every 3 to 4 days in 75-cm² flasks. Primary bovine aortic endothelial cells (Cell Applications, Inc., CA) were maintained in 10% fetal bovine serum/Dulbecco's modified Eagle's medium, pH 7.4, with antibiotics and not used beyond passage 8.

Cell Proliferation Assay

SMCs (2000 cells/well) and endothelial cells (3000 cells/well) were seeded into 96-well titer plates and rendered growth-quiescent by incubation in medium containing 0.05% fetal bovine serum for 24 hours and transfected with 0.1 or 0.3 μ mol/L PDGF-R α antisense (AS) oligonucleotide (ODN) or its size-matched scrambled (SCR) counterpart, PDGF-R α SCR ODN, using FuGENE6 (3 μ l/ μ g). After 72 hours, the cells were trypsinized, resuspended in Isoton II, and quantitated using a Coulter Z1 counter (Beckman). PDGF-R α AS and PDGF-R α SCR ODN sequences were 5'-GTTCTCCTCGGTTCT-3' and 5'-GTGTCTCCTCCTGTC-3', respectively, based on analysis of rat and bovine PDGF-R α in MFOLD.

Plasmid Constructs

pLuc-a2²⁷ containing the promoter region of PDGF-R α was a generous gift from Dr. Yutaka Kitami (Ehime University School of Medicine, Ehime, Japan). pLuc-a2m.Sp1 and pLuc-a2m.Ets were produced using the QuikChange XL site-directed mutagenesis kit (Stratagene) in accordance with the manufacturer's instructions. The primers were pLuc-a2m.Sp1²⁴ forward 5'-TTTATTTTGAAGAGACCATT-TTTTTTCTTCATTTCTGACAGCT-3' and pLuc-a2m.Ets forward 5'-GGGGGGGGCTTCATGGAAATGCAGTATTTAC-3'. CMV-Sp1 was received from Dr. Robert Tjian (Howard Hughes Medical Institute, University of California, Seattle, WA). CMV-gutless was generated by excising Sp1 cDNA from CMV-Sp1. Ets-1 cDNA was excised from pKCR3-Ets-1 (received from Dr. Ian Cassidy, Department of Biochemistry and Molecular Biology, University of Queensland, Australia) and cloned into pcDNA3. pcDNA3-Fli-1, used in co-transfection experiments, was a gift of Dr. Michael Eisbacher (Centre for Vascular Research, University of New South Wales, Sydney, Australia).²⁸

Transient Transfection Analysis

For reporter gene analysis, SMCs were transfected with 10 μ g of pLuc-a2, pLuc-a2.mSp1, or pLuc-a2.mEts. Cells

were also transfected with 1 μ g of pRL-null (Promega) to correct for transfection efficiency. *Firefly* luciferase activity was normalized to *Renilla*. Transient transfections were performed using FuGENE6 (Roche). After incubation at 22°C for 10 minutes, the DNA/FuGENE6 mixture was added to cells containing 10 ml complete medium. Twenty-four hours after transfection cell lysates were prepared for assessment of luciferase activity in which the dual luciferase assay reporter system (DLR) was performed on a manual luminometer, (model TD-20/20; Turner Designs, Quantum Science). Hydrogen peroxide (Sigma) was incubated for 24 hours after transfection for a further 24 hours and cell lysates were prepared as above.

mRNA Expression Analysis

Cells were washed two times with ice-cold phosphate-buffered saline (PBS) and harvested with 4.5 ml of TRIzol reagent (Gibco) in accordance with the manufacturer's instructions. cDNA synthesis from total RNA was performed using Superscript II RT (Promega) in accordance with the manufacturer's instructions. Thermal cycling conditions were as follows: PDGF-R α : 94°C for 10 seconds, 62°C for 30 seconds, 72°C for 1.5 minutes for 40 cycles; GAPDH: 94°C for 30 seconds, 60°C for 30 seconds, 68°C for 2 minutes for 18 cycles; and u-PA: 94°C for 30 seconds, 64°C for 30 seconds, 72°C for 1 minute for 33 cycles. PDGF-R α primers were: forward 5'-AGAT-AGCTTCATGAGCCGAC-3' and reverse 5'-GGAACAG-GGTCAATGTCTGG-3', GAPDH primers: forward 5'-AC-CACAGTCCATGCCATCAC-3' and reverse 5'-TCCAC-CACCCTGTTGCTGTA-3'; u-PA primers: forward 5'-GCCAAAGAAATTCAAAGGGGAG-3' and reverse 5'-CGT-ATCTTCAGCAAGGCTATG-3'. Densitometric analyses were performed using Quantity One V4.1.0 (Bio-Rad).

Preparation of Nuclear Extracts

SMC monolayers were washed two times with ice-cold PBS, pH 7.4, then scraped into 10 ml of cold PBS. The cells were pelleted by centrifugation at 250 \times *g* for 10 minutes at 4°C. Cells were lysed by the addition of ice-cold hypotonic solution (buffer A) consisting of 10 mmol/L HEPES, pH 8.0, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 200 mmol/L sucrose, 0.5% Nonidet P-40, 0.5 mmol/L phenylmethyl sulfonyl fluoride (PMSF), and 1 μ g/ml aprotinin. The suspension was recentrifuged and the nuclei were lysed in an ice-cold solution (buffer C) consisting of 20 mmol/L HEPES, pH 8.0, 100 mmol/L KCl, 0.2 mmol/L ethylenediamine tetraacetic acid (EDTA), 20% glycerol, 1 mmol/L dithiothreitol, 0.5 mmol/L PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin. The nuclear fraction was combined with an equal volume of buffer D (20 mmol/L HEPES, pH 8.0, 100 mmol/L KCl, 0.2 mmol/L EDTA, 20% glycerol, 1 mmol/L dithiothreitol, 0.5 mmol/L PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin) and stored at -80°C.

Electrophoretic Mobility Shift Assays (EMSAs)

Binding reactions for gel shift assays were performed in 20 μ l of 10 mmol/L Tris-HCl, 50 mmol/L NaCl₂, 1 mmol/L EDTA, 2 mmol/L dithiothreitol, 5% glycerol, 0.5% Nonidet P-40, 1 mg/ml bovine serum albumin, ³²P-labeled oligonucleotide probe (150,000 cpm), and 1 μ l of relevant recombinant protein or 2 μ l of nuclear extract. The recombinant protein reaction was incubated for 20 minutes at 22°C. In supershift studies, the appropriate affinity-purified anti-peptide polyclonal antibody (Santa Cruz Biotechnology) was incubated with binding mix for 20 minutes at 37°C. Bound complexes were separated from free probe by loading samples onto a 6% nondenaturing polyacrylamide gel and electrophoresing at 120 V for 2.5 hours. The gels were vacuum-dried at 80°C and subjected to autoradiography overnight at -80°C. Ets consensus oligonucleotide sequence: Ets cons: 5'-ATC AGA AAA TTG TGG GCG GAA ACT TCC AGG-3'.¹⁸ Generation of recombinant Ets-1 protein was previously described.¹⁸ Recombinant Sp1 was purchased from Promega (Madison, WI). Sp1 consensus oligonucleotide sequence: Oligo A represents proximal PDGF-A promoter and interacts with Sp1.²⁹ Oligo A: 5'-GGG GGG GGC GGG GGC GGG GGC GGG GGA GG-3'.

Chromatin Immunoprecipitation Analysis

SMCs were washed in PBS, pH 7.4, incubated with 1% formaldehyde for 10 minutes, and quenched with glycine (final concentration, 0.1 mol/L). The cells were washed with PBS, trypsinized, then resuspended and incubated in buffer A (100 mmol/L Tris-HCl, pH 9.4, 10 mmol/L dithiothreitol) for 15 minutes at 30°C. The cells were precipitated by spinning at 14,000 \times *g* for 1 minute, washed sequentially in cold PBS, buffer I (0.25% Triton X-100, 10 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L HEPES, pH 6.5), buffer II (200 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 50 mmol/L HEPES, pH 6.5), lysed in lysis buffer [1% sodium dodecyl sulfate (SDS), 10 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.1, 0.5 mmol/L PMSF, 0.5 μ g/ml leupeptin, 2 μ g/ml aprotinin] and sonicated. After spinning, the supernatant was mixed with dilution buffer (1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.1). Protein A- and G-Sepharose (previously incubated with salmon sperm DNA and preimmune serum) was added to the supernatant and gently mixed at 4°C for 2 hours. The suspension was evenly divided and 5 μ g of rabbit polyclonal antibodies to Sp1 and Ets-1 (Santa Cruz Biotechnology) were added or no antibody was added, and incubated overnight at 4°C. The suspensions were washed sequentially in TSEI (0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl, pH 8.1, 150 mmol/L NaCl), TSEII (0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl, pH 8.1, 500 mmol/L NaCl), buffer III (0.25 mol/L LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.1), 1 \times TE, and elution was performed in elution buffer (1% SDS, 0.1 mol/L NaHCO₃) at 65°C for 8 hours.

The suspension was treated with proteinase K at 37°C overnight, before phenol-chloroform extraction and ethanol precipitation in the presence of tRNA as a carrier. Primer sequences for PDGF-R α were: forward 5'-GCA-CACCATCTCACAATCCA-3' and reverse 5'-AGTCATC-CTCCGAAAATAATCA-3'. The fragment of PDGF-R α promoter was amplified by polymerase chain reaction. Thermal cycling conditions were as follows: 94°C for 30 seconds, 56°C for 10 seconds, 72°C for 45 seconds for 46 cycles, generating a 628-bp product.

Western Immunoblot Analysis

Cells treated with 10 nmol/L H₂O₂ for designated times were washed two times with ice-cold PBS before being lysed on ice in 1× RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing protease inhibitors (2 mmol/L PMSF, 5 mmol/L EDTA, 10 μg/ml leupeptin, and 1% aprotinin). Cell lysates were collected after centrifugation at 14,000 rpm for 20 minutes at 4°C and the protein concentration was determined by BCA protein assay (Pierce). Lysates containing 10 μg or 20 μg of protein were prepared in SDS sample buffer (50 mmol/L Tris, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, and 30 mmol/L dithiothreitol) and boiled for 5 minutes with 0.5 mol/L iodoacetamide added before loading on a 10% SDS-polyacrylamide gel. Gels were blotted onto Immobilon-P (polyvinylidene difluoride) membranes (Millipore) and blocked overnight at 4°C in 5% skim milk powder, 0.05% Tween 20, and PBS. Ets-1, Sp1, and PDGF-R α protein were detected using rabbit polyclonal antibodies (Santa Cruz Biotechnology) and chemiluminescence detection (NEN Life Sciences Products) according to the manufacturer's instructions. Densitometric analyses were performed using Quantity One V4.1.0 (Bio-Rad).

Results

PDGF-R α Is Required for SMC Growth in Vitro

To demonstrate the reliance of cultured SMCs on PDGF-R α for cell growth, we exposed WKY12-22 cells (SMCs bearing remarkable similarity to SMCs of the synthetic phenotype^{30,31}) to antisense oligonucleotides targeting PDGF-R α . These agents blocked SMC growth in both a dose-dependent and sequence-specific manner (Figure 1). In contrast, these agents had no effect on endothelial cell replication (Figure 1), consistent with the poor expression of PDGF-R α in the latter cell type.^{32,33}

PDGF-R α , Ets-1, and Sp1 Are Expressed by SMCs in Human Atherosclerotic Plaque

Immunohistochemical analysis on sister sections of formalin-fixed, paraffin-embedded human atherosclerotic carotid artery specimens obtained by endarterectomy revealed that PDGF-R α , and the transcription factors

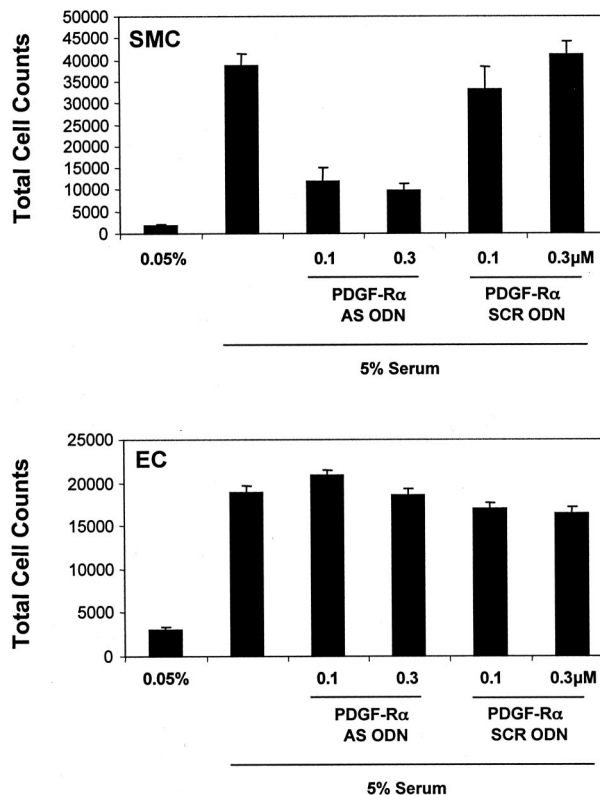


Figure 1. Anti-sense oligonucleotides targeting PDGF-R α block SMC growth *in vitro*. WKY12-22 SMCs were incubated overnight in 0.05% fetal bovine serum/Waymouth's medium and transfected with 0.1 or 0.3 μmol/L PDGF-R α AS ODN or its size-matched scrambled counterpart PDGF-R α SCR ODN. The medium was changed to 0.05% fetal bovine serum/Waymouth's and after 2 days the cells were trypsinized, resuspended in Isoton II, and quantitated using a Coulter counter. Endothelial cells (EC) grown in Dulbecco's modified Eagle's medium with the equivalent amounts of serum were treated identically. The data are representative of two experiments.

Ets-1 and Sp1 are expressed in SMCs in these lesions (Figure 2). Unlike the spatial pattern of PDGF-R α and α -SMA staining, Ets-1 and Sp1 expression was confined mainly to cell nuclei (Figure 2), consistent with their roles as transcription factors.

Ets-1 Induces PDGF-R α mRNA Expression and Transcription

Because PDGF-R α and Ets-1 are expressed in SMC after balloon injury^{12,14,34} and in atheroma (Figure 2), we explored whether Ets-1 regulates PDGF-R α in this cell type. PDGF-R α mRNA expression was assessed 24 hours after transfection of SMCs with pcDNA3-Ets-1 or its backbone control pcDNA3. Semiquantitative reverse transcriptase-polymerase chain reaction analysis showed that Ets-1 up-regulated endogenous steady-state PDGF-R α mRNA (Figure 3A, top left). Urokinase-type plasminogen activator (u-PA) is a known transcriptional target of Ets-1.³⁵ Levels of endogenous u-PA, like those of PDGF-R α , increased twofold 24 hours after the forced expression of Ets-1 (Figure 3A, top right) confirming therefore, that PDGF-R α expression is positively influenced by Ets-1. Corresponding GAPDH transcript levels demonstrated

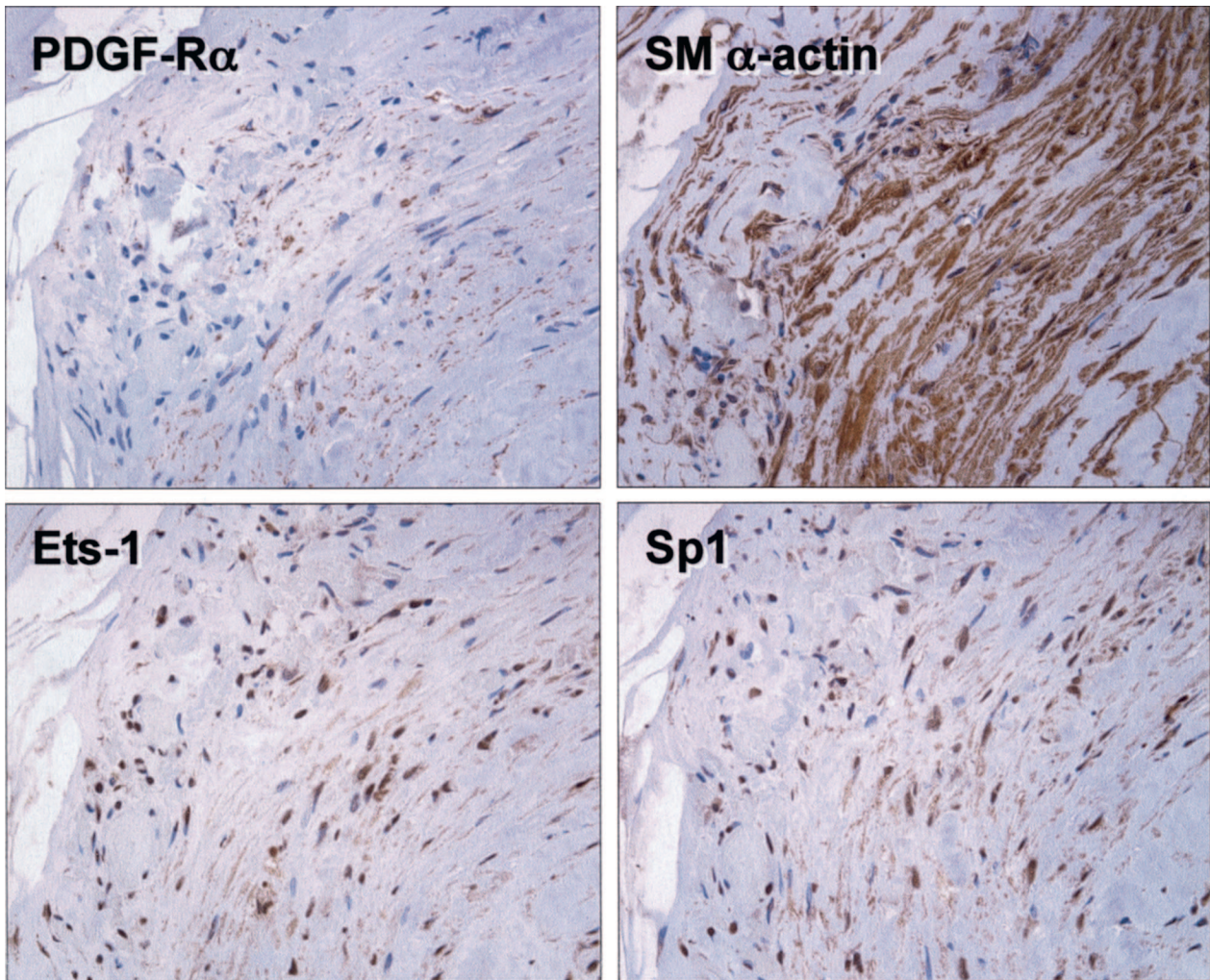


Figure 2. PDGF-R α , Ets-1, and Sp1 are expressed in SMCs of human carotid atheroma. Paraffin sections of formalin-fixed human carotid atherosclerotic plaques were stained with polyclonal antibodies to PDGF-R α , Ets-1, Sp1, and α -SMA. No staining was observed in the absence of primary antibody.

unbiased sample loading (Figure 3A, middle). Additionally, we assessed the effect of overexpressing the cell-restricted Ets family member Fli-1³⁶ on PDGF-R α transcription. Figure 3B demonstrates that although Ets-1 activates the PDGF-R α promoter, Fli-1 was inactive.

We also assessed the effect of overexpressing Ets-1 on PDGF-R α transcription. SMCs were co-transfected with pcDNA3-Ets-1 and pLuc-a2, a *Firefly* luciferase-based reporter construct driven by 1.3 kb of PDGF-R α promoter.²⁷ Luciferase activity 24 hours after transfection revealed that Ets-1 activates PDGF-R α transcription (Figure 3B). The proximal region of the PDGF-R α promoter contains a putative reverse Ets-binding motif (⁵TTCC³) located at -45/-42 bp relative to the transcriptional start site (Figure 3C).

Abrogation of Ets-1 Induction of PDGF-R α Transcription on Mutation of a Putative Reverse Ets Motif (⁻⁴⁵TTCC⁻⁴²)

To determine the functional relevance of the ⁻⁴⁵TTCC⁻⁴² element in the PDGF-R α promoter we mu-

tated ⁻⁴⁵TTCC⁻⁴² to ⁻⁴⁵AAGG⁻⁴² in pLuc-a2 plasmid to generate pLuc-a2.mEts. We assessed the effect of the mutation on PDGF-R α transcription by co-transfecting SMCs with pLuc-a2 or pLuc-a2.mEts and pcDNA3-Ets-1. Luciferase activity 24 hours after transfection revealed that Ets-1 no longer up-regulated PDGF-R α promoter-dependent expression when the reverse Ets binding element was disrupted (Figure 4A).

The Ets Recognition Element (⁻⁴⁵TTCC⁻⁴²) in the PDGF-R α Promoter Is Not Required for Sp1-Inducible PDGF-R α Transcription

We have previously shown that Sp1 regulates PDGF-R α transcription in SMCs via an atypical Sp1 binding element located at ⁻⁶¹G₁₀⁻⁵².²⁴ Our previous studies in SMCs reveal that endogenous Ets-1 and Sp1 physically interact.¹⁸ Because co-operative regulation of PDGF-R α transcription has not been described, we hypothesized that Ets-1 and Sp1 together mediate expression of this gene.

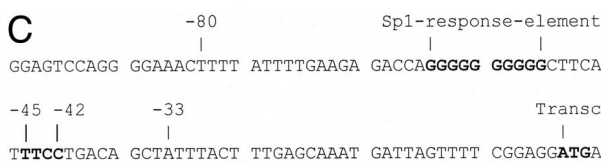
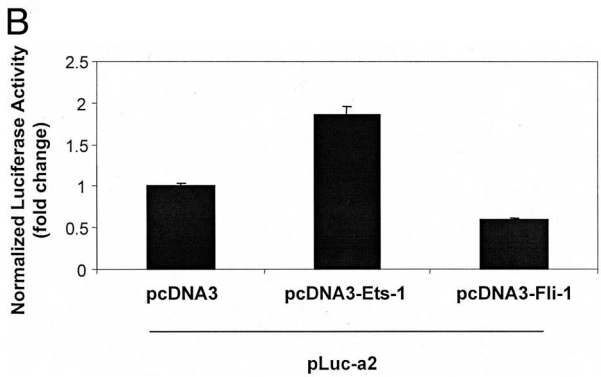
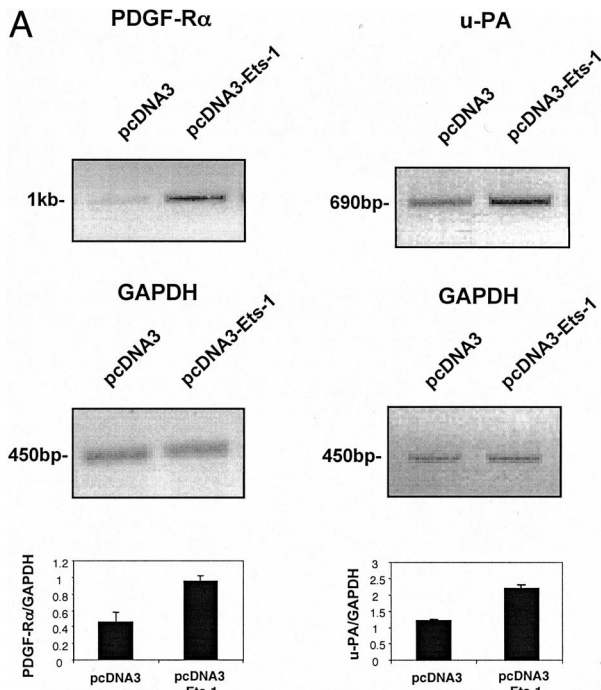


Figure 3. Ets-1 induces PDGF-Rα mRNA expression and transcription. **A:** Cells were transfected with 20 μg of pcDNA3-Ets-1 or pcDNA3 and PDGF-Rα or u-PA mRNA expressions were assessed by reverse transcriptase-polymerase chain reaction after 24 hours. The expected amplification product of PDGF-Rα is 1017 bp, GAPDH is 450 bp, and u-PA is 690 bp. Densitometric analysis was performed on PDGF-Rα, u-PA, and GAPDH mRNA bands. The result is representative of at least two separate determinations performed in duplicate. **B:** Ets-1 activates the PDGF-Rα promoter, whereas Fli-1 does not. SMCs were co-transfected with the PDGF-Rα promoter-reporter construct pLuc-a2, pRL-Null, and either pcDNA3-Ets-1 or pcDNA3-Fli-1. Luciferase activity was assessed by luminometry after 24 hours. **C:** Nucleotide sequence of the PDGF-Rα proximal promoter. The transcriptional (transc) start site (ATG)²⁷ is indicated. The reverse Ets (-45⁺TTCC⁻⁴²) binding site, that is the subject of this study, and the upstream Sp1 element (-61/-52)²⁴ are indicated. Oligo -80/-33 contains both putative Ets and Sp1 sites. The sequence was obtained from EMBL RN 13172 *Rattus norvegicus*.

To determine whether Sp1 required the -45TTCC-42 element for Sp1-inducible PDGF-Rα promoter-dependent transcription, we co-transfected SMCs with either pLuc-a2 or pLuc-a2.mEts, and CMV-Sp1 or its backbone

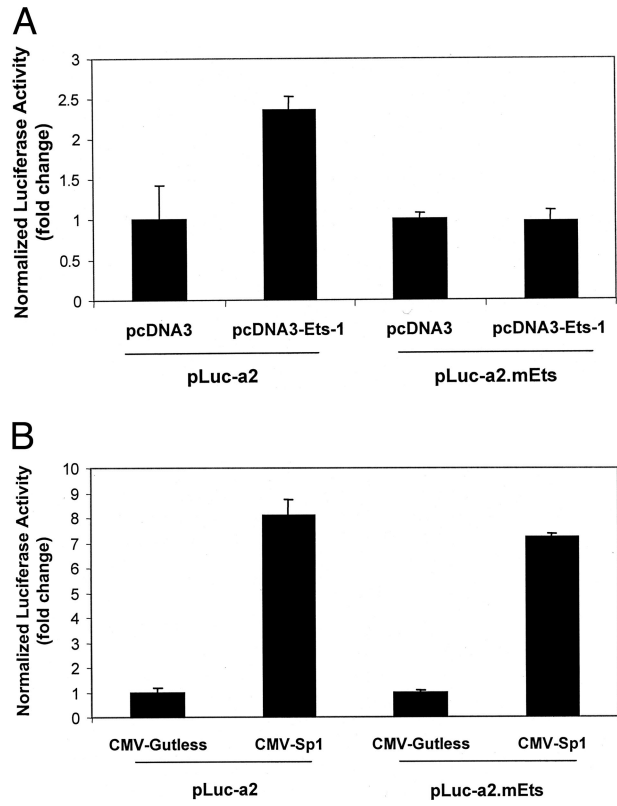


Figure 4. Ets-1 induction of PDGF-Rα is abolished on mutation of the Ets (-45⁺TTCC⁻⁴²) motif whereas Sp1 induction is unaffected. **A:** Cells were transfected with 10 μg of pLuc-a2 or pLuc-a2.mEts and 1 μg of pcDNA3-Ets-1 or pcDNA3. *Firefly* luciferase was determined in cell lysates after 24 hours. The y axis indicates the ratio of *Firefly* luciferase activity over *Renilla* to normalize for transfection efficiency. **B:** Sp1 does not require the Ets site to up-regulate PDGF-Rα transcription. The cells were transfected with 10 μg of pLuc-a2 or pLuc-a2.mEts and 3 μg of CMV-Sp1 or CMV-gutless. *Firefly* luciferase was determined in cell lysates after 24 hours. The y axis represents the ratio of *Firefly* luciferase activity over the *Renilla* to normalize for transfection efficiency. The result is representative of at least two independent observations.

control CMV-gutless. Luciferase activity, determined 24 hours after transfection, demonstrated that integrity of the Ets binding motif (-45/-42 bp) is not essential for Sp1-stimulated PDGF-Rα transcription (Figure 4B).

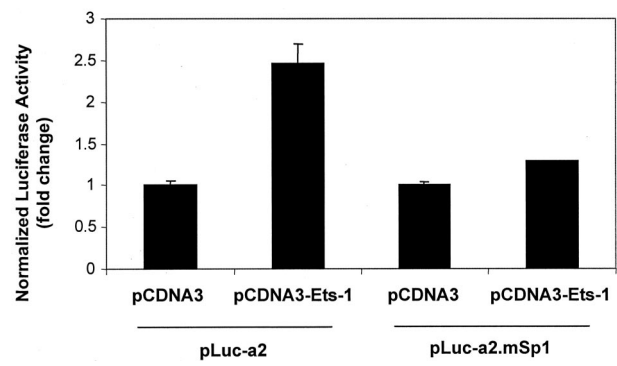


Figure 5. Ets-1 induction of PDGF-Rα is abolished by mutation of the proximal Sp1 (-61G₁₀⁻⁵²) element in PDGF-Rα promoter. This site (-61G₁₀⁻⁵²) mediates Sp1-inducible PDGF-Rα expression.²⁴ Cells were transfected with 10 μg of pLuc-a2 or pLuc-a2.mSp1 and 1 μg of pcDNA3-Ets-1 or pcDNA3. *Firefly* luciferase was determined in cell lysates after 24 hours. The y axis represents the ratio of *Firefly* luciferase activity over *Renilla* to normalize for transfection efficiency. The result is representative of at least two independent observations.

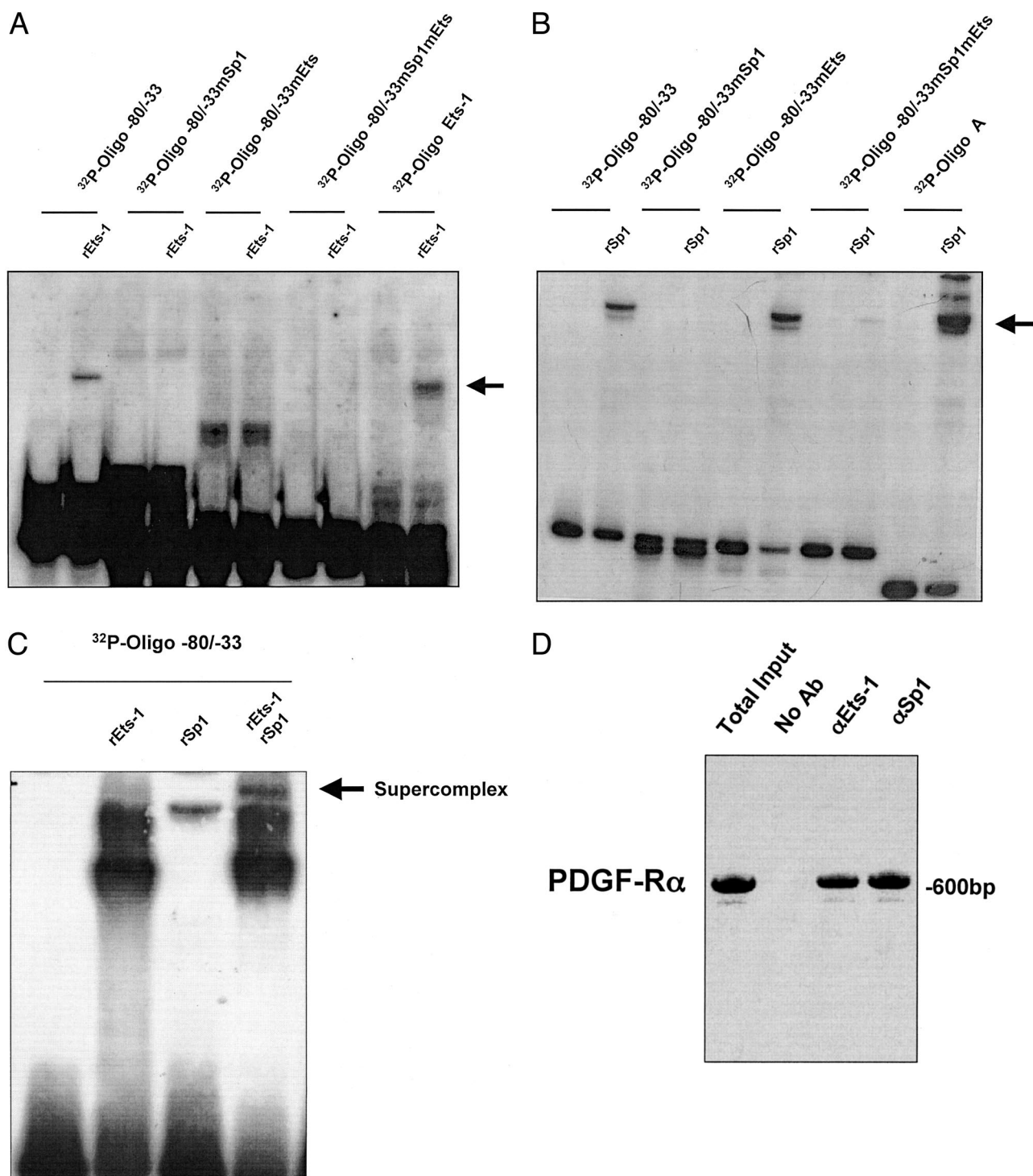


Figure 6. Interaction of Ets-1 and Sp1 with -80/-33 region of the PDGF-R α promoter requires ⁻⁴⁵TTCC⁻⁴² and ⁻⁶¹G₁₀⁻⁵². Oligonucleotides spanning the -80/-33 region of the PDGF-R α proximal promoter were incubated with indicated recombinant (r) protein. EMSA using rEts-1 (**A**), rSp1 (**B**), or rEts-1 and rSp1 (**C**). The **arrow** indicates the nucleoprotein complex. Wild-type ³²P-Oligo -80/-33, or ³²P-Oligo -80/-33mEts (where ⁻⁴⁵TTCC⁻⁴² was changed to ⁻⁴⁵TACA⁻⁴²), an Sp1 mutant, ³²P-Oligo -80/-33mSp1 (where ⁻⁶¹G₁₀⁻⁵² was changed to ⁻⁶¹T₁₀⁻⁵²) and a double mutant, ³²P-Oligo -80/-33mSp1mEts (where both sites were mutated). **D**: Chromatin immunoprecipitation analysis demonstrating amplification of the PDGF-R α promoter in the total input, without antibody pull-down (no Ab), or pulled down with Sp1 and Ets-1 antibodies. The data are representative of at least two independent determinations.

Ets-1 Induction of PDGF-R α Transcription Is Critically Dependent on an Intact Sp1-Response Element

When we addressed the question of Sp1/Ets-1 interplay in the opposite manner we were surprised by the outcome. Ets-1 failed to transactivate the PDGF-R α promoter if the Sp1 response element ($^{-61}G_{10}^{-52}$) was disrupted ($^{-61}G_{10}^{-52}$ changed to $^{-61}T_{10}^{-52}$ in construct pLuc-a2.mSp1).²⁴ PDGF-R α transcription in this experiment was assessed in cells co-transfected with pLuc-a2 or pLuc-a2.mSp1 and pcDNA3-Ets-1 or pcDNA3. Luciferase activity 24 hours after transfection revealed Ets-1 no longer altered PDGF-R α promoter-dependent transcription (Figure 5).

Interaction of Ets-1 with the Oligo -80/-33 Is Critically Dependent on Both Proximal Sp1 and Ets Sites

To provide a mechanistic explanation for these observations we performed EMSA studies using Ets-1 and Sp1 and this proximal region of the PDGF-R α promoter (Figure 3C). Recombinant Ets-1 (rEts-1) formed a nucleoprotein complex with ³²P-Oligo -80/-33 (Figure 6A, lane 2). rEts-1 no longer bound the promoter fragment when ³²P-Oligo -80/-33 bore a mutation (mEts) in the Ets motif (Figure 6A, lane 6). rEts-1 also failed to bind the oligonucleotide when ³²P-Oligo -80/-33 carried a mutation (mSp1) in the Sp1 motif (Figure 6A, lane 4). These findings support our transient transfection studies in which Ets-1 failed to up-regulate PDGF-R α transcription when either the Ets or Sp1 elements were mutated (Figures 4A and 5). Mutation of both elements (mSp1mEts) in ³²P-Oligo -80/-33 failed to support formation of a rEts-1 nucleoprotein complex (Figure 6A, lane 8). The binding of rEts-1 protein to a previously used Ets-binding oligonucleotide (³²P-Oligo Ets-1)²⁹ served as a positive control (Figure 6A, lane 10) in this analysis.

Sp1 Requires the Sp1 Element but Not the Ets Site to Bind the PDGF-R α Promoter

Our PDGF-R α promoter-dependent reporter studies revealed that the Ets binding motif (-45/-42 bp) is not required for Sp1-inducible PDGF-R α transcription (Figure 4B). This, together with binding analyses using rEts-1 (Figure 6A), led us to investigate the capacity of recombinant Sp1 (rSp1) to bind Oligo -80/-33. ³²P-Oligo A,²⁹ which bears multiple consensus Sp1 recognition elements served as a positive control for rSp1 binding (Figure 6B, lane 10). rSp1 formed a nucleoprotein complex with ³²P-Oligo -80/-33 (Figure 6A, lane 2). ³²P-Oligo -80/-33 bearing a mutation in the Sp1 element (mSp1), as expected, did not bind rSp1 (Figure 6B, lane 4). When the Ets site in ³²P-Oligo -80/-33 was mutated (mEts), rSp1 binding was unaffected (Figure 6B, lane 6) indicating therefore, that Sp1 does not require the Ets site to bind the PDGF-R α promoter. In

contrast, both sites are required for Ets-1 binding and PDGF-R α transactivation (Figures 4A, 5, and 6A). rSp1 failed to bind ³²P-Oligo -80/-33 when both the Ets and Sp1 sites were disrupted (Figure 6B, lane 8). A supercomplex formed when Ets-1 and Sp1 were incubated simultaneously with ³²P-Oligo -80/-33 bearing both respective sites (Figure 6C). Chromatin immunoprecipitation analysis shows that the authentic PDGF-R α promoter is indeed occupied by Ets-1 and Sp1 (Figure 6D). Ets-1/Sp1 binding and transactivation of the PDGF-R α promoter are summarized in tabular form in Table 1. Cooperative interactions between Ets-1 and Sp1 mediate the expression of genes such as Fas ligand,¹⁸ CD53,³⁷ SERA3,³⁸ and fgl2,³⁹ and more recently PDGF-A.⁴⁰

Hydrogen Peroxide Stimulates Ets-1 and Activates PDGF-R α Expression via the $^{-45}TTCC^{-42}$ and $^{-61}G_{10}^{-52}$ Elements in the Proximal Promoter

The preceding studies demonstrate that Ets-1 binds to and activates the PDGF-R α promoter in an Sp1-dependent manner. These findings alone, however, do not establish whether the Ets site plays an important role in the context of a pathophysiologically relevant agonist. Reactive oxygen species are produced in human atheroma⁴¹ and in the balloon-injured artery wall.⁴² Western immunoblot analysis revealed that low concentrations of H₂O₂ (10 nmol/L) stimulate Ets-1 expression in SMCs within 4 hours (Figure 7A) without affecting Sp1 levels (Figure 7A), consistent with previously demonstrated H₂O₂ up-regulation of Ets-1 but not Sp1, albeit in endothelial cells.⁴³ PDGF-R α protein levels increased in SMCs exposed to the same concentration of H₂O₂ after 4 hours (Figure 7B).

EMSA performed using nuclear extracts of SMCs incubated with H₂O₂ for 4 hours revealed a single peroxide-inducible nucleoprotein complex that was eliminated in supershift analysis using antibodies to Ets-1 and Sp1 (Figure 7C). The Ets-1 elimination was achieved under conditions different from those previously reported.²⁴ This complex was unaffected by antibodies to YY1, GATA6, or several other Ets family members including Ets-2 (Figure 7C and data not shown). Transient transfection analysis revealed that

Table 1. PDGF-R α Promoter Transactivation and Binding Analyses to Ets-1 and Sp1

WT and mutant EMSA probes or reporter constructs	Ets-1 activation	Sp1 activation	Ets-1 binding	Sp1 binding
WT	+	+	+	+
mEts	-	+	-	+
mSp1	-	-	-	-

The table summarizes the results of transient transfection and binding studies with wild-type (WT) and mutant PDGF-R α promoter oligonucleotides (Oligo -33/-80) and reporter constructs (pLuc-a2). PDGF-R α promoter interactions and transactivation by Ets-1 or Sp1 are indicated by + or -.

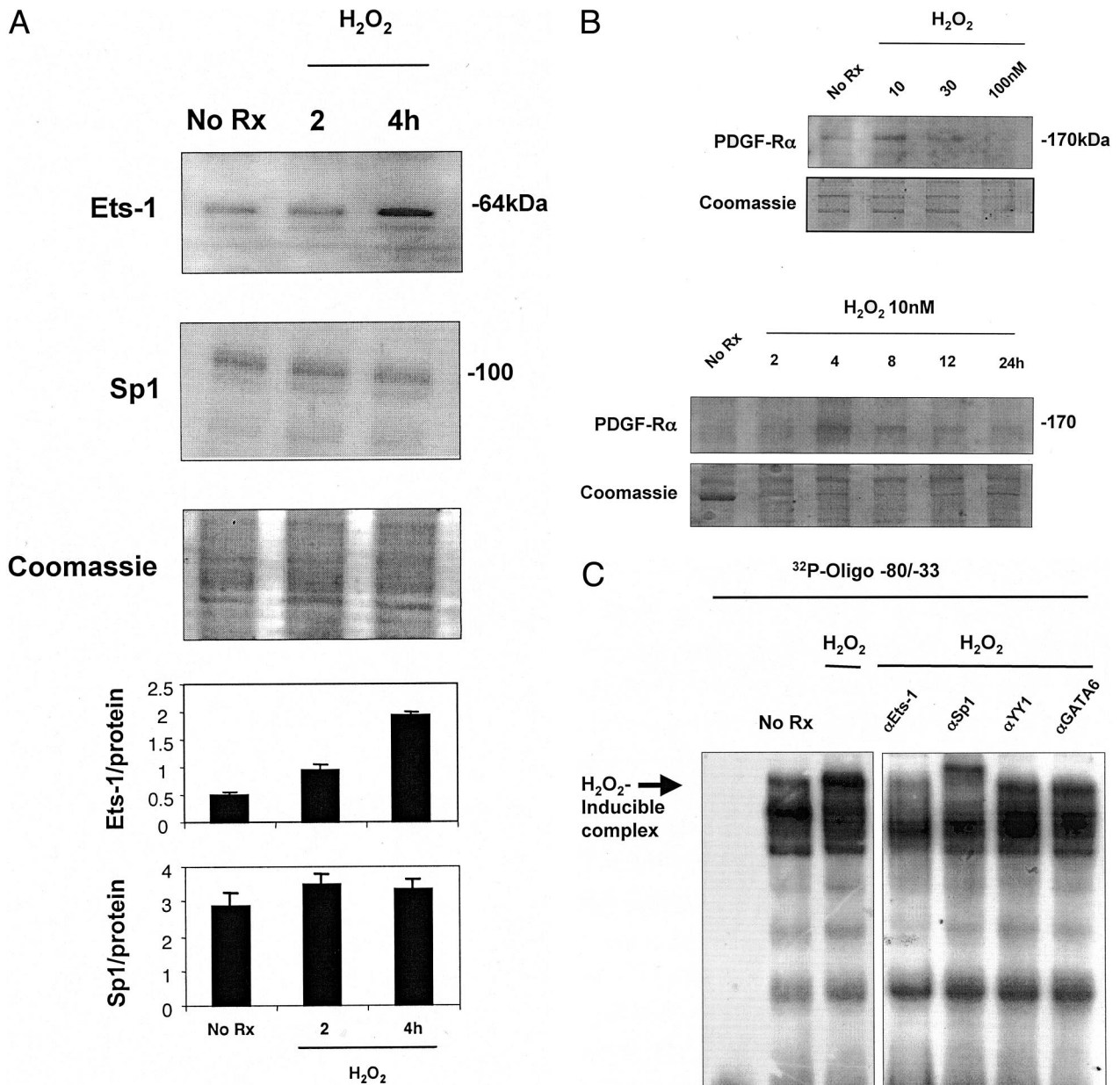
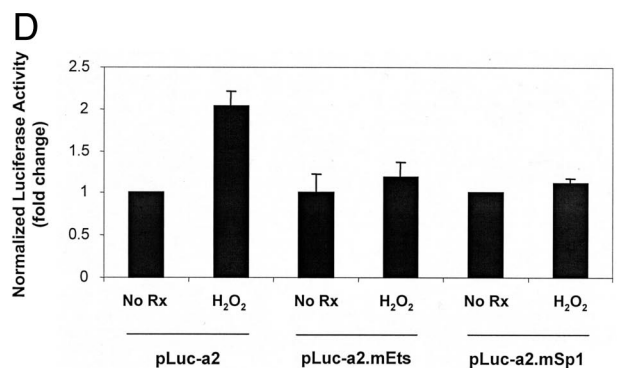


Figure 7. H₂O₂ induces Ets-1 protein expression and PDGF-R α transcription via the ⁻⁴⁵TTCC⁻⁴² motif in the proximal promoter. **A:** Growth quiescent cells were incubated with 10 nmol/L H₂O₂ for 2 and 4 hours. Ets-1 and Sp1 protein levels were assessed by Western immunoblot analysis (20 μ g total protein for Ets-1 analysis and 10 μ g for Sp1). Ets-1 protein levels increased compared to basal levels with addition of 10 nmol/L H₂O₂ compared to untreated cells. Sp1 protein levels were unchanged compared to basal levels with addition of 10 nmol/L H₂O₂ compared to untreated cells. Coomassie-stained gel demonstrates unbiased loading. Densitometric analysis was performed on bands of interest and relative to Coomassie-stained protein. **B:** SMCs were incubated with various concentrations of H₂O₂ for different times and PDGF-R α protein levels assessed by Western immunoblot analysis (10 μ g of total protein). Coomassie-stained gels demonstrate unbiased loading. **C:** EMSA using nuclear extracts of SMCs that had been incubated with 10 nmol/L H₂O₂ for 4 hours. The H₂O₂-inducible nucleoprotein complex was eliminated by antibodies to Ets-1 and Sp1, but not YY1 or GATA6. **D:** SMCs were transfected with 10 μ g of either pLuc-a2, pLuc-a2.mEts, or pLuc-a2.mSp1 for 24 hours followed by the addition of 10 nmol/L H₂O₂ for 24 hours. *Firefly* luciferase was determined in cell lysates after 24 hours. The y-axis represents the fold change in PDGF-R α promoter activity. The result is representative of at least two independent observations.



H₂O₂ activated PDGF-R α promoter-dependent expression within 24 hours (Figure 7D). Mutation of the ⁻⁴⁵TTCC⁻⁴² element in construct pLuc-a2.mEts completely blocked H₂O₂ activation of the PDGF-R α promoter (Figure 7D). Moreover, H₂O₂ activation of PDGF-R α transcription was absolutely dependent on element ⁻⁶¹G₁₀⁻⁵² (Figure 7D). This represents the first demonstration of the capacity of H₂O₂ to modulate PDGF-R α transcription in any cell type.

Discussion

The capacity of H₂O₂ to induce PDGF-R α expression in vascular SMCs has implications to PDGF-R α -dependent hyperplasia in the injured artery wall. That synthetic WKY12-22 SMCs were used in the present study may account for the nanomolar concentrations of H₂O₂ that stimulated Ets-1 and PDGF-R α expression. Ets-1, PDGF-R α , and reactive oxygen species are found in arterial lesions (present study).^{12,23,34,44} Superoxide dismutase, glutathione peroxidase, and catalase are intensely expressed by migrating SMCs in human atherosclerotic lesions.⁴¹ There is evidence that reactive oxygen species can influence SMC accumulation in the artery wall as a consequence of increased migration and proliferation.^{41,45} Accordingly, a reactive oxygen species/Ets-1/PDGF-R α axis may influence the formation of the SMC-rich neointima, just as Ets-1 serves as a transcriptional mediator of peroxide-inducible Ets-1-dependent changes in the endothelial cell phenotype.⁴³ This is particularly relevant to vascular complications of diabetes wherein reactive oxygen species are generated under hyperglycemic conditions.⁴⁵

The functional interplay of transcription factors like Ets-1 in the promoter regions of key genes exerts a profound phenotypic influence in the vessel wall. This study provides greater insight into the transcriptional regulation of PDGF-R α expression by demonstrating Ets-1 and Sp1 co-occupancy and the critical requirement of Sp1 in Ets-1-inducible PDGF-R α transcription. That Ets-1 and Sp1 also modulate transcription of one of the ligands of PDGF-R α in vascular SMCs,⁴⁰ which is also inducibly expressed in vascular lesions,^{34,46} suggests a more general theme in the transcriptional control of the PDGF ligand-receptor system.

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