Zinc Finger Transcription Factors Mediate High Constitutive Platelet-derived Growth Factor-B Expression in Smooth Muscle Cells Derived from Aortae of Newborn Rats

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Platelet-derived growth factor (PDGF) B-chain gene is differentially expressed in smooth muscle cells (SMCs) derived from the rat aortic wall. SMCs cultured from two week-old rats (pups) express high levels of PDGF-B mRNA, whereas cells isolated from three month-old rats (adults) express low levels of PDGF-B. Nuclear run-off experiments indicate that increased PDGF-B gene expression in pups is mediated, at least in part, at the transcriptional level. We used electrophoretic mobility shift assays and Western blot analysis to demonstrate that levels of Sp1 and Sp3, two zinc finger transcription factors which mediate basal expression of the PDGF-B gene, are elevated in pup nuclei compared with adult nuclei. The immediate-early transcription factor, Egr-1, which footprints the PDGF-B promoter, is also constitutively expressed in these cells. Transient transfection and binding studies show that these factors interact with a region in the proximal PDGF-B promoter key for basal expression in pup cells. Mutation of this proximal element in transfected pup cells attenuates reporter gene expression to levels observed in adult cells. Conversely, overexpression of Sp1 in adult cells augments PDGF-B promoter-dependent expression. Elevated PDGF-B expression in cultured newborn rat SMCs may therefore require high constitutive expression of a number of zinc finger transcription factors and their specific interactions with the proximal PDGF-B promoter.

Platelet-derived growth factor (PDGF)1 consists of an A-chain and a B-chain held together in heterodimeric or homodimeric configuration by disulfide bonds (1). Multiple cell types, including vascular smooth muscle cells (SMCs) (2), produce and respond to this potent mitogen and chemotactant in culture. Seifert et al. (3) observed over a decade ago that PDGF may be developmentally regulated in these cells. SMCs cultured from the aortae of 2-week-old rats (pups) (WKY12–22 cells) secreted almost 200-fold greater levels of PDGF than cells isolated from 3-month-old rats (adults) (WKY3M-22 cells). Subsequent Northern blot analysis determined that PDGF-B gene expression is at least 60-fold greater in cultured pup SMCs than adult cells (4). Evidence that differential PDGF-B expression is mediated at the level of transcription was obtained from nuclear run-off analysis (5). Several other genes, such as tropoelastin and α1-procollagen (type I), were found to be overexpressed in pup cells relative to adult cells by cDNA library screening strategies. Cloning pup cells by dilution plating revealed that subpopulations expressed high levels of elastin, CYPIA1 and osteopontin mRNA (6). Cultured pup and adult SMCs also display marked differences in morphology. The epithelioid and cobblestone shape of pup cells contrasts with the spindle-like, elongated bipolar nature of adult SMCs (4).

Basal and inducible expression of the PDGF-B gene is mediated by nucleotide elements located approximately 40 bp upstream of the Tata box. We and others showed that Sp1 binds to the 5′-CCACCC-3′ motif and drives basal expression in vascular endothelial (7) and osteosarcoma cells (8). This is consistent with in vivo footprinting studies which indicate that this region is occupied by nuclear factors in intact cells (9). The related zinc finger transcription factor, Sp3, also interacts with this region of the promoter and activates transcription (8, 10). Egr-1, also known as TIS8, zif268, and NGFI-A (11), plays a positive regulatory role in PDGF-B promoter-dependent expression in cells exposed to phorbol esters or subjected to mechanical injury (12, 13). Egr-1 binds to a cryptic recognition element in the PDGF-B promoter and can displace Sp1 from overlapping binding sites (13). This region also mediates inducible PDGF-B promoter-dependent gene expression in endothelial cells exposed to thrombin (14); however, functional trans-acting factors mediating this process have not yet been identified.

We hypothesized that the restricted expression of PDGF-B in subpopulations of cultured SMCs may be regulated by functional interactions of key zinc finger transcription factors with nucleotide elements in the proximal PDGF-B promoter. Here, we report that Sp1, Sp3, and Egr-1 are constitutively elevated in pup SMCs compared with adult cells. Promoter deletion and transient transfection analysis in pup cells revealed that the region bound by these factors is critical for basal expression directed by the PDGF-B promoter. Mutation of this region attenuates expression to levels comparable with those in adult cells. These findings provide mechanistic insights into the differential expression of PDGF-B gene in SMCs.
Mechanisms Underlying High Basal PDGF-B Expression in SMCs

EXPERIMENTAL PROCEDURES

Cell Culture—Pup and adult SMC lines were cultured in Waymouth’s MB752/1 medium (Life Technologies, Inc.), 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. Cultures were passaged every 3–4 days in 75 cm² flasks. Cells seeded for preparation of nuclear extracts were grown to 80% confluence and washed twice in phosphate-buffered saline (PBS), pH 7.4, then returned to the incubator in 1% growth medium for 24 h prior to extraction.

Transient Cell Transfections—SMCs were seeded in 100-mm tissue culture plates 2 days prior to transfection. When approximately 60% confluent, the cells were transfected with 10 μg of the indicated promoter-reporter plasmid along with 2 μg of pTKGH to correct for transfection efficiency. In overexpression studies, SMCs were cotransfected with 3 μg of either Sp1-pcDNA3 (CMV promoter based), 5 μg of Egr-1-pcDNA3, or pcDNA3 alone. Transfections were performed using Superfect (Qiagen). A precipitate was formed using 5 μl of Superfect reagent/μg of plasmid DNA and the transfection mix made up to 300 μl with serum- and antibiotic-free Waymouth’s medium. After incubation at 22 °C for 10 min, 3 ml of complete Waymouth’s growth medium was added, and the entire mixture was then added to cells previously washed once in PBS, pH 7.4. Following a 3-h incubation at 37 °C in an atmosphere of 5% CO₂, the precipitate was gently aspirated, and the monolayers were washed once with 4 ml of PBS, pH 7.4, prior to the addition of 10 ml of complete Waymouth’s growth medium. For overexpression experiments, SMCs were refed with 2% Waymouth’s medium. Two days post-transfection, the conditioned medium was collected for determination of soluble growth hormone by enzyme-linked immunosorbent assay (Biocline Australia), and cell lysates were prepared for assessment of chloramphenicol acetyltransferase activity as described (7).

Preparation of Nuclear Extracts—SMCs were washed twice with PBS, pH 7.4, at 4 °C and removed from the culture dish by scraping. The cells were pelleted by centrifugation at 1200 rpm for 10 min at 4 °C. The cell pellet was resuspended in cold PBS, pH 7.4, and the suspension was added, and the entire mixture was then added to cells previously washed once in PBS, pH 7.4. Following a 3-h incubation at 37 °C in an atmosphere of 5% CO₂, the precipitate was gently aspirated, and the monolayers were washed once with 4 ml of PBS, pH 7.4, prior to the addition of 10 ml of complete Waymouth’s growth medium. For overexpression experiments, SMCs were refed with 2% Waymouth’s medium. Two days post-transfection, the conditioned medium was collected for determination of soluble growth hormone by enzyme-linked immunosorbent assay (Biocline Australia), and cell lysates were prepared for assessment of chloramphenicol acetyltransferase activity as described (7).

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Western Blot Analysis—Electrophoresis, transfer, and immunodetection were performed as described previously (13).

Oligonucleotide Synthesis, Purification, and Radiolabeling—Oligonucleotides were synthesized with trityl groups off and purified by gel electrophoresis. Oligonucleotides were radiolabeled with [γ-¹³⁵]ATP (Bresatec) using T₄ polynucleotide kinase (New England Biolabs) and purified by centrifugation in Chromaspin-10 columns (CLONTECH).

Electrophoretic Mobility Shift Assay (EMSA)—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), 5% sucrose, 1 mg of poly(dI-dC)-poly(dI-dC) (Sigma), ¹³⁵P-labeled oligonucleotide probe (150,000 cpm), and 3 μg of nuclear extract (determined by Bio-Rad Protein Assay). The reaction was incubated for 35 min at 22 °C. In supershift studies, 2 μl of the appropriate affinity-purified antipeptide polyclonal antibody (Santa Cruz Biotechnology) was incubated with the binding mix for 10 min before addition of the probe. These antibodies do not cross-react with the others’ protein target. Bound complexes were separated from free probe by loading samples onto a 6% non-denaturing polyacrylamide gel and electrophoresing at 200 V for 2.5 h. The gels were vacuum dried at 80 °C and subjected to autoradiography overnight at −80 °C.

RESULTS

Certain Zinc Finger Transcription Factors Are More Abundant in Cultured Pup Aortic SMCs than Adult SMCs—Previous investigations by our group (7, 13) and others (8, 14, 15) determined that PDGF-B gene expression in cell types other than SMCs is under the transcriptional control of the zinc finger nuclear factors, Sp1, Egr-1, and Sp3. Since the PDGF-B gene is expressed at higher levels in cultured pup rat aortic SMCs than SMCs derived from adult rats, we hypothesized that elevated PDGF-B gene expression may be due to elevated levels of Sp1, Egr-1, and Sp3 in the former cell type. Using a ¹³⁵P-labeled double-stranded oligonucleotide bearing consensus sites for this superfamily of regulatory proteins together with nuclear extracts from cultured pup or adult aortic SMCs, we observed the formation of several distinct nucleoprotein complexes, designated a-d (Fig. 1). Differences were observed in several of these complexes between the two cell subtypes. By densitometric assessment, complexes a, b, c, and d were approximately 12-, 11-, 4-, and 5-fold more abundant using extracts from pup cells as compared with adult cells, respectively.

Antibody inhibition experiments were performed to identify the protein components of these complexes. Inclusion of polyclonal antibodies directed against Sp1 in the binding reaction attenuated formation of complex a (Fig. 2A), the most intense of these complexes. Antibodies raised against Egr-1 eliminated complex b (Fig. 2B), while antibodies recognizing Sp3 completely abolished the formation of complex c (Fig. 2C). Interestingly, the Sp1 antibody slightly attenuated the intensity of complex c while the Sp3 antibody weakly inhibited the formation of complex a (Figs. 2, A and C). This raises the possibility that Sp1 and Sp3 occupy the promoter fragment simultaneously in these cells. Indeed, the incomplete supershift obtained using the Sp1 antibody (Fig. 2A) has been observed previously by several groups (16–19).

Several criteria indicate that differences in Sp1, Egr-1, and Sp3 binding activity observed between pup and adult SMCs was not a consequence of unequal protein content in each
preparation of extract. First, identical amounts of pup and adult nuclear extracts were used for each EMSA. Second, the intensity of nucleoprotein complexes was similar between pup and adult samples. Third, the nucleoprotein complex obtained when a 32P-labeled oligonucleotide bearing a serum response element in the egr-1 promoter was incubated with these extracts was slightly more intense when adult samples were used (Fig. 2D).

We performed Western blot analysis to provide confirmatory evidence that Sp1, Egr-1, and Sp3 are present in greater levels in nuclear extracts from pup cells than adults since DNA binding activity and levels of protein may not necessarily correlate. Findings from immunoblot analysis indicate that Sp1, Egr-1, and Sp3 levels are indeed greater in pup nuclei than adults (Fig. 3), consistent with observations from gel and supershift studies (Fig. 2). The Coomassie Blue-stained gel indicates equal protein loading (Fig. 3). Taken together, these results demonstrate that Sp1, Egr-1, and Sp3 are constitutively expressed in pup SMCs and that these levels exceed those in adult cells.

**Pup Aortic SMCs Support High Basal Expression of a Reporter Gene Driven by the Zinc Finger Transcription Factor**

**FIG. 2.** Sp1, Egr-1, and Sp3 binding activity is greater in nuclei of pup aortic SMCs than adult SMCs. 32P-oligo GC was incubated with pup or adult SMCs, and nucleoprotein complexes were resolved by gel electrophoresis. Polyclonal antibodies directed toward Sp1 (A), Egr-1 (B), or Sp3 (C) were incubated with the extracts 10 min prior to the addition of 32P-oligo GC. Nucleoprotein complexes containing Sp1, Egr-1, and Sp3 are indicated by arrows. D, Nuclear proteins binding to the serum response element of the egr-1 promoter are more abundant in adult SMCs than pup cells. Pup and adult SMCs were incubated with 32P-Egr-1 prom (5'-AGGATCATCCTTGCCTATTTGGCAGCATCC-3', sense strand), and the nucleoprotein complex was resolved by gel electrophoresis as described under “Experimental Procedures”. The nucleoprotein complex is indicated by the arrow.
We used EMSA and supershift in Cultured Pup Aortic SMCs—pup cells. d75 contain nucleotide elements crucial for basal expression in expression in pup SMCs. A32P-oligonucleotide spanning the region in the PDGF-B promoter responsible for high basal expression driven by this construct was low in adult cells (Fig. 4). The higher levels of reporter gene expression in pup cells obtained using this construct are thus consistent with the greater abundance of factors binding the element in this cell type (Fig. 1).

Nucleotide Elements in the Proximal PDGF-B Promoter Are Required for High Basal PDGF-B Promoter-dependent Expression in Pup Aortic SMCs—Nucleotide regulatory elements mediating basal expression of the PDGF-B gene have not yet been defined in vascular SMCs. Transient transfection analysis was performed in pup cells with a series of PDGF-B promoter fragments cloned upstream of a promoterless CAT cassette (7). CAT activity directed by construct d26, which extends 153 bp upstream of the TATA box, was comparable with expression generated from construct d77, which bears 82 bp of promoter sequence (Fig. 5). However, CAT expression produced by construct d75, which bears 19 bp of promoter sequence, was only 9% that of d77 (Fig. 5). These findings indicate that the region between the 5' end points of the PDGF-B fragments in d77 and d75 contain nucleotide elements crucial for basal expression in pup cells.

Sp1, Egr-1, and Sp3 Bind to the Proximal PDGF-B Promoter in Cultured Pup Aortic SMCs—We used EMSA and supershift studies to determine whether Sp1, Egr-1, and Sp3 interact with the region in the PDGF-B promoter responsible for high basal expression in pup SMCs. A 32P-oligonucleotide spanning the −30/−13 region of the PDGF-B promoter in competition with its unlabeled cognate revealed the formation of several specific nucleoprotein complexes (Fig. 6, complexes a-d, compare lanes 2 and 4). Complexes a-c were barely detectable when adult nuclei were used in the EMSA (lane 3). Complexes a-d were abrogated in the presence of a molar excess of oligo GC (lane 6), whereas an irrelevant oligonucleotide, E74, was without effect (lane 5). The oligo GC result strongly suggested the involvement of zinc finger transcription factors in these complexes. Antibody inhibition experiments provided definitive evidence that Egr-1 (lane 7), Sp3 (lane 8), and Sp1 (lane 9) associate with this region of the PDGF-B promoter preferentially in pup nuclei. Interestingly, the partial supershift obtained using the Sp1 antibody alone was completely abrogated by coincubation with the Sp3 antibody (lane 10). These findings indicate that Sp1, Egr-1, and Sp3 bind to the proximal PDGF-B promoter in pup cells although, of all these factors, Sp1 associates with this region of the promoter most abundantly.

The Proximal Region of the PDGF-B Promoter Required for Basal Expression in Pup SMCs Supports Inducible Expression in Adult Cells—Transient of construct d77 into pup SMCs resulted in high levels of PDGF-B promoter-dependent gene expression (Fig. 7A, column 1). In comparison, reporter gene expression driven by this construct was low in adult cells (column 3). Mutation of the region bound by these factors in the PDGF-B promoter resulted in an 87% decrement in normalized CAT activity in pup cells (column 2). Interestingly, reporter gene expression directed by the mutant construct in pup cells was comparable with levels obtained using the wild-type construct in adult cells (column 3). Mutation of the zinc finger binding site (−34/−18) only slightly attenuated promoter-dependent expression in adult cells (column 4). When wild type d77 was cotransfected with a cytomegaloviral promoter-driven Sp1 expression construct in adult cells, reporter expression increased to levels comparable with those in pup cells (column 5). Overexpression of Egr-1, either alone or in combination with Sp1, also resulted in inducible PDGF-B promoter-dependent expression.
expression (Fig. 7B). These binding, mutational, and overexpression studies demonstrate that elevated PDGF-B expression in cultured pup SMCs is influenced by zinc finger nuclear factors binding to regulatory elements in the proximal region of the PDGF-B promoter.

DISCUSSION

In this paper, we have investigated the transcriptional mechanisms underlying the high constitutive expression of PDGF-B in SMCs isolated from the aortae of pup rats. Curiously, SMCs cultured from adult rats of the same strain express low levels of PDGF-B. EMSA and Western blot analysis revealed that levels of Sp1 and two other zinc finger transcription factors, Sp3 and Egr-1, are markedly elevated in the nuclei of pup SMCs compared with adult cells. 5' deletion and transient transfection analysis defined the proximal region of the PDGF-B promoter as that which mediates high basal expression of the gene in pup cells. EMSA using extracts from pup cells revealed that Sp1, Egr-1, and Sp3 bind to this region of the promoter in a specific manner. Mutation of the proximal element abolished basal PDGF-B promoter-dependent expression in these cells. Conversely, overexpression of Sp1 and/or Egr-1 in adult cells increased reporter gene expression. These studies define a critical role for the proximal region of the PDGF-B promoter in the elevated expression of the gene in pup SMCs and identifies several nuclear proteins that bind to these elements in a specific and functional manner.

While the basic structure of the rat aorta is already formed at birth, the vessel wall continues to develop within the first three months postpartum. SMCs proliferate and differentiate most actively in the first few weeks and deposit large amounts of connective tissue matrix components into the extracellular space while the artery undergoes remodeling (20). The migratory and proliferative effects of PDGF-BB on cultured aortic SMCs (2) and the chemotactic effects of PDGF-BB on SMCs in rat (21) and porcine (22) models suggest that the PDGF B-chain may play a developmental role. A thinner aortic wall, among other abnormalities, in PDGF-B null mice is consistent with a role for PDGF-B in blood vessel development (23). High constitutive PDGF-B expression in pup SMCs that decline in older mice lends support to this notion *prima facie*. However, Northern blot analysis indicates that PDGF-B mRNA is present only in low amounts in aortic SMCs in pup and adult rats (24). Placement of pup SMCs in culture results in a dramatic change in PDGF-B phenotype, so much so that abundant levels of PDGF-B mRNA are observed after six passages (4). Moreover, SMCs isolated from the neointima of adult rats 2 weeks after balloon catheter injury have an epitheloid morphology strongly resembling that of pup SMCs (25). Steady-state PDGF-B mRNA levels in these pup-like neointimal cells also increase with time in culture (26). *In situ* hybridization using rat-specific cDNA probes and an en face technique shed light on this
apparent conundrum. It appears that only a subpopulation of SMCs can actually express PDGF-B in the aortic media. SMC cultures expressing high levels of PDGF-B after successive passages may arise from clonal expansion of the PDGF-B-expressing SMC subpopulation (27). Cloning pup SMCs by dilute plating have generated isolates that either express or do not express PDGF-B mRNA (6). Aortic SMCs are, therefore, a heterogeneous population with diverse patterns of growth and gene expression (6). Such gross differences in PDGF-B expression between cultured pup and adult SMCs provide a valuable resource to investigate transcriptional mechanisms that contribute to cell-specific gene expression. In addition, the similarity of the PDGF-B phenotype between cultured pup SMCs and SMCs isolated from the balloon-injured rat adult artery wall (26) implicates a role for zinc finger transcription factors in the vascular response to injury.

Differences in nuclear transcription factor content have previously been suggested as a regulatory mechanism underlying cell-specific gene expression. For example, interleukin-2 expression in murine Th1 cells, but not Th2 cells, may be due to the presence of NFAT in the former cell type (28). Second, expression of Bruton’s agammaglobulinemia tyrosine kinase (Btk) gene in B-cells, but not K562 cells or Jurkat T-cells, may be due to the presence of both Spi-1/PU.1 and Spi-B in B-cells (29). Third, murine surfactant protein A (SP-A) promoter-dependent expression in mouse lung epithelial (MLE-15) cells, but weakly, if at all, in HeLa, 3T3, or H441 cells, may be due to the preferential expression of thyroid transcription factor-1 (TTF-1) in the former cell type (30). Finally, Myod, the skeletal muscle lineage control gene product that converts adult SMCs to skeletal muscle myoblasts, is unable to alter the phenotype of pup SMCs (31), suggesting that pup and adult SMCs differ in nuclear factors able to interact with Myod (6). The present study also extends recent findings demonstrating no major differences between the content of NFκB and AP-1 in the nuclei of pup and adult cells (32). Our findings also demonstrate that overexpression of Sp1 and Egr-1 could augment PDGF-B promoter-dependent expression in rat SMCs and that these effects were not additive under these conditions. Gel shift studies indicate that in addition to Sp1 and Egr-1, Sp3 and other factors, yet to be defined, bind specifically to the proximal PDGF-B promoter (Fig. 6). While overexpression studies are helpful in determining the functional importance of transcription factors, simultaneous overexpression of multiple factors with a reporter may not necessarily approximate the expression pattern of the authentic gene. This process is likely to be influenced by the nature, concentration, stoichiometry, and even the order of assembly of nuclear factors on the promoter.

While the present findings indicate that the proximal region of the PDGF-B promoter is required for high basal PDGF-B promoter-dependent expression in pup cells and that this region is bound by a large number of nuclear factors, elevated PDGF-B expression may not be mediated by these trans-acting factors alone. For example, basal and inducible PDGF-A transcription is, like PDGF-B, dependent upon specific interactions of Sp1, Sp3, and Egr-1 with the proximal promoter (33). Unlike PDGF-B, however, steady-state PDGF-A mRNA levels are reportedly similar in pup and adult SMCs but do increase with time in culture (4, 6). This may result from differences in the methylation state of the authentic promoter, or combinatorial interactions between transcription factors over promoter elements. Methylation of the herpes simplex virus thymidine kinase promoter results in loss of DNase I hypersensitivity and inhibition of transcription (34). Proactin promoter-dependent gene expression in pituitary tumor cells is inversely correlated with CpG methylation of the promoter and the ability of transcription factors to bind (35). The G+C-rich nature of the PDGF-A promoter adds support to this possibility (36). Second, transcription factors undergo ordered cooperative interactions over promoter elements to form stereospecific enhancer complexes that augment rates of transcription (37). Sp1 binds DNA upon promoter binding (38) and interacts synergistically with YY1 (39), Ets-1 (40), HIV Tat (41), GATA-1 (42, 43), and p53 (44). Differences in the nature of or content of other regulatory factors may, therefore, influence this highly selective process and contribute to the preferential expression of PDGF-B in cultured pup SMCs.

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