Platelet-derived growth factor (PDGF) is a potent mitogen and chemotactant for vascular smooth muscle cells (SMCs) whose biological activity is mediated via its high affinity interaction with specific cell surface receptors. The molecular mechanisms governing the expression of PDGF receptor-α (PDGFR-α) are poorly understood. Here we demonstrate that PDGFR-α protein and transcriptional regulation in SMCs is under the positive regulatory influence of the zinc finger nuclear protein, Sp1. Electrophoretic mobility shift, competition, and supershift analysis revealed the existence of an atypical G-rich Sp1-binding element located in the PDGFR-α promoter −61 to −52 bp upstream of the transcriptional start site. Mutation of this sequence ablated endogenous Sp1 binding and activation of the PDGFR-α promoter. PDGF transactivation, mRNA, and protein expression were repressed in SMCs exposed to fibroblast growth factor-2 (FGF-2). This inhibition was rescued by the blockade of extracellular signal-regulated kinase-1/2 (ERK1/2). FGF-2 repression of PDGFR-α transcription was abrogated upon mutation of this Sp1-response element. FGF-2 stimulated Sp1 phosphorylation in an ERK1/2- but not p38-dependent manner, the growth factor enhancing Sp1 interaction with the PDGFR-α promoter. Mutation of residues Thr\textsuperscript{453} and Thr\textsuperscript{739} in Sp1 (amino acids phosphorylated by ERK) blocked FGF-2 repression of PDGFR-α transcription, and phosphorylates ERK1/2-dependent Sp1 phosphorylation, thereby repressing PDGFR-α transcription via the −61/−52 element in the PDGFR-α promoter. Phosphorylation triggered by FGF-2 switches Sp1 from an activator to a repressor of PDGFR-α transcription, a finding previously unreported in any Sp1-dependent gene.

Platelet-derived growth factor (PDGF)\textsuperscript{1} is a family of potent mitogenic and chemotactic proteins (1) whose biological activity is mediated via high affinity interactions with specific protein-tyrosine kinase cell surface receptors, PDGFR-α and -β. PDGFs are secreted as disulfide-linked bivalent ligands that induce receptor dimerization, trans-phosphorylation, and the activation of signal transduction cascades (2). PDGFR-α and -β have different ligand binding capabilities for their ligands. PDGFR-α binds PDGF-A, PDGF-B, and PDGF-C, whereas PDGFR-β binds PDGF-B and PDGF-D (3). Assembly of the receptor dimer combinations (PDGFR-αα, αβ, ββ) is dependent on the isoform of the dimeric ligand (3). Consequently, PDGF-αA causes αα receptor complex formation, PDGF-AB induces αα or αβ, and PDGF-BB stimulates αa, αβ, or ββ (4–8). PDGF and fibroblast growth factor-2 (FGF-2) have long been recognized to play a regulatory role in vascular pathologies such as atherosclerosis and postangioplasty restenosis involving smooth muscle cell (SMC) growth. Both these growth factors and their receptors are expressed in SMCs. SMC replication and matrix deposition lead to lesion formation and thickening of the artery wall (9). Conversely, SMC apoptosis can weaken the atherosclerotic plaque and lead to its rupture (10, 11). Interestingly, as the plaque develops from early to late stage, FGF-2 levels decrease (12), whereas PDGFR-α levels increase (13). Beyond this, however, an interrelationship between FGF-2 and PDGFR-α has not been established.

The molecular mechanisms governing the transcription of PDGFR-α are poorly understood. The PDGFR-α gene lacks a typical TATA box but contains GATA motifs and a CCAAT box. It also contains potential sites for AP-1, AP-2, Oct-1, and Oct-2 transcription factors (14). CCAAT/enhancer-binding proteins C/EBP-α and C/EBP-β bind to the PDGFR-α promoter and modulate its activity both positively and negatively (15, 16). For example, IL-1β can induce PDGFR-α mRNA expression via activation of C/EBP-α (15, 16). PDGFR-α is under the positive transcriptional influence of NF-κB (17). The proto-oncogene product Cbl negatively regulates PDGFR-α by enhancing ubiquitination and degradation (18). Deletion of PDGFR-α in mice produces severe cardiovascular abnormalities among many other defects, leading to embryonic death at day 8–16 (19).

Our previous studies have demonstrated that the zinc finger

\textsuperscript{1} The abbreviations used are: PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; FGF-2, fibroblast growth factor-2; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; SMC, smooth muscle cell; C/EBP, CCAAT/enhancer-binding proteins; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; CMV, cytomegalovirus; Luc, luciferase.

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transcription factor Sp1 regulates transcription of the PDGF-A and PDGF-B genes in SMCs and in other vascular cell types (20–22). Here we show that PDGFR-α expression in SMCs is under the positive regulatory influence of Sp1, via an atypical recognition element located 61–52 bp upstream of the transcriptional start site. This site mediates FGF-2 repression of PDGFR-α expression in an ERK1/2-dependent manner. Phosphorylation of Sp1 upon exposure to FGF-2 switches Sp1 from an activator to a repressor of PDGFR-α transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—WKY12-22 rat aortic SMCs were cultured in Waymouth’s MB752/1 medium (Invitrogen) supplemented with 10% fetal calf serum, 30 μg/ml L-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C and 5% CO₂. The cells were passaged every 3–4 days in 75-cm² flasks.

**Plasmid Constructs**—The plasmid pLuc-a2 (23), containing the promoter region of platelet-derived growth factor receptor-α, was kindly donated by Dr Yutaka Kitami from Ehime University School of Medicine, Ehime, Japan. pLuc-a2.Sp1m3 and CMV-Sp1.mThr453/mThr739 were produced using the QuikChange® XL site-directed mutagenesis kit (Stratagene) in accordance with the manufacturer’s instructions. Thr⁴⁵³ was converted to Ala⁴⁵³, and Thr⁷³⁹ was changed to Ala⁷³⁹. The primers are as follows: Sp1m3 forward, 5’-TTATAAGCGCGTTTCTGTTACTCCTATC-3’; Sp1m3 reverse, 5’-AGCTGGTCAAGAATGAGAAAAAATGTCCTCTG-3’. The sequence was obtained from EMBL RN13172 Rattus norvegicus.

**Transient Transfections**—For reporter gene analysis, WKY12-22 cells were transfected with 10 μg of pLuc-a2, pLuc-a2.Sp1m3, or CMV-Sp1.mThr453/mThr739 using Lipofectamine 2000 (Invitrogen). Firefly luciferase activity was normalized to Renilla. Error bars represent S.E. The data are representative of two or more independent determinations.

**FIG. 1.** Sp1 stimulates PDGFR-α protein and promoter-dependent expression. A, Western blot analysis for PDGFR-α in SMCs 24 h after transfection with CMV-Sp1 or pcDNA3. The non-specific (ns) band of lower molecular mass demonstrates unbiased loading. As shown in B, CMV-Sp1 increases PDGFR-α promoter activity in a dose-dependent manner. The backbone control, pcDNA3, has no effect. Firefly luciferase activity was normalized to Renilla. Error bars represent S.E. The data are representative of two or more independent determinations.

**FIG. 2.** Nucleotide sequence of the PDGFR-α proximal promoter. The transcriptional (transc) start site (ATG) (23) is indicated. The novel Sp1-binding element that is the subject of this study (−61/−52) is indicated. Consensus elements for Sp1 do not appear in this region of the promoter. The C/EBP element and putative site for AP-1 are indicated. The sequence was obtained from EMBL RN13172 Rattus norvegicus.

**FIG. 3.** Atypical Sp1-binding element in the PDGFR-α promoter interacts with endogenous Sp1. This figure shows an electrophoretic mobility shift assay using 32P-labeled oligonucleotide spanning the −80/−33 region (32P-Mutant PDGFR-α Oligo −80/−33) of the PDGFR-α promoter or the 32P-labeled mutant (G10T10) oligonucleotide and SMC nuclear extracts. Supershift analysis with Sp1 antibodies abrogates complexes C₁ and C₂ and decreases intensity of C₃ and C₄, whereas Ets-1 antibodies have no effect. UL, unlabeled oligonucleotide.
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Sp1.mThr283/Thr289. Cells were also transfected with 0.5 μg of pRL-TK to correct for transfection efficiency. Firefly luciferase activity was normalized to Renilla. Transient transfections were performed using FuGENE6 (Roche Applied Science) where 3 μl of FuGENE6/μg of transfected DNA was added, and the transfection mix was made up to 1 ml with serum-free medium. After incubation at 22 °C for 10 min, the DNA/FuGENE6 mixture was added to cells containing 10 ml of complete medium. Twenty-four h after transfection, cell lysates were prepared for assessment of luciferase activity where the dual luciferase assay reporter system (Promega DLR™) was performed on a manual luminometer (model TD-20/20 Turner Designs, Quantum Science).

RNA Expression Analysis—Cells were washed two times with ice-cold PBS and harvested with 4.5 ml of TRIzol reagent (Invitrogen) in accordance with the manufacturer’s instructions. cDNA synthesis from total RNA was performed using Superscript II reverse transcriptase (Promega, WI) in accordance with the manufacturer’s instructions. Thermal cycling conditions as follows: PDGFR-α 94 °C for 10 s, 62 °C for 30 s, 68 °C for 1.5 min for 40 cycles; GAPDH 94 °C for 30 s, 60 °C for 30 s, 68 °C for 2 min for 18, 24, and 28 cycles. The PDGFR-α primers are: 5′-AGATAGCTTCATGAGCCGAC-3′ (forward) and 5′-GGAAAGGCTTCTGG-3′ (reverse); the GAPDH primers are: 5′-ACCACAGTCAATGTCGTCTGG-3′ (forward) and 5′-TCCACACCTGTTGGTCTGTA-3′ (reverse).

Western Immunoblot Analysis—Transfected cells were washed two times with ice-cold PBS before being lysed on ice in 1× radioimmunoprecipitation analysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 10 μg/ml leupeptin, and 1% aprotinin). 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 dithiothreitol (DTT)) and boiled for 5 min with 0.5 μl iodoacetamide added before loading onto an 8% SDS-polyacrylamide gel. Gels were blotted onto Immobilon-P (polyvinylidene difluoride) membranes (Millipore, Bedford, MA) and blocked overnight at 4 °C in 5% skim milk powder, 0.05% Tween 20, and PBS. Sp1 was detected using rabbit polyclonal antibodies (Santa Cruz Biotechnology) and chemiluminescence detection (PerkinElmer Life Sciences) according to the manufacturer’s instructions.

Preparation of Nuclear Extracts—SMC monolayers were washed two times with ice-cold PBS, pH 7.4, and then scraped into 10 ml of cold PBS. The cells were pelleted by centrifugation at 250 × g for 10 min at 4 °C. Cells were lysed by the addition of ice-cold hypotonic solution (Buffer A) consisting of 10 mM HEPES, pH 8.0, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 200 mM sucrose, 0.5% Nonidet P-40, 0.5 mM PMSF, 1 μg/ml aprotinin. The suspension was recentrifuged, and the nuclei were lysed in an ice-cold solution (Buffer C) consisting of 20 mM HEPES, pH 8, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin. The nuclear fraction was combined with an equal volume of Buffer D (20 mM HEPES, pH 6.0, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin).

Electrophoretic Mobility Shift Assay—Binding reactions for gel shift assays were performed in 20 μl of 10 mM Tris-HCl, 50 mM MgCl2, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1 mM PMSF, 1 μg of salmon sperm DNA (Sigma), 32P-labeled oligonucleotide probe (150,000 cpm), and 3 μg of nuclear extract (determined by Pierce protein assay). The reaction was incubated for 35 min at 22 °C. In supershift studies, 2 μl of the appropriate affinity-purified anti-peptide polyclonal antibody (Santa Cruz Biotechnology) was incubated with the binding mix for 10 min before the addition of the probe. Bound complexes were separated from free probe by loading samples onto a 6% non-denaturing polyacrylamide gel and electrophoresing at 120 V for 2.5 h. The gels were vacuum-dried at 80 °C and subjected to autoradiography overnight at −80 °C.

RESULTS AND DISCUSSION

Sp1 Positively Regulates PDGFR-α Transcription and Protein Expression—Our previous investigations demonstrated a positive regulatory role for Sp1 in the transcriptional regulation of PDGF A- (22) and B-chain (20, 21) via cis-acting ele-

![Figure 4](Image 59x333 to 306x531)

**FIG. 4.** Mutation of -68G10 to -72 abolates Sp1-inducible PDGFR-α promoter activity. Transient transfection analysis in SMCs using pLuc-a2 or pLuc-a2.Sp1m3 together with CMV-Sp1 or pcDNA3 demonstrates that Sp1-inducible PDGFR-α expression is no longer observed in pLuc-a2.Sp1m3. Firefly luciferase activity was normalized to Renilla. Error bars represent S.E. The data are representative of two or more independent determinations.

![Figure 5](Image 64x84 to 558x258)

**FIG. 5.** FGF-2 inhibits PDGFR-α expression. As shown in A, FGF-2 inhibits PDGFR-α promoter-dependent activity. SMCs were transfected with pLuc-a2 and treated with the indicated concentration of FGF-2 for 24 h prior to the assessment of luciferase activity. As shown in B, FGF-2 inhibits PDGFR-α mRNA expression. Reverse-transcriptase-PCR of PDGFR-α using SMCs was performed following FGF-2 treatment. GAPDH expression demonstrates unbiased loading. Firefly luciferase activity was normalized to Renilla. Error bars represent S.E. The data are representative of two or more independent determinations.
ments in the proximal promoter regions of these genes. Unlike the PDGFR-A and -B promoters, however, consensus elements for Sp1 do not appear in the proximal PDGFR-α promoter. Whether Sp1 controls PDGFR-α expression has not been investigated in any cell type. We assessed levels of PDGFR-α protein in vascular SMCs 24 h after transfection with the CMV-based Sp1 expression vector, CMV-Sp1. Western immunoblot analysis revealed that Sp1 overexpression produced a discreet band of molecular mass 170 kDa (Fig. 1A), which was barely apparent in cells transfected with the backbone vector, pcDNA3 (Fig. 1A). To confirm these observations at the level of transcription, we co-transfected SMCs with CMV-Sp1 and pLuc-a2, a Firefly luciferase-based reporter construct driven by 1.3 kb of PDGFR-α promoter (23). The cells were also transfected with the Renilla luciferase-based construct to correct for transfection efficiency. Normalized luciferase activity 24 h after transfection revealed dose-dependent induction of PDGFR-α transcription by Sp1 (Fig. 1B).

An Atypical Sp1-binding Motif in the PDGFRα Promoter Serves as a Functional Sp1-response Element—The proximal region of the PDGFR-α promoter does not contain a consensus Sp1-binding motif (5’-GGCCGG-3’). However, a G-box comprising 10 consecutive guanines was present at position -61G10-52 relative to the transcriptional start site (Fig. 2). To determine whether this site could support an interaction with Sp1, we performed an electrophoretic mobility shift assay using total cell extracts of SMCs exposed to FGF-2 for 8 or 24 h is shown. Cells were incubated with PD98059 (10 μM) or SB202190 (500 nM) for 1 h prior to the addition of FGF-2. As shown in the right panel, the ERK1/2 inhibitor rescues the PDGFR-α promoter from repression by FGF-2. Luciferase activity was measured following 24 h of FGF-2/PD98059 treatment. Firefly luciferase activity was normalized to Renilla. Error bars represent S.E. The data are representative of two or more independent determinations.

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Fig. 6. FGF-2-repression of PDGFR-α is ERK1/2-dependent. As shown in the left panel, FGF-2 suppression of PDGFR-α is rescued following treatment with PD98059. Western immunoblot analysis using total cell extracts of SMCs exposed to FGF-2 for 8 or 24 h is shown. Cells were incubated with PD98059 (10 μM) or SB202190 (500 nM) for 1 h prior to the addition of FGF-2. As shown in the right panel, the ERK1/2 inhibitor rescues the PDGFR-α promoter from repression by FGF-2. Luciferase activity was measured following 24 h of FGF-2/PD98059 treatment. Firefly luciferase activity was normalized to Renilla. Error bars represent S.E. The data are representative of two or more independent determinations.

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To establish the functional significance of this atypical Sp1-binding element in the context of 1.3 kb of the PDGFR-α promoter, we performed co-transfection analysis with CMV-Sp1 or pcDNA3 in SMCs together with mutant pLuc-a2.Sp1m3 to determine whether this site could support an interaction with Sp1, we performed an electrophoretic mobility shift assay using total cell extracts of SMCs exposed to FGF-2 for 8 or 24 h is shown. Cells were incubated with PD98059 (10 μM) or SB202190 (500 nM) for 1 h prior to the addition of FGF-2. As shown in the right panel, the ERK1/2 inhibitor rescues the PDGFR-α promoter from repression by FGF-2. Luciferase activity was measured following 24 h of FGF-2/PD98059 treatment. Firefly luciferase activity was normalized to Renilla. Error bars represent S.E. The data are representative of two or more independent determinations.

Fig. 7. The -61G10-52 element in proximal PDGFR-α promoter is critical for FGF-2 repression of PDGFR-α transcription. Transient transfection analysis in SMCs using pLuc-a2 and pLuc-a2.Sp1m3 together with FGF-2 was performed. Firefly luciferase activity was normalized to Renilla. Error bars represent S.E. The data are representative of two or more independent determinations.

no effect (Fig. 3). Thus, endogenous Sp1 protein interacts with this atypical Sp1 element in the PDGFR-α promoter in a sequence-specific manner.

To establish the functional significance of this atypical Sp1-binding element in the context of 1.3 kb of the PDGFR-α promoter, we performed a co-transfection analysis into pLuc-a2 (pLuc-a2.Sp1m3). Co-transfection analysis with CMV-Sp1 or pcDNA3 in SMCs together with mutant pLuc-a2.Sp1m3 completely abrogated Sp1 induction of the PDGFR-α promoter (Fig. 4). These findings thus demonstrate the existence of a novel Sp1-response element in the proximal region of the PDGFR-α promoter.

FGF-2 Represses PDGFR-α Transcription, mRNA, and Protein Expression—PDGFR-α levels progressively decrease in the...
growing atheroma as levels of FGF-2 increase (12, 13). We hypothesized that FGF-2 may negatively influence PDGFR-H9251 expression. Whether a growth factor can repress the expression of the receptor of another has not been demonstrated previously. Luciferase activity in SMCs transfected with pLuc-a2 was reduced by FGF-2 in a dose-dependent manner within 24 h (Fig. 5A). Semiquantitative reverse-transcriptase-PCR analysis confirmed these data. FGF-2 repressed endogenous PDGFR-H9251 mRNA expression 24 h after exposure of the cells to the growth factor (Fig. 5B, left panel). Corresponding GAPDH transcript levels demonstrated unbiased sample loading (Fig. 5B, right panel). Western blot analysis further revealed FGF-2 suppression of PDGFR-H9251 protein expression, after 8 and 24 h of incubation with FGF-2 (Fig. 6, left panel). These findings indicate that FGF-2 inhibits PDGFR-H9251 gene expression at the level of mRNA and protein.

**FGF-2 Repression of PDGFR-H9251 Gene Expression Is Dependent on ERK1/2 but Not p38 MAP Kinase**

We and others have demonstrated that a major signaling pathway used by FGF-2 in SMCs is the extracellular signal-regulated kinase-1/2 (ERK1/2) cascade (24–27). We hypothesized that FGF-2 repression of PDGFR-H9251 involves the ERK1/2 pathway. Western blot analysis further revealed FGF-2 suppression of PDGFR-H9251 protein expression, after 8 and 24 h of incubation with FGF-2 (Fig. 6, left panel). Corresponding GAPDH transcript levels demonstrated unbiased sample loading (Fig. 5B, right panel). Western blot analysis further revealed FGF-2 repression of PDGFR-H9251 protein expression, after 8 and 24 h of incubation with FGF-2 (Fig. 6, left panel). These findings indicate that FGF-2 inhibits PDGFR-H9251 gene expression at the level of mRNA and protein.

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**FGF-2 Represses PDGFR-α Transcription via Sp1 Phosphorylation**

We and others have demonstrated that a major signaling pathway used by FGF-2 in SMCs is the extracellular signal-regulated kinase-1/2 (ERK1/2) cascade (24–27). We hypothesized that FGF-2 repression of PDGFR-α involves the ERK1/2 pathway. Western blot analysis revealed that FGF-2 repression of PDGFR-α protein expression was rescued by the MEK/ERK inhibitor PD98059 (10 μM) at 8 and 24 h (Fig. 6, left panel). In contrast, the p38 kinase inhibitor SB202190 (500 nM) failed to modulate FGF-2 downregulation of PDGFR-α expression (Fig. 6, left panel). Reversibility of FGF-2 inhibition by the ERK1/2 inhibitor, but not the p38 kinase inhibitor, was examined. The results indicate that FGF-2 repression of PDGFR-α gene expression is dependent on the ERK1/2 pathway but not on the p38 MAP kinase pathway.
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Fibroblast growth factor-2 (FGF-2) is a mitogenic factor for smooth muscle cells (SMCs) and can stimulate proliferation and migration. It also plays a role in the regulation of cell differentiation and the expression of specific genes. Transient transfection analysis in SMCs transfected with the CMV-Sp1 double mutant (CMV-Sp1.mThr453/mThr739) revealed that FGF-2 could not suppress the gastrin gene promoter activity, which was virtually abrogated by the mutation in the Sp1-binding site (Fig. 7). This novel Sp1-response element therefore mediates FGF-2 repression of the PDGFR-α promoter. The addition of FGF-2 stimulated Sp1 phosphorylation.

To determine whether FGF-2 stimulates Sp1 phosphorylation via ERK1/2, SMCs were treated with PD98059 prior to the addition of FGF-2. Densiometric analysis after Western blotting for Sp1 revealed that FGF-2 phosphorylation of Sp1 increases its molecular mass; the hyper- and hypo-phosphorylated forms can be readily resolved by polyacrylamide gel electrophoresis (30, 31). Western blot analysis for Sp1 using nuclear extracts from SMCs treated with FGF-2 for 8 and 24 h demonstrated increased levels of Sp1 phosphorylation (Fig. 8A). Incubation of extracts from FGF-2-treated cells with calf intestinal phosphatase (30, 31) was performed to determine the capacity of a growth factor to regulate the expression of the receptor of another, as has been observed for the insulin-like growth factor-I receptor with PDGF (33) and FGF-2 (34). It is thus tempting to speculate that the inverse relationship between the expression of FGF-2 and PDGFR-α in the complex milieu of a developing atheroma may be mediated by the phosphorylation status of Sp1 and this novel recognition element in the PDGFR-α promoter.

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