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Platelet-Derived Growth Factor-BB Inhibits Rat α1D-Adrenergic Receptor Gene Expression in Vascular Smooth Muscle Cells by Inducing AP-2-Like Protein Binding to α1D Proximal Promoter Region

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ABSTRACT

We have previously found that, in addition to mediating contraction of vascular smooth muscle, activation of α1D-adrenergic receptors (AR) induces smooth muscle cell (SMC) hypertrophy. Despite their importance, little is known about how α1D-AR expression is regulated. Recently, we demonstrated that platelet-derived growth factor (PDGF)-β receptor stimulation, but not various other growth factors, inhibits transcription of α1D-, but not α1A- or α1B-ARs, resulting in reduced norepinephrine-mediated SMC growth. To investigate this inhibitory mechanism, herein we cloned and characterized 1.6 kb of the 5′-flanking region of the rat α1D-AR gene. Reporter gene transfection assays in rat aorta and vena cava SMCs showed that this 5′-flanking region, which lacks a TATA-box, possesses strong promoter activity. Two transcription initiation sites and their flanking promotor regions were identified, wherein the proximal promotor mediated PDGF-BB inhibition of transcription. Gel mobility shift assays suggested that Sp1 binds constitutively at two consensus sites within the −399 base pair (bp)/−349-bp region of the proximal promotor. This constitutive binding was unaffected by PDGF-BB. In contrast, a flanking motif (−384 bp/−349 bp), possessing putative Sp1/activator protein-2 (AP-2) overlapping binding sites and located upstream of the proximal transcription initiation site, was required for PDGF-BB inhibition of α1D transcription. PDGF-BB increased AP-2 binding to the distal AP-2 site in this region in the context of SMCs. Furthermore, overexpression of AP-2 protein, by transgene transfection, dose-dependently inhibited α1D-AR activity driven by this motif. Thus, PDGF-BB may increase AP-2 binding within the proximal promoter to cause down-regulation of α1D-AR expression in SMCs when PDGF is elevated, such as in the postnatal growing vascular wall and in vascular hypertrophic diseases.

Three α1-adrenergic receptor (AR) subtypes have been cloned, characterized, and designated as α1A-AR, α1B-AR, and α1D-AR. All three subtypes are expressed by vascular smooth muscle cells (SMCs), with heterogeneity evident among vessel types supplying different tissues within the same and different species. Among the few vessels examined, the α1D-AR is strongly expressed in rat aorta (Ping and Faber, 1993; Eckhart et al., 1996) and mediates α1-AR constriction of aorta (Guarino et al., 1996) and resistance vessels of rat skeletal muscle (Leech and Faber, 1996). α1-ARs also may mediate sympathetic control of SMC growth in normal vessel maturation and in certain vascular diseases. Augmented SMC growth is central to the pathogenesis of atherosclerosis, neointimal growth after angioplasty and coronary artery bypass, and hypertensive wall hypertrophy. Denervation studies have suggested a direct role for adrenergic nerves in normal thickening of the SMC layer in growing vessels (Lee et al., 1987), as well as in excessive wall hypertrophy of arteries in animal models of human essential hypertension (Head, 1991). Administration of α1-AR agonists and antagonists have suggested that α1-AR stimulation accelerates the development of and worsens the severity of atherosclerosis (Kukreja et al., 1981). Finally, α1-AR blockade attenuates SMC growth and neointimal lesion development that cause restenosis after balloon angioplasty (Fingerle et al., 1991; Vashisht et al., 1992). We have demonstrated that α1-AR stimulation induces hypertrophy of cultured SMCs and increases protein synthesis of the media of intact aorta (Chen et al., 1995). At least in cultured SMCs, this hypertrophy is mediated by the α1D-AR, with subsequent coupling to the mitogen-activated protein kinase kinase-dependent pathway (Xin et al., 1997),

This study was supported by National Institutes of Health Grant HL52610. The GenBank accession number for the α1D-adrenergic receptor promotor sequence reported herein is AF071014. Sequence scanning revealed no significant relatedness to other sequences except the α1D gene of other species.

ABBREVIATIONS: AR, adrenergic receptor; SMC, smooth muscle cell; PDGF, platelet-derived growth factor; PKC, protein kinase C; NE, norepinephrine; SSC, standard saline citrate; kb, kilobase; bp, base pair; RPA, RNase protection assay; PCR, polymerase chain reaction; SV40, simian virus 40; GMSA, gel mobility shift assay; NPE, nuclear protein extract; HIF, hypoxia-inducible factor-1.

suggesting a direct contribution of α1D-AR stimulation to hypertrophic vascular diseases.

Despite the importance of the α1D-AR, very little is known about regulation of its expression. Compared with adult rats, α1D-AR expression appears to be suppressed in the growing aorta of immature rats (Gurdal et al., 1995; Ibara et al., 1997) at a time when various growth factors, notably platelet-derived growth factor (PDGF)-B, are strongly up-regulated (Majesky et al., 1990; Rafty and Khachigian, 1998). Recently, we demonstrated in cultured rat aorta SMCs and intact ex vivo aorta that activation of the PDGF-β receptor, but not PDGF-α receptor or several other peptide and G protein-coupled growth agonists, sharply reduces α1D- (but not α1A- or α1B-) AR transcription and receptor density. This is dependent on a protein kinase C (PKC) mechanism, and results in attenuation of norepinephrine (NE)-induced SMC growth (Xin et al., 1999). PDGF-BB-dependent α1D-AR down-regulation may provide a negative feedback mechanism which, if compromised, could result in combined PDGF and noradrenergic growth stimulation that could worsen progression of vascular hypertrophic disease. Thus, the purpose of this study was to determine how α1D-AR gene transcription is repressed by PDGF-BB.

Materials and Methods

Cloning and Sequencing of 5′-Flanking Region of Rat α1D-AR Gene. A rat liver genomic library, kindly provided by Dr. E. M. Wilson (University of North Carolina), was expressed in LE392 host cells and screened with a [^{32}P]dCTP-labeled *Bam*HI/*Sac*I fragment derived from the rat α1D-AR cDNA (Lomasney et al., 1991). Nitrocellulose filters were hybridized overnight at 42°C in 50% formamide, 6× standard saline citrate (SSC; 1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt's solution, 100 μg/ml sheared salmon sperm DNA, and 1% SDS. Filters were washed three times with 1× SSC-0.1% SDS at 55°C until Geiger counter-detected radioactivity had decreased to an acceptable level. Filters were subjected to autoradiography on Kodak XAR-5 film with double intensifying screens overnight at −80°C. Phage plaques that hybridized positively were plaque-purified by secondary screening procedures.

Restriction endonuclease fragments of genomic DNA were identified by Southern hybridization with the same probe as used for filter screening. A 4.6-kilobase (kb) fragment was subcloned into pBluescript II SK$^+$ vector at *Eco*RI/*Hin*dIII sites. DNA sequencing was performed in both directions (Sequenase; US Biochemical Corp., Cleveland, OH) (Sambrook et al., 1989). Correct orientation of the fragment was confirmed according to overlapping sequence from the reported cDNA sequence. This fragment contained 1596 base pairs (bp) of the α1-AR 5′-flanking region.

Primer Extension Analysis. Primers were synthesized by Gibco BRL (Paisley, Scotalnd). P1 corresponded to −265 bp/−235 bp (5′-GCGGTGGCTGCGGAGTCACAAGGAAAGAAGG-3′); P2 corresponded to −173 bp/−141 bp (5′-GCTGCAGGGGAGCAGTGCTGCAG GTAGAGCAGG-3′). Both probes were end-labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase. 30 to 60 μg of rat SMC RNA (or yeast tRNA as the negative control) were annealed to 10^6 cpm of the primers and extended with 200 U of reverse transcriptase (Superscript II; Life Technologies, Inc., Grand Island, NY) (Sambrook et al., 1989). Products were analyzed on 7 M urea, 6% polyacrylamide gels, in parallel with sequencing reactions carried out on the full-length 5′-flanking region of the α1D clone with the same primers. Because of GC-rich regions in the α1D 5′-flanking sequence, some experiments included parallel sequencing carried out on M13 phage single-strand DNA with −40 primer (Life Sciences, St. Petersburg, FL).

RNase Protection Assay (RPA). Briefly, different sized riboprobes that covered either both putative transcription initiation start sites or the proximal transcription initiation site were generated by restriction enzymes or polymerase chain reaction (PCR) and subcloned into pBluescript SK$^+$ (Strategene, Inc., La Jolla, CA) and transcribed by T3 or T7 polymerase. 10^4 cpm of RNA probe for rat cyclophilin (Ambion, Inc., Austin, TX) transcribed by T7 RNA polymerase in the presence of [α-^{32}P]CTP was added to the same RPA to help identifying the product size. RPA was performed as described in Chen et al. (1995).

Reporter Gene Constructs and Expression Vectors. A 1782 bp (−1596 bp to +186 bp, relative to translation start site) *Eco*RI/*Hin*dIII fragment of the α1D-AR genomic DNA was blunted with Klenow fragment and then subcloned into the *Sma*I site of pGL3 basic vector (Promega Biotec, Madison, WI) with 5′ to 3′ orientation. The pGL3 basic vector lacks eucharyotic promotor or enhancer sequences; thus, luciferase expression above the basel level of vector alone depends on proper insertion of functional putative regulatory sequences upstream of the luciferase gene. Different deletion mutants were made by restriction endonuclease digestion or PCR. All constructs were purified by double CsCl gradient centrifugation and verified by sequencing. The human AP-2 expression plasmids SPRSV-AP2 (containing the full-length AP-2 cDNA coding region) and empty expression plasmid (Sp-72; Promega Biotech, with RSV-LTR and simian virus 40 (SV40) polyA) were generously provided by Dr. Trevor Williams (Williams and Tjian, 1991).

Rat Vascular SMC Culture and Transient Transfection Assay. Preparation of primary culture of adult rat thoracic aorta and vena cava SMCs was performed as described previously (Chen et al., 1995). Cells were cultured in 100-mm dishes in M-199 supplemented with 10% fetal bovine serum, 200 mg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. SMCs were passaged at 95% confluence with 0.10% trypsin-EDTA every 5 to 7 days and seeded at 3000 to 5000 cells/cm^2. For transfection assays, cells from passage four or five were split onto 6-well plates and cultured to confluence. Unless otherwise noted, SMCs were then transfected for 8 h with 5 μg/well of reporter plasmid and 2 μg/well of the pSV-β-galactosidase reference plasmid (Promega Biotech) with calcium phosphate precipitation (Eckhart et al., 1997). After 8 h of transfection, cells were rinsed with PBS and received fresh media. Before addition of PDGF-BB, media was replaced with serum-defined media (containing 50% Dulbecco's modified Eagle's medium/50% F-12 media supplemented with 5 mg/l transferrin, 35.2 mg/l ascorbic acid, 6 μg/ml selenium, 100 U/ml penicillin, and 100 μg/ml streptomycin). After 24 h, cells were harvested in reporter lysis buffer (Promega Biotec), and lysates were obtained by centrifugation. Luciferase and β-galactosidase activities were detected as described in Eckhart et al. (1997). All transfections were conducted in duplicate with at least two different preparations of each construct. Each experiment was repeated at least four times.

Preparation of Nuclear Protein Extract and Gel Mobility Shift Assays (GMSAs). Rat aorta SMCs were cultured to confluence and made quiescent by growing in serum-free defined media for 24 h. Nuclear protein extracts from PDGF-BB-treated or -untreated cells were collected as described in Yang et al. (1997). Sequences of oligonucleotides used for GMSAs were as follows: P2 (−384 bp/−349 bp), 5′-TCCGTCCCGCCCCCGCGCGGAGTCCCCAGCGCTGCA-3′; P1 (−399 bp/−384 bp), 5′-CTCGACCGCCCCTCCT-3′; mut1 (−384 bp/−349 bp), 5′-TCCGTCCatgatCCGCGCGG AGTCCCCAGCGCT-GCA-3′; and mut2 (−384 bp/−349 bp), 5′-TCCGTCCCGC-CCCCGCGCGGAtgataCAGCGCTGCA-3′. Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP. GMSAs were conducted as described previously (Yang et al., 1997). Briefly, 1 μl of nuclear protein extract (3 μg of protein) was mixed with labeled probe (1 ng; 10^5 cpm) in a 10-μl reaction mixture containing 4 μg of polydeoxyinosinic-deoxycytidylic acid, 10 mM HEPES, pH 7.9, 10% glycerol, 2% Ficoll-400, 40 mM NaCl, and 2 nM dithiothreitol. The reaction was carried out on ice for 30 min after addition of Sp1 and AP-2 consen-

sus (Life Technologies, Inc.) or other oligonucleotide competitors. Sp1 and AP-2 antibodies (sc-59-x and sc-184-x, Santa Cruz Biotechnologies, Santa Cruz, CA) were added 15 min before adding probe. DNA-protein complexes were separated on 5% nondenaturing polyacrylamide gels.

Results

Cloning and Sequencing of 5′-Flanking Region of Rat α1D gene. Four positive clones were obtained by initial and secondary screening. Sequence analysis of 1596 bp of 5′-flanking DNA that encompasses the translation initiation site (ATG, designated as +1 below) and extending through +186bp of the coding region revealed absence of a TATA-box, but presence of several GC-rich regions (GenBank accession no. AF071014). Comparison with sequences in the Transcription Factors DataBase (Genetics Computer Group, Madison, WI) identified a number of DNA consensus and putative sequences for trans-acting factors, notably, an Sp1/AP-2 cluster between −479 bp and −349 bp (Fig. 1A).

Deletion Analysis of α1D Promoter. To identify DNA regions and elements important for constitutive expression of the α1D gene, a series of truncated α1D-AR luciferase reporter plasmids (in the promoter/enhancer-less pGL3 basic vector) were constructed and examined (Fig. 1B). In rat aorta SMCs, the full-length 1.6-kb α1D fragment drove luciferase expression (β-galactosidase-corrected) 11-fold higher than basal activity of the pGL3 Basic vector; this activity was intermediate to the 22-fold level of luciferase expression driven by the pGL3 control vector, which contains the SV40 promotor and enhancer sequences (Fig. 1C). The 1.6-kb α1D fragment also drove expression 5-fold higher than pGL3 basic in rat thoracic vena cava SMCs, wherein pGL3-control drove expression at levels comparable to aorta SMCs (data not shown; $n = 4$). This lower level of α1D promotor activity in vena cava SMCs is consistent with the lower levels of α1D-AR mRNA present in both cultured vena cava SMCs and fresh vena cava compared with cultured SMCs and fresh medial layer from aorta (Eckhart et al., 1996). In the context of aorta SMCs, serial 5′ deletion from −1596 bp to −914 bp caused no significant change in promoter activity (Fig. 1C), suggesting motifs located in this region may have little regulatory effect on endogenous constitutive α1D promoter activity. Further deletion from −914 bp to −657 bp, and then to −597 bp, reduced α1D activity to 50 and 30%, respectively, of the full length 5′-flanking region, suggesting the presence of basal positive *cis*-acting elements in this region. Further deletion of sequence to −479 bp caused a recovery of activity, suggesting the presence of negative regulatory elements between −597 bp and −479 bp. Additional deletion to −185 bp eliminated all α1D promoter activity, suggesting that positive regulatory elements are between −479 bp and −185 bp. The activity pattern of these positive and negative regulatory regions was not changed by 3′ deletion from +186 bp to −349 bp to test for removal of the CCAAT-box at −200 bp. These results indicate that positive regulatory element(s) are located between −914 bp and −597 bp and between −479 bp and −185 bp. Importantly for subsequent analyses below, the −479-bp/−349-bp region fully drove luciferase transcription 12-fold above basal (pGL3-basic) at a level equivalent to the −1.6-kb full DNA flanking region (compare with −1596-bp/+186-bp and −1596-bp/−349-bp constructs). And the −399-bp/−349-bp region conferred activity 8-fold above basal activity. These results suggest the presence of promotor elements between −399 bp and −349 bp. The pattern of transcriptional activity of these chimeric constructs in aorta SMCs (Fig. 1C) was similar in the context of vena cava SMCs (data not shown; $n = 4$).

Transcription Initiation Site(s) of Rat α1D-AR Gene. Deletion analysis showed positive regulatory activity upstream of the reported (Lomasney et al., 1991; Perez et al.,

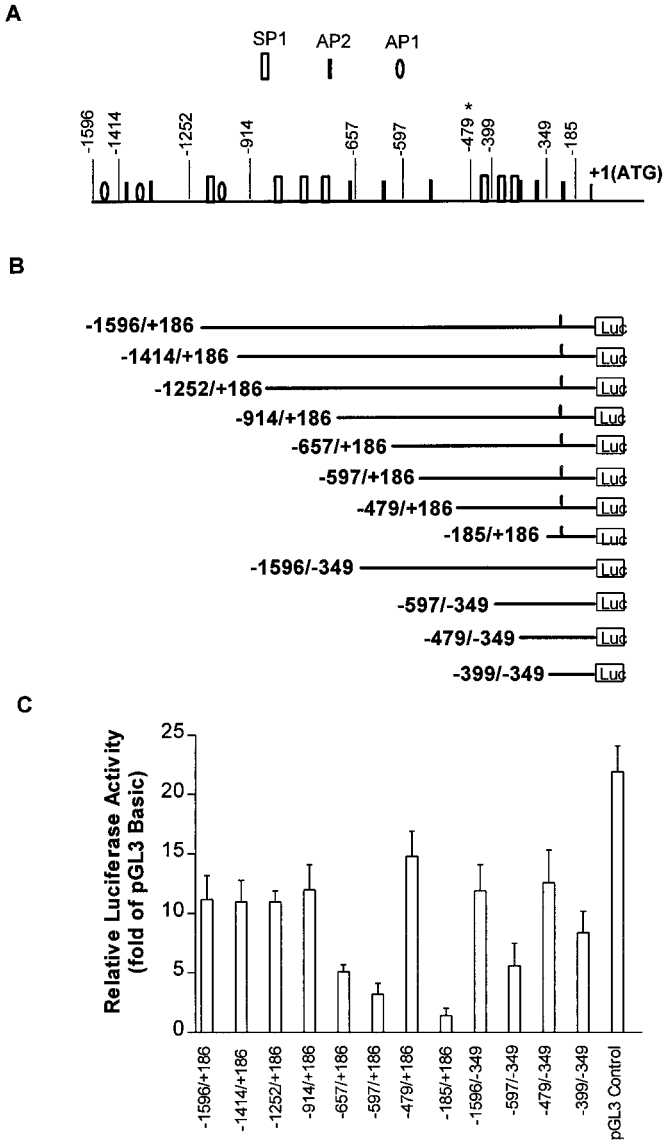

Fig. 1. Determination of rat α1D-AR gene promoter region in SMCs. A, deletion sites used to make deletion constructs and schematic location of putative Sp1, AP-2, and AP-1 transcription factor-binding sites (consensus sites for other transcription factors not shown). * denotes 5′-end of cDNA (Lomasney et al., 1991; Perez et al., 1991). B, schematic of α1D-AR-luciferase chimeric constructs. Sequential restriction enzyme digests of the 5′-flanking region of α1D-AR gene were subcloned into the promoter/enhancer-less luciferase expression vector pGL3 basic and were transiently transfected into aorta SMCs. C, luciferase activity in SMCs transfected with the chimeric constructs, normalized for β-galactosidase expression, and expressed as a fold of pGL3 basic vector activity. Transfection with the pGL3 control vector containing the SV40 promoter and enhancer was included in each experiment (last bar). The up-tick at far right in the (A) and (B) models represents the location of the translation ATG codon. Data are means ± S.E. from at least four independent transfections performed in duplicate.

1991) cDNA start site at −479 bp (Fig. 1C). However, as identified above, promoter activity also was driven strongly by the −479-bp/−349-bp construct (Fig. 1C), suggesting the existence of an additional promoter (s). We therefore performed primer extension assays with two probes corresponding to −265 bp/−235 bp (P1) and −173 bp/−141 bp (P2) to map the transcription initiation site(s). One extended product (244 bp) was detected by P1 (Fig. 2A), demonstrating transcription initiation at the −479 bp "C" that is identical with the reported cDNA start site identified by overlapping genomic DNA and rat cerebral cortex cDNA mapping (Lomasney et al., 1991; Perez et al., 1991). However, P2 indentified another extended product (147 bp), indicating use of a second start site at the −288-bp "C" (Fig. 2B) in SMCs. This proximal initiation site matched the deletion construct data (Fig. 1C) showing that the −479-bp/+186-bp fragment still possesses full activity.

Northern blot analysis was used in an attempt to confirm the primer extension results. mRNA isolated from rat cerebral cortex and cultured SMCs was analyzed with the same probe as used to clone the α1D promoter. However, we only detected a diffuse band with a size near 3 kb (data not shown), which is similar to that reported by Lomasney et al. (1991) from Northern analysis of rat cerebral cortex. The small size difference between the predicted products (190 bases) of the two transcription initiation sites (Fig. 2) probably prevented their resolution by Northern analysis. We then performed RPAs with an antisense riboprobe that extended from −497 bp to −87 bp (Fig. 2D; RP). A protected 200-bp band was identified that corresponded to the proximal transcription initiation site (Fig. 2C). However, only a faint band of the expected size (393 bp) for the distal start site at −479 bp was present in both RPAs (Fig. 2C) and also was seen using cerebral cortex RNA. This faint band could not be brought out by assay or probe modifications, presumably due to secondary structure in this GC-rich region. We also used different sized riboprobes that extend to both transcription initiation sites or only to the proximal initiation site in RPAs, and confirmed the 200-bp product. An RPA with P1 was not run because the −497/−87-bp riboprobe clearly showed use of the proximal start site. These results demonstrate that both proximal and distal transcription initiation sites exist in the α1D gene and are used by rat SMCs.

Identification of PDGF-BB-Responsive Motif in Rat α1D-AR Gene Promoter. To locate the *cis*-DNA element(s) responsible for PDGF-BB down-regulation of α1D transcription, SMCs were transfected with the −1.6-kb α1D 5′ sequence or the series of deletion mutant constructs (Fig. 1B),

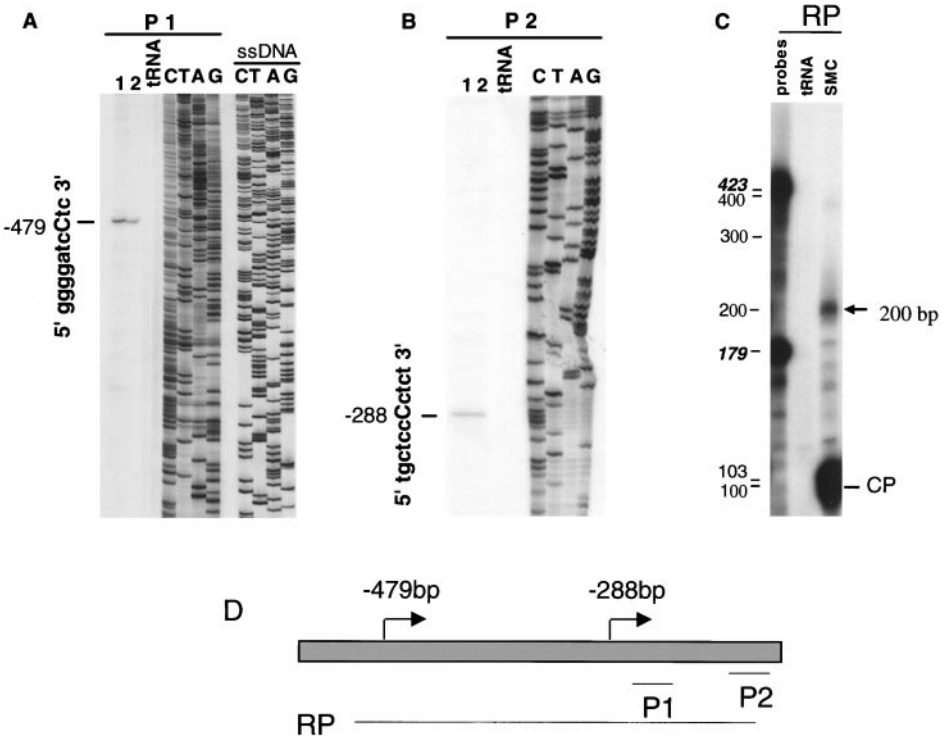

Fig. 2. Rat α1D-AR gene transcription initiation sites in SMCs. Primer extension assays were performed with antisense oligonucleotide primers P1 (A and D) and P2 (B and D). Sequences of P1 and P2 are described in *Materials and Methods*. Total RNA (40–60 μg) from rat aorta SMCs was hybridized with γ-^{32}P-labeled P1 or P2 primers at 42°C, and reactions were carried out as described in *Materials and Methods*. Lanes 1 and 2 in (A) and (B) represent two independent RNA samples. Yeast tRNA was included as negative controls. Reaction products were analyzed for size on 6% polyacrylamide sequencing gels. CTAG are sequencing ladders from parallel sequencing reactions performed on the full-length α1D 5′-flanking region construct with the same primer as used for the extension reaction (A, left ladder and B), and on M13 single-stranded DNA template with the −40 primer (A, right ladder). Because of high GC content in the −479-bp region, two types of sequencing ladders with different resolutions were used to confirm the identity of the distal initiation site at −479. The corresponding nucleotide transcription start sites (marked as capital letters) are shown on the left side in each reaction. The size of the P1 and P2 products were 244 and 147 bp, respectively. This sequence of the product in (A) also was confirmed against a smaller run of sequencing ladder. C, a representative RPA confirming the −288-base primer extension product. A fragment (from −497 bp to −87 bp), spanning both initiation sites that were identified by primer extensions, was subcloned into pBluescript SK$^+$ vector and in vitro transcribed into a 423-base (bold type) riboprobe (RP, in D). RNA markers (not shown in the figure) and a 179-base (bold type) cyclophilin riboprobe were included to help determine the product size, after primer extensions showed that no extension product around 103 bp was present. A 200-base-protected transcript corresponding to the proximal transcription initiation site (B) was identified in two independent experiments.

followed by exposure to 20 ng/ml PDGF-BB or vehicle (control) for 24 h. An almost identical pattern of activity was obtained in the presence of vehicle (Fig. 3, control), compared with Fig. 1C data, confirming the robustness of these construct activity assays. PDGF-BB caused ~50% inhibition of luciferase activity of 8 out of 12 deletion constructs (Fig. 3), and 3′-deletions to −349 bp had no effect on PDGF-BB inhibition. Notably, PDGF-BB decreased by ~50% the 12-fold and 8-fold activities of the −479-bp/−349-bp and −399-bp/−349-bp constructs. The −399-bp/−349-bp region lacks restriction sites and has high GC content. Therefore, we were unable, with various PCR strategies, to make additional deletion constructs between −399 bp and −349 bp (with either +186 bp or −349 3′ ends). As controls, PDGF-BB had no effect on luciferase expression of pGL3 basic or pGL3 control vectors (cotransfected with β-gal for correction): Luciferase activity in cells transfected with pGL3 basic or pGL3 control and treated for 24 h with vehicle [reporting 149 ± 7 (n = 3) and 3267 ± 435 (n = 6) U of luciferase activity, respectively], was not different from cells treated for 24 h with 20 ng/ml PDGF-BB [176 ± 26 (n = 3) and 3488 ± 332 (n = 6), respectively]. Collectively, Fig. 3 data narrow the minimal responsive region for PDGF-BB down-regulation of α1D transcription activity to the −399-bp/−349-bp region.

Constitutive Sp1 Binding to −399-bp/−349-bp Region Is Unaffected by PDGF-BB. There are two Sp1 and two AP-2 consensus sites in the −399-bp/−349-bp PDGF-BB responsive region (Figs. 1A and 4A). To determine if PDGF-BB affects nuclear protein binding to these sites, GMSAs were performed with rat aorta SMC nuclear protein extracts (NPEs). Two binding complexes were detected in control NPE by probe P1, which contains a putative Sp1 binding site (Fig. 4). These complexes were competed by cold consensus Sp1 oligonucleotide and by cold excess P1, but not by an unrelated oligonucleotide (hypoxia-inducible factor-1, HIF-1), demonstrating binding specificity. PDGF-BB did not substantially alter binding activity of P1. In control and PDGF-BB-treated NPE incubated with probe P1, Sp1 polyclonal antibody (noncross-reactive with Sp2, Sp3, or Sp4) also reduced binding activity of the upper and lower complexes and induced a supershifted band (Fig. 5, left). The complexes also were again competed by cold Sp1 consensus oligonucleotide. Antibodies to the estrogen receptor and to AP-2 (AP-2 antibody also used below in Fig. 6) had no effect on the upper and lower complexes of P1 (n = 2 independent experiments for each antibody). The same effects were seen for the upper and lower complexes detected by probe P2 (Fig. 5, right) (except AP-2 antibody, discussed below for Fig. 6). The intensity of the P2 middle complex (Fig. 5, arrow), identified as putative AP-2 activity in Figs. 6 and 7, was not competed or

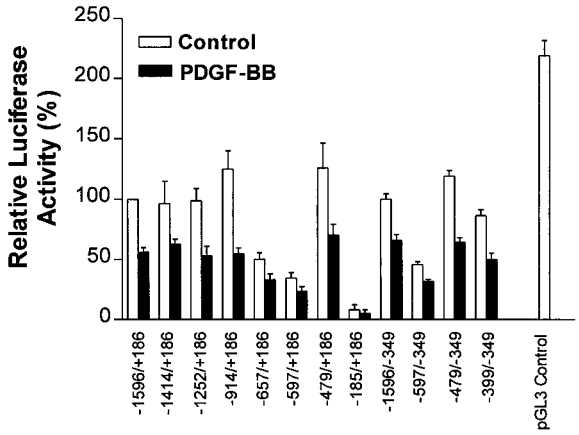

Fig. 3. PDGF-BB responsive motif in the α1D-AR 5′-flanking region. Deletion constructs described in Fig. 1B were transiently transfected into confluent aorta SMCs, along with β-galactosidase expression vector for normalization to transfection efficiency. After an 8-h transfection, serum-free defined media (without insulin) was replaced, and PDGF-BB (20 ng/ml) (black bars) or vehicle (white bars, control) was added for 24 h. Vehicle-treated luciferase activity was expressed as a percentage of the full-length construct, and PDGF-BB-treated luciferase activity was expressed as a percentage of that for the same constructs with vehicle treatment. Transfection with the positive control vector (pGL3 control) was included in each experiment. Data are represented as means ± S.E. for at least four independent transfections done in duplicate.

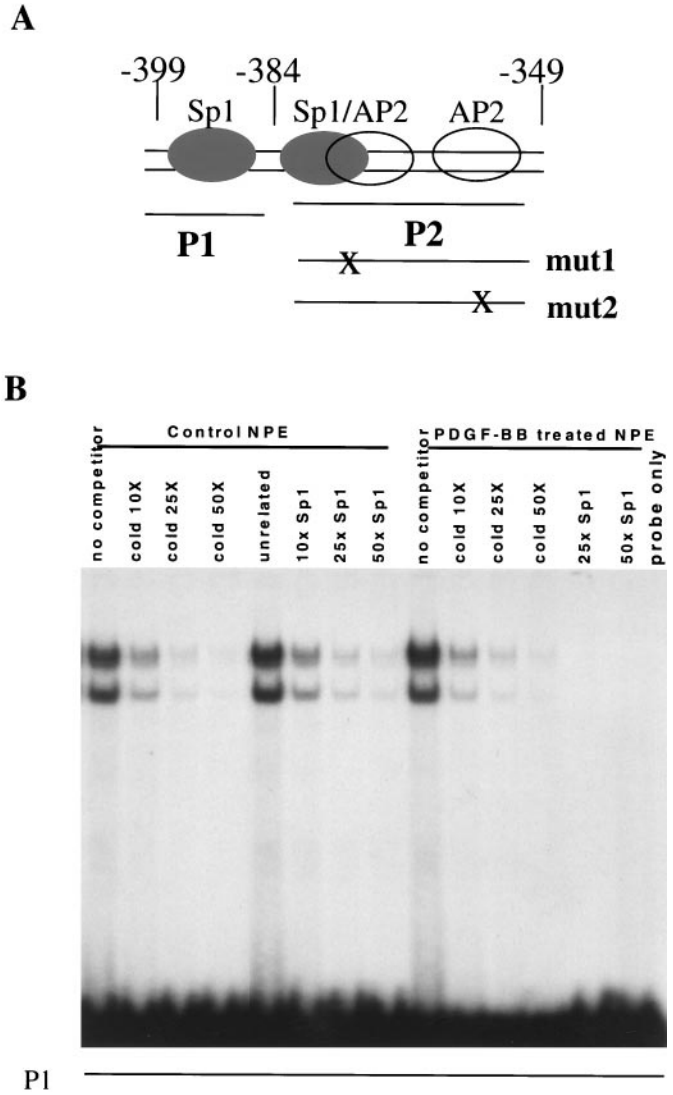

Fig. 4. PDGF-BB does not affect constitutive binding to the −399-bp/−384-bp region of the α1D promoter containing a consensus Sp1 element. GMSAs were used to examine transcription factor binding to putative binding sites in the −384-bp/−349-bp region with P1 and P2 (A) oligonucleotide probes (sequences in *Materials and Methods*). Mut1 and mut2 refer to Fig. 7 experiments. B, NPE isolated from vehicle-treated (control) SMCs or PDGF-BB-treated (20 ng/ml; 24 h) cells were incubated with P1. Competition was performed with various amounts of unlabeled P1 (cold), unrelated oligonucleotides (HIF-1; 50×), or consensus Sp1 oligonucleotide. GMSA results are representative of at least three independent experiments. Same abbreviations used in Figs. 5–7.

supershifted by Sp1 consensus oligo or Sp1 antibody. Doubling the antibody concentration caused no additional competition or supershifting. We did not examine whether widely expressed Sp1-related family members (Sp2, Sp3) or other GC-box binding proteins represent the residual upper and lower band binding in Fig. 5 in the presence of Sp1 antibody. However, cold consensus Sp1 oligo dose-dependently competed binding of the upper and lower complexes in both regions (Figs. 4 and 6). Yet Sp2 protein does not bind to consensus Sp1 oligo, and Sp1-DNA complex migrates more slowly than Sp3-DNA complex (Kingsley and Winoto, 1992). Thus, Sp4 or a non-Sp family protein, rather than Sp2 or Sp3, may represent the residual binding insensitive to Sp1 antibody. These data suggest that Sp1 constitutively binds to its consensus elements in the −399-bp/−349-bp region. Indeed, this putative Sp1 band pattern is consistent with the Sp1 band pattern in other genes (Yang et al., 1995; Ye et al., 1996). Like P1, PDGF-BB did not substantially alter the upper or lower binding to P2. These data suggest that PDGF-BB does not alter constitutive Sp1 binding to the two Sp1 elements in the −399-bp/−349-bp region.

PDGF-BB Increases AP-2-Like Protein Binding to −384-bp/−349-bp Region. PDGF-BB increased the middle binding complex of P2 (Figs. 5–7, arrow). The presence of a faint middle band in control NPE cannot reflect exogenous PDGF-BB because SMCs were maintained in serum-free defined media. It is not known whether it reflects low levels of SMC-derived PDGF-BB. The middle band of P2 augmented by PDGF-BB was competed by cold P2 but not by unrelated (HIF-1) oligonucleotide, indicating binding specificity (Figs. 6 and 7). It also was competed by consensus AP-2 oligonucleotide and AP-2 antibody, but not by consensus Sp1 oligonucleotide or antibody (Figs. 5 and 6). These data indicate that PDGF-BB increases binding of AP-2 or a closely related protein. In control NPE, the upper and lower bands of P2 were competed by cold P2, AP-2 antibody, and AP-2 consensus oligonucleotide (not shown for control NPE, but shown in Fig. 6 for PDGF-BB-treated NPE). These data suggest that an Sp1-AP-2-like protein (s), in addition to SP1, may constitutively bind to this region. AP-2 consensus oligo and AP-2 antibody did not affect the upper and lower binding to P1.

PDGF-BB Increases AP-2-Like Protein Binding to Distal AP-2 Site in −384-bp/−349-bp Region. The above-mentioned results suggest that PDGF-BB increases AP-2 protein binding to one or both of the putative AP-2 sites in

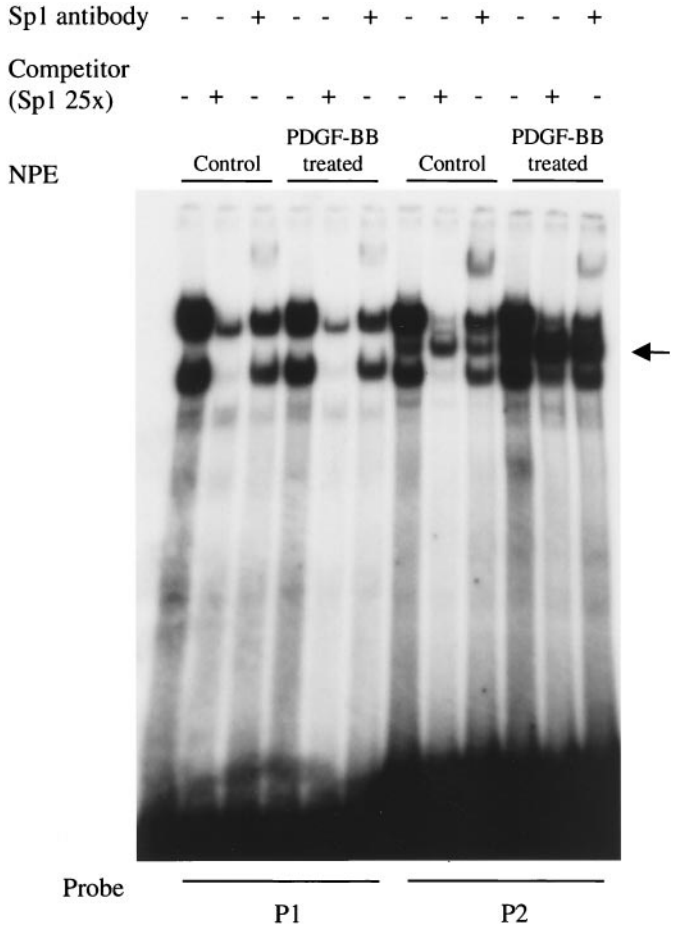

Fig. 5. Sp1 binds constitutively to the proximal and distal consensus Sp1 elements in the −399-bp/−349-bp region of the α1D promotor. PDGF-BB does not alter this binding, but increases non-Sp1 binding activity. GMSA showing competition by consensus Sp1 oligo and Sp1 antibody (1 μl in 9-μl reaction volume) of P1 and P2 upper and lower binding activities. The autoradiogram was overexposed to aid detection of the binding complex supershifted by Sp1 antibody. Although the P2-binding activity of intermediate mobility (arrow) is better resolved in Figs. 6 and 7, as in those figures, PDGF-BB only increased the middle band which was unaffected by Sp1 oligo or antibody. The GMSA is representative of three independent experiments.

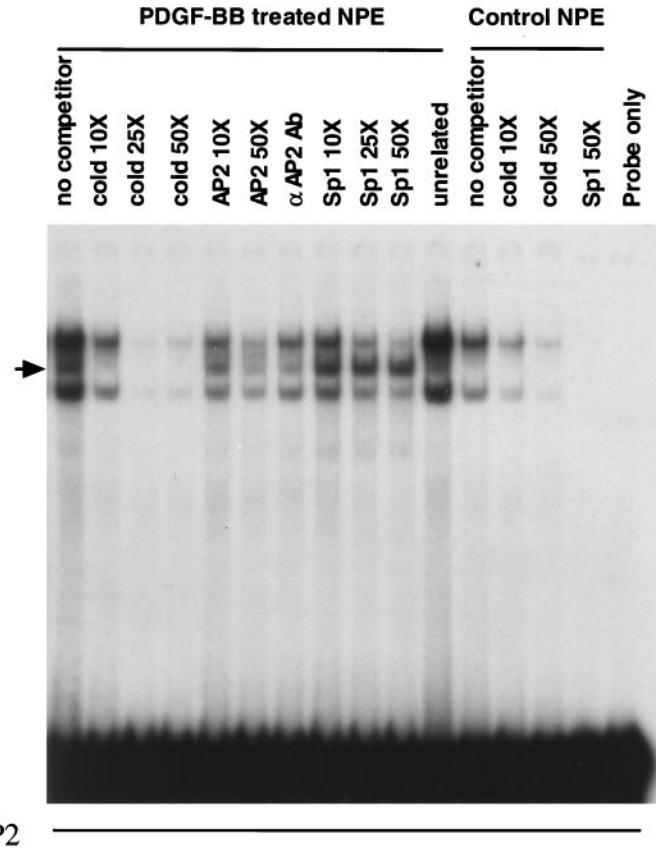

Fig. 6. PDGF-BB increases AP-2-like protein binding to the −384-bp/−349-bp region. Representative GMSA with P2 and NPE from control or PDGF-BB-treated SMCs. Competition performed with various amounts of unlabeled P2 (cold), consensus AP-2 oligonucleotide, anti-AP-2 antibody, consensus Sp1 oligonucleotide or unrelated oligonucleotide (HIF-1; 50×). Arrow indicates PDGF-BB-induced protein-DNA binding. Results are representative of at least three independent experiments.

the −384-bp/−349-bp region of the α1D adrenergic receptor proximal promotor. To determine which one or if both sites show increased binding during PDGF-BB treatment, two mutated P2 oligonucleotides (mut1 and mut2, sequences in *Materials and Methods*) (Fig. 4A) were synthesized. GMSAs with labeled P2 were performed with cold mut1 or mut2 competition (Fig. 7). Specificity was confirmed by competition with cold P2 and absence of competition with unrelated oligonucleotide. In control NPE, mut1 failed to compete with binding, whereas mut2 completely blocked the binding complexes. These results indicate that the distal AP-2 binding site (or Sp1 site because AP-2/Sp1 sites overlap here) in the −384-bp/−349-bp region is required to form the constitutive binding complexes in control SMC nuclear extracts. Similarly, in PDGF-BB-treated nuclear extracts the increased protein-binding complex (middle band) and upper and lower bands were not affected by cold mut1, whereas cold mut2 competed them away (Fig. 7). These results suggest that PDGF-BB increases AP-2 binding to the distal putative AP-2 site at −376 bp.

PDGF-BB Represses Activity of −384-bp/−349-bp Region of α1D-AR Promoter. An additional transient transfection assay was performed with a more delineated construct to confirm that promoter activity of this region is inhibited by PDGF-BB. As shown in Fig. 8 left, PDGF-BB inhibited activity of the −384-bp/−349-bp construct to the same degree (50% inhibition) as the −479-bp/−349-bp construct. Therefore, the −384-bp/−349-bp fragment of the α1D-AR gene functionally mediates PDGF-BB inhibition. To further confirm the dependence of PDGF-BB repression of α1D promotor activity on the distal AP-2 site, transient transfection assays were performed with mut1 of Fig. 4. Mutation of the distal AP-2 site completely abolished all activity (was the same as pGL3 basic), and, as expected, 24-h treatment with 20 ng/ml PDGF-BB had no effect (Fig. 8, right).

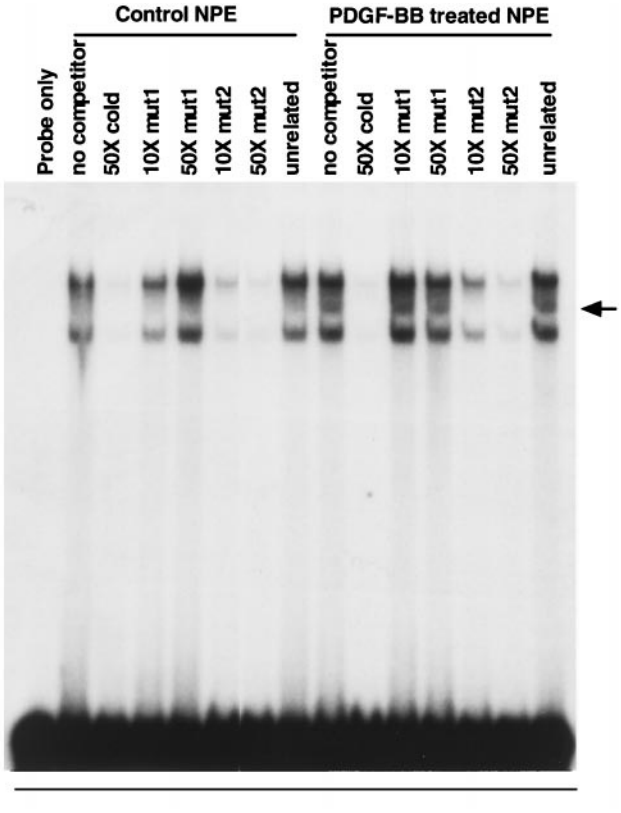

Fig. 7. PDGF-BB increases AP-2-like protein binding to the distal AP-2 site in the −384-bp/−349-bp region. GMSAs with P2 (Fig. 4A) and competitions with cold P2, mut1, or mut2 (sequences of mut1 or mut2 were described in *Materials and Methods* and schematically represented in Fig. 4A), or unrelated oligonucleotide (HIF-1; 50×). NPEs were isolated from control or PDGF-BB-treated SMCs. Results are representative of at least three independent experiments.

Expression of Exogenous AP-2 Protein Dose-Dependently Inhibits α1D-AR Promoter Activity in SMCs. Cotransfection assays were performed to investigate whether overexpression of native AP-2 protein, presumably through binding to the −384-bp/−349-bp region, represses α1D-AR promoter activity. SMCs were transiently transfected with the −479-bp/−349-bp construct and various amounts of expression vector encoding AP-2 protein (SPRSV-AP-2) (Fig. 9). Expression of exogenous AP-2 (0.125–5.0 μg of plasmid DNA/well) dose-dependently inhibited activity of the −479-bp/−349-α1D construct. As expected, the empty AP-2 expression plasmid did not induce any luciferase activity. These data further strengthen the conclusion from the previous studies that PDGF-BB promotes AP-2 protein which in turn represses α1D-AR promoter activity.

Discussion

We recently showed that PDGF-BB, acting through the PDGF-β receptor, selectively inhibits α1D-AR gene transcription and receptor density in vitro and ex vivo through a PKC but not mitogen-activated protein kinase kinase or phosphatidylinositol 3-kinase-dependent pathway that is not dependent on new protein synthesis; functionally, this suppresses NE-induced SMC growth (Xin et al. 1999) that we have shown is α1D-AR mediated (Xin et al., 1997). To investigate how endogenous α1D-AR transcription might be reduced by PDGF-BB, in the present study we cloned and characterized the 1.6-kb 5′-flanking region of the rat α1D-AR gene. The rat α1D-AR promoter lacks a TATA-box but has several GC-rich regions. Reporter deletion analysis suggested that SMCs use two transcription initiation sites, which were then identified by primer extension assays and RPAs. Furthermore, GMSAs mapped apparent PDGF-BB-augmented AP-2 protein binding to the distal putative AP-2 site at −376 bp in the promoter region for the proximal start site. Constitutive putative Sp1 binding to two consensus elements in this region was unaffected by PDGF-BB. Transfection mapping analysis confirmed that this region confers PDGF-BB inhibition of α1D-AR gene promotor-luciferase construct activity. Moreover, transfection of an AP-2 expression vector into SMCs dose-dependently repressed α1D-AR construct activity. These results suggest that PDGF-BB inhibits α1D-AR gene transcription by promoting AP-2 protein binding to the distal Sp1/AP-2 overlapping site at −376 bp in the α1D proximal promoter region.

The existence of multiple transcription initiation sites has been observed in other α1-AR genes. Human and rat α1B-AR and human α1A-AR genes, that lack TATA-box and CCAAT-box motifs, can use two or three different transcription initiation sites depending on cell context (Gao and Kunos, 1994; Eckhart et al., 1996; Razik et al., 1997). In the present study, we found with primer extension that the distal site mapping

to −479 bp is identical with the start site suggested from results of rat cerebral cortex cDNA cloning strategies (Lomasney et al., 1991; Perez et al., 1991). However, our RPAs (and PCR strategies) failed to unambiguously detect this transcript. This may reflect incomplete hybridization of riboprobes and PCR primers with the several highly GC-rich regions present downstream from the distal initiation site. An additional proximal transcription start site located at −288 bp was identified in rat SMCs. The identification of this start site was supported by the following evidence. First, transfection analysis with deletion mutants indicated existence of additional positive regulatory element(s) downstream of the reported cDNA starting site (Figs. 1 and 3). Second, primer extension assays and RPAs revealed a transcript arising from an initiation site located downstream of the rat cortex cDNA start site (Fig. 2). Third, GMSAs showed two constitutive Sp1 binding activities in the −399-bp/−349-bp region. These Sp1 binding sites are located ~100 bp upstream of the proximal transcription initiation site. This location and their apparent importance to constitutive α1D-AR promoter activity (Figs. 5–7) is consistent with the reported primary role of the Sp1 protein in specifying accurate transcription initiation from so-called TATA-less promoters (Azizkhan et al., 1993). Sp1 binding sites present at one-to-several hundred base pairs upstream of transcription initiation sites have been proposed to serve as anchoring factors to position the basal transcription complex at the proper downstream initiation site (Azizkhan et a., 1993).

In deletion reporter transfection assays, PDGF-BB down-regulation of α1D-AR promoter activity was mapped to the −399-bp/−349-bp region of the gene. Sequence analysis showed that this GC-rich region contains two Sp1 and two AP-2 consensus-binding sites. GMSAs and Sp1 antibodies demonstrated constitutive Sp1 binding to the distal Sp1 element at −390. However, the constitutive binding at the proximal Sp1/AP-2 overlapping site at −376 was inhibited by both consensus Sp1 and AP-2 oligonucleotides and antibodies (Figs. 5 and 6). More studies are needed to define the nature of this "AP-2/Sp1-like" constitutive-binding activity.

PDGF-BB induced binding in the −384-bp/−349-bp region was competed by consensus AP-2 oligonucleotide and was significantly inhibited by anti-AP-2 antibody. However, we did not observe supershift of the PDGF-BB-induced binding complex by AP-2 antibody, as is often the case depending on antibody used, species differences in targeted protein, and interference by antibody of protein binding to DNA. Separate mutations of the distal and proximal putative AP-2 binding sites suggested that PDGF-BB increased AP-2 binding to the distal rather than proximal AP-2 binding site in the −384-bp/−349-bp region. More importantly, cotransfection of the −479-bp/−349-bp construct with various amounts of AP-2 expression vector into SMCs showed that expression of exogenous AP-2 could dose-dependently inhibit activity of this α1D-AR construct. Additional studies are needed to determine whether possible concomitant depletion of cofactors (Kannan et al., 1994) or displacement of the "AP-2/Sp1-like" protein that constitutively binds to this region (Figs. 5–7) is necessary for AP-2 *trans*-activation of α1D-AR repression by PDGF-BB.

The AP-2 protein plays important roles in positive and negative regulation of gene expression in many cell types. For example, induction of AP-2 binding by transforming growth factor-α increases transcription of the vascular endothelial growth factor gene (Gille et al., 1997). 5-Lipoxygenase-specific inhibitor induces AP-2 binding that is adjacent to an nuclear factor-1 site, resulting in suppression of type I collagen gene expression in stellate cells (Chen et al., 1996). Interestingly, a nuclear factor-1 site (−461 bp) is also in close proximity to several AP-2 sites in the α1D proximal promoter. Also, vasopressin gene transcription is inhibited by AP-2 binding (Iwasaki et al., 1997). The mechanism of AP-2 induction, but not repression, is beginning to be clarified. Three major pathways [activation of the retinoic acid receptor (Luscher et al., 1989), cAMP-dependent protein kinase A (Imagawa et al., 1987), and PKC activation (Hyman et al., 1989)] have been linked to AP-2 induction and subsequent stimulation of gene expression. In Xin et al. (1999), we demonstrated that PDGF-BB-induced α1D-AR down-regulation was abolished by PKC inhibition. Thus, PDGF-BB inhibition of α1D-AR expression may rely on PKC-dependent increase in AP-2 binding. In that same study, we also found that cycloheximide partially attenuated PDGF-BB reduction of

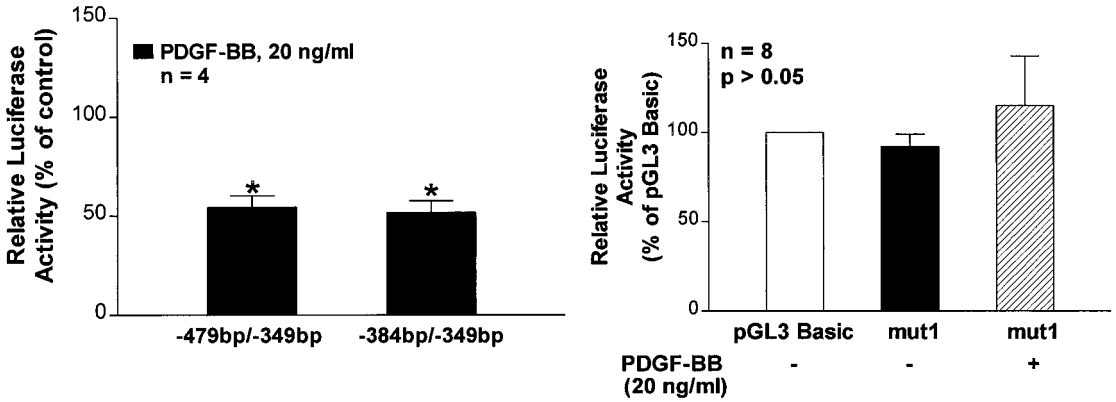

Fig. 8. PDGF-BB represses activity of the −384-bp/−349-bp fragment of the α1D-AR promoter. Left, the −479-bp/−349-bp and −384-bp/−349-bp fragments were subcloned into pGL3 basic vector in 5′ to 3′ orientation. After cotransfection with β-galactosidase for 8 h, SMCs were incubated for 24 h with 20 ng/ml PDGF-BB in serum-free, defined media. Luciferase activities from PDGF-BB-treated cells are represented as percentage of activity relative to each untreated (control) construct, where control luciferase activity was 4- to 5-fold above pGL3 basic vector level. Data are means ± S.E. from four independent transfections performed in duplicate per construct. *$p < .05$ versus control (one-tailed t test). Right, the −384-bp/−349-bp mut1 fragment of Fig. 4, along with pGL3 basic, was examined with the same methods described for (left) for eight independent duplicate transfections for each construct.

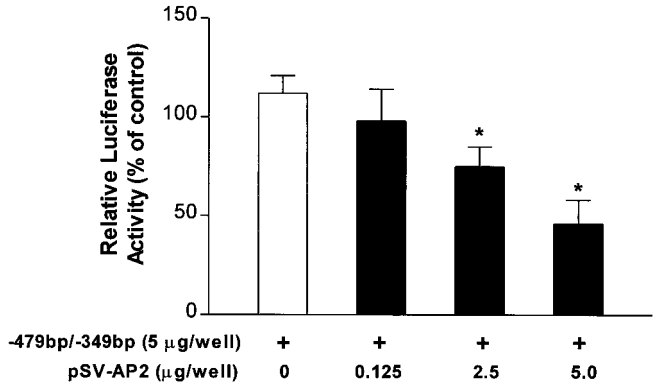

Fig. 9. Expression of exogenous AP-2 protein dose-dependently inhibits α1D-AR promoter activity in SMCs. Aorta SMCs were transiently transfected with the −479-bp/−349-bp construct (5 μg/well) and cotransfected with various amounts of pSV-AP-2 or control vector, as well as β-galactosidase vector. Luciferase activities represented as fold relative to the −479-bp/−349-bp construct. Data are means ± S.E. from three independent experiments. *$p < .05$ versus control (ANOVA and Bonferroni test).

α1D expression. Whether this reflects a partial requirement of new protein synthesis, as well as "activation/mobilization" by PDGF-BB of an existing pool of AP-2, requires additional study.

The α1D-AR is the dominant α1-AR subtype expressed by rat arterial SMCs in adult aorta (Ping and Faber, 1993; Eckhart et al., 1996) and carotid artery (Yang et al., 1999). Activation of α1D-AR causes arterial constriction (Clarke et al., 1995; Piascik et al., 1995; Leech and Faber, 1996) and medial SMC hypertrophy (Chen et al., 1995; Xin et al., 1997). Although little is known concerning regulation of α1D-AR expression, we have recently observed, with quantitative RT-PCR of intima and medial layers of the carotid artery, that α1D-AR mRNA (and α1ARs by radioligand binding assay) rapidly decreases and remains sharply down-regulated at 4, 21, and 42 days after balloon injury (Faber et al., 1999). This may reflect increased PDGF activity in the vascular wall during medial repair and neointimal lesion formation that could modulate α1D-AR expression. PDGF is a key factor governing neointimal growth after balloon angioplasty (Schwartz et al., 1995), where PDGF-BB and β-receptor induction follow a time course similar to this inhibition of α1D-AR mRNA (Scott et al., 1996; Uchida et al., 1996; Panek et al., 1997). Down-regulation of α1D-ARs by PDGF also may underlie an ontogenic change in α1D-AR expression. Compared with α1B, α1D-AR expression appears to be low in the postnatal growing rat aorta, but up-regulates greatly by adulthood (Gurdal et al., 1995; Ibara et al., 1997). PDGF-B ligand is high, compared with adult, in the growing artery of young animals and in SMCs cultured from aorta of rat pups (Majesky et al., 1990; Rafty and Khachigian, 1998). Thus, an inverse relationship between PDGF and α1D-AR expression is suggested by these two examples. The physiological significance of reduction of vascular α1D-AR expression induced by PDGF-β receptor stimulation in the normal growing vascular wall or during repair after injury constitute important areas for future investigation.

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