

Nuclear Factor- κ B Interacts Functionally with the Platelet-derived Growth Factor B-Chain Shear-Stress Response Element in Vascular Endothelial Cells Exposed to Fluid Shear Stress

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Abstract

Hemodynamic forces, such as fluid shear stress, that act on the endothelial lining of the cardiovascular system can modulate the expression of an expanding number of genes crucial for homeostasis and the pathogenesis of vascular disease. A 6-bp core element (5'-GAGACC-3'), defined previously as a shear-stress response element is present in the promoters of many genes, including the PDGF B-chain, whose expression is modulated by shear stress. The identity of the nuclear protein(s) binding to this element has not yet been elucidated. Using electrophoretic mobility shift assays and in vitro DNase I footprinting, we demonstrate that nuclear factor- κ B p50-p65 heterodimers, which accumulate in the nuclei of cultured vascular endothelial cells exposed to fluid shear stress, bind to the PDGF-B shear-stress response element in a specific manner. Mutation of this binding motif abrogated its interaction with p50-p65 and abolished the ability of the promoter to mediate increased gene expression in endothelial cells exposed to shear stress. Transient cotransfection studies indicate that p50-p65 is able to activate PDGF-B shear-stress response element-dependent reporter gene expression in these cells. These findings thus implicate nuclear factor- κ B in the transactivation of an endothelial gene responding to a defined fluid mechanical force. (*J. Clin. Invest.* 1995. 96:1169-1175.)

Key words: platelet-derived growth factor • fluid shear stress • nuclear factor- κ B • vascular endothelial cells

Introduction

The endothelial lining of blood vessels is continually subjected to fluid shear stresses by virtue of its contact with flowing blood.

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Cultured vascular endothelial cells that are exposed to laminar shear stresses comparable in magnitude to those generated by blood flow in the arterial circulation develop morphologic and functional alterations (1). Many of these endothelial responses to hemodynamic shear stresses involve changes in gene expression (2-11). Previously, we used 5' deletion and electrophoretic mobility shift analysis (EMSA)¹ to define a 6-bp core element (GAGACC) as a shear-stress response element (SSRE) in the PDGF B-chain promoter (12). This element is also present in many other genes whose expression in vascular endothelial cells is modulated by fluid shear stress (13).

Using the PDGF B-chain as a model, we asked whether modulation of gene expression by shear stress could be mediated by the direct interaction of a transcription factor with the SSRE. A recent report demonstrated increased levels of the p65 subunit of the pluripotent transcription factor nuclear factor- κ B (NF- κ B) in the nuclei of vascular endothelial cells exposed to fluid shear stress (14). In this study, we used EMSA and supershift analysis, in vitro DNase I footprinting with recombinant NF- κ B, and functional assays to show that p50-p65 heterodimers interact with the PDGF-B SSRE. Cotransfection studies indicate that recombinant NF- κ B can also transactivate PDGF-B SSRE-dependent reporter gene expression in these cells. These findings thus provide a correlative link between a transcription factor and a gene it transactivates in endothelial cells responding to a defined fluid mechanical force.

Methods

Cell culture and shear-stress conditions. Bovine aortic endothelial cells (BAEC) were isolated (15) and cultured in DME (Gibco BRL, Gaithersburg, MD) containing 10% calf serum (BioWhittaker, Inc. Walkersville, MD), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cells were passaged using trypsin/EDTA (BioWhittaker) and maintained in a humidified atmosphere of 5% CO₂/air at 37°C. Laminar shear stress was applied to confluent monolayers of BAEC (passage number < 5) at 10 dyn/cm² for 1 h using a cone-and-plate flow apparatus as previously described (12).

EMSA. Nuclear extracts were prepared as previously described (16) and incubated with 100,000 cpm of ³²P-P-SSRE in binding buffer (10 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 mg/ml

1. **Abbreviations used in this paper:** BAEC, bovine aortic endothelial cells; CAT, chloramphenicol acetyltransferase (EC 2.3.1.28); EMSA, electrophoretic mobility shift assay; NF- κ B, nuclear factor- κ B; SSRE, shear-stress response element; VCAM-1, vascular cell adhesion molecule-1.

salmon sperm DNA, and 1 mg/ml poly dI.dC-poly dI.dC) in a final volume of 20 μ l. After 30 min at 4°C, nondenaturing sample buffer was added to the binding reaction and loaded onto a 5% polyacrylamide/Tris-borate-EDTA gel and run at 200 V for 2 h at 22°C. ³²P-labeled PDI was incubated with nuclear extracts from cells exposed to shear stress (10 dyn/cm², 1 h) or treated with recombinant human TNF- α (200 U/ml, 1 h). In competition studies, unlabeled oligonucleotide was added 10 min before the addition of probe. In supershift studies, rabbit polyclonal anti-peptide antibodies (generous gift of Dr. N. Rice) were added 10 min before the addition of the probe. When recombinant proteins were used in EMSA, the binding buffer contained 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mg/ml BSA, 0.5% NP-40, and 1 mM DTT.

In vitro DNase I footprinting. A single end-labeled fragment of the human PDGF-B promoter was prepared by digesting construct d24-chloramphenicol acetyltransferase (CAT) (17) with EcoRI. The DNA was ³²P-labeled and cut with HindIII to yield two labeled fragments of 280 and 360 bp. The larger fragment was isolated by electrophoresis and purified using glass beads. G ladders were generated by methylation of the probe with dimethyl sulfate and piperidine cleavage. Binding reactions and electrophoresis were performed as previously described (17).

Plasmid construction and transfection procedure. Construct d26mSSRE-CAT was made by subcloning a PCR fragment bearing a block mutation (indicated by lower case lettering) in the SSRE with 5'-TGAAGGTTGCTCGGCTCTacactgtagcaTAAGCG-3' as the 5' primer and 5'-TGGGCTGCAGGTCGACTCTAG-3' as the 3' primer and d24-CAT (17) as the template. The sequence spanned by the PCR fragment included the PDGF-B core promoter (17) and elements upstream. The fragment was blunt-end subcloned into the SmaI site of pCAT3 (12). SSRE.SV40-CAT hybrid constructs were made by subcloning oligonucleotide inserts into the EcoRI-BglII sites of pCAT-promoter (Promega Corp., Madison, WI). All constructs were sequenced before use by the dideoxynucleotide termination protocol (18). BAEC were transfected with 8 μ g of construct and 8 μ g of cytomegalovirus β -galactosidase as the normalizing plasmid using the calcium phosphate precipitation technique (17). CAT activity was assessed 48 h after the change in the culture medium and was corrected for the efficiency of transfection after assessment of β -galactosidase levels in the cell lysate.

SSRE.SV40-CAT and vascular cell adhesion molecule-1 (VCAM-1).SV40-CAT were constructed by subcloning double-stranded oligonucleotide inserts into the blunt-ended BglII site of pCAT-promoter. All constructs were sequenced before use. BAEC were transfected with 10 μ g of construct and 2 μ g of pTKGH (Nichols Institute, San Juan Capistrano, CA) as the normalizing plasmid using the calcium phosphate precipitation technique (17). For cotransfection experiments, 0.04 μ g of p50 and p65 in pcDNA (Invitrogen, San Diego, CA) or 0.04 μ g of pcDNA alone were used. CAT activity was assessed 48 h after the change in the culture medium (with or without 200 U/ml of TNF- α) and corrected for the efficiency of transfection after assessment of growth hormone levels in the culture supernatant by radioimmunoassay.

Results

An oligonucleotide bearing the PDGF-B SSRE interacts with nuclear proteins from vascular endothelial cells exposed for 1 h to physiological levels of fluid shear stress. Cultured BAEC were exposed to a physiological level of laminar shear stress (10 dyn/cm²) for 1 h, using a well-characterized in vitro fluid mechanical system (19). A distinct nucleoprotein complex was detected in an EMSA in which nuclear extracts from these cells were incubated with a radiolabeled oligonucleotide (P-SSRE) whose sequence spans the PDGF-B SSRE (Fig. 1 A). Excess unlabeled cognate competed with this complex, whereas an oligonucleotide bearing the consensus nucleotide recognition sequence for the transcription factor AP-1, whose protein compo-

nents (*c-fos* and *c-jun*) are themselves induced by shear stress in vascular endothelial cells (7, 9, 14), did not compete (Fig. 1 A). The rapid induction of nascent and steady-state PDGF-B transcripts, as detected by transcriptional run-off (Khachigian, L. M., and T. Collins, unpublished data) and Northern blot analysis (4), respectively, and the appearance of the nucleoprotein complex by EMSA 10 min (Resnick, N., and M. A. Gimbrone, Jr., unpublished data) after the application of the fluid mechanical force suggests that an early response transcription factor might be responsible for the modulation of PDGF-B gene expression by shear stress. Upon inspection, the PDGF-B SSRE bears resemblance to the consensus nucleotide recognition sequences for the p50 (NF- κ B1) and p65 (Rel A) subunits of NF- κ B (20) (Fig. 1 B). Ferguson analysis (17, 21, 22) revealed that the combined size of the nuclear proteins from shear-stressed endothelial cells that bound ³²P-P-SSRE was ~ 111 kD (Fig. 1 C). Collectively, these findings suggested that members of the NF- κ B/Rel family may interact with the SSRE.

Shear-activated and recombinant nuclear factor- κ B p50-p65 heterodimers bind to the PDGF-B SSRE. Nuclear extracts from shear-stressed cells, but not from static cells, bound specifically to an oligonucleotide bearing a consensus NF- κ B motif from the positive regulatory domain (PDI) of the E-selectin promoter (23) (Fig. 2). Mutation of the oligonucleotide sequence in the NF- κ B binding site (PDI_m) (23) abrogated its ability to compete with the probe (Fig. 2). The electrophoretic mobility of the shear-induced band was identical to that obtained with nuclear extracts from endothelial cells stimulated by TNF- α (Fig. 2), a potent inducer of NF- κ B binding activity (24), or when ³²P-P-SSRE was substituted for ³²P-PDI (Fig. 2). To establish the identity of the shear-induced nuclear proteins that bound ³²P-P-SSRE, we used antibodies to peptides of specific NF- κ B/Rel subunits. The shear-induced band was eliminated by the presence of either anti-p50 or anti-p65 in the binding reaction, whereas anti-c-Rel or preimmune serum failed to affect the formation of this complex (Fig. 2). Accordingly, the identity of the nuclear protein that bound ³²P-P-SSRE as a consequence of shear stress was the p50-p65 heterodimeric form of NF- κ B. The rapid translocation of NF- κ B from the endothelial cytoplasm to the nucleus was further indicated by the nuclear accumulation of immunoreactive p65 as early as 10 min after the application of shear (Resnick, N., and M. A. Gimbrone, Jr., unpublished data). Using recombinant proteins, ³²P-P-SSRE was bound by p50-p65 (Fig. 3 A) and p50-p50 (Fig. 3 A) but not c-Rel (Khachigian, L. M., and T. Collins, unpublished data) in a specific manner. Unlabeled P-SSRE and an oligonucleotide bearing a consensus palindromic binding site for NF- κ B, κ B-PD (23) could compete with the interaction (Fig. 3 A), whereas the AP-1 consensus oligonucleotide did not compete (Fig. 3 A). Furthermore, in vitro DNase I footprint analysis revealed that both p50-p65 (Fig. 3 B) and p50-p50 (Khachigian, L. M., and T. Collins, unpublished data) bound specifically to a larger fragment of the PDGF-B promoter containing the SSRE in a dose-dependent manner. An interaction between NF- κ B and the PDGF-B promoter has hitherto not been reported.

NF- κ B binding site in the PDGF-B promoter is required for shear-induced reporter gene expression. To determine whether the NF- κ B binding site was crucial for shear-induced SSRE-dependent gene expression, we mutated the element in the context of the intact PDGF-B promoter as well as in isolation in a heterologous expression vector. A series of mutant forms of P-SSRE were generated and screened by EMSA for their

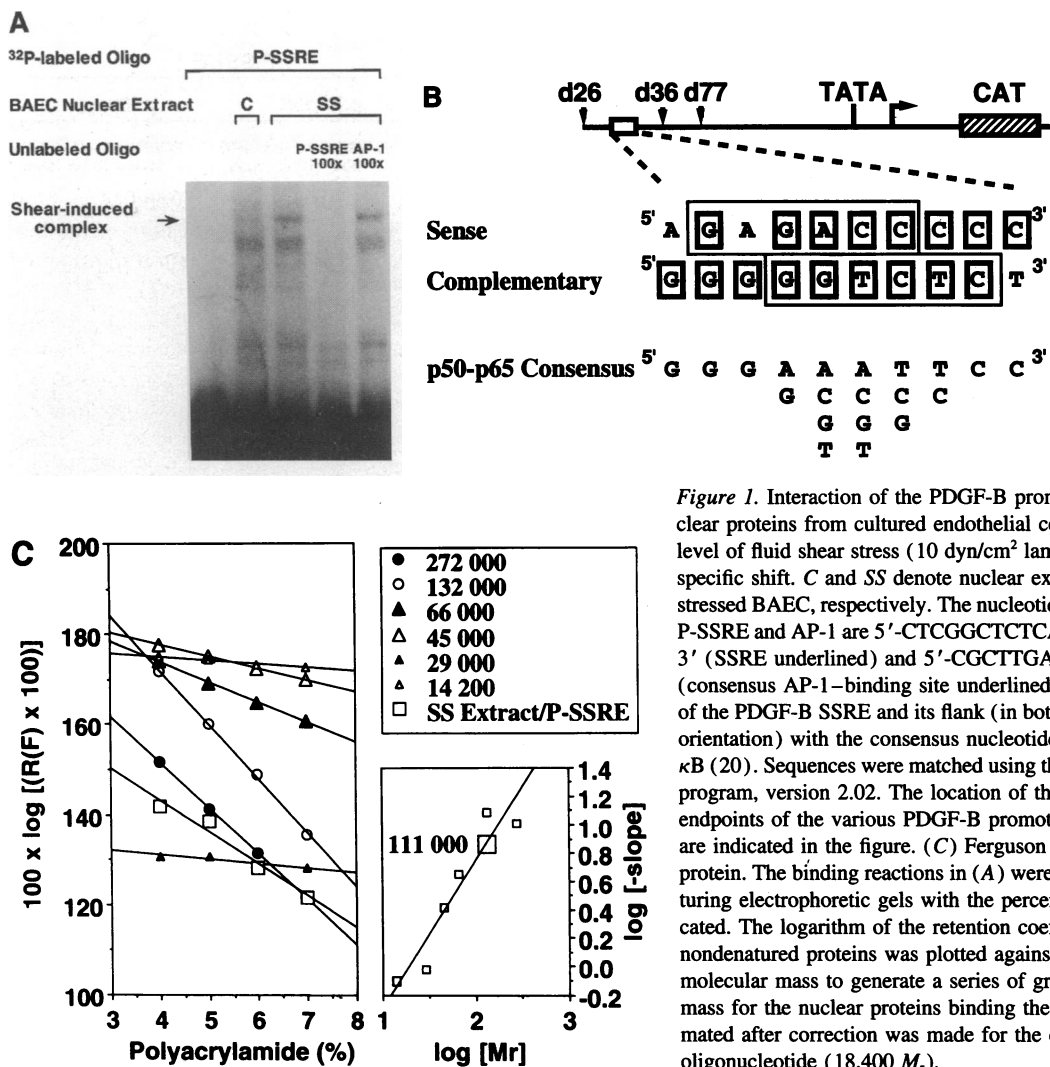


Figure 1. Interaction of the PDGF-B promoter oligonucleotide with nuclear proteins from cultured endothelial cells exposed to a physiological level of fluid shear stress (10 dyn/cm² laminar flow). (A) Shear-induced specific shift. C and SS denote nuclear extracts from static and shear-stressed BAEC, respectively. The nucleotide sequences of oligonucleotide P-SSRE and AP-1 are 5'-CTCGGCTCTCAGAGACCCCTAAGCGCC-3' (SSRE underlined) and 5'-CGCTTGATGACTCAGCCGGAA-3' (consensus AP-1-binding site underlined), respectively. (B) Alignment of the PDGF-B SSRE and its flank (in both the sense and complementary orientation) with the consensus nucleotide recognition sequence for NF- κ B (20). Sequences were matched using the IBI Pustell sequence analysis program, version 2.02. The location of the SSRE and the 5' deletion endpoints of the various PDGF-B promoter-reporter constructs (12, 17) are indicated in the figure. (C) Ferguson analysis of the SSRE-binding protein. The binding reactions in (A) were applied to a series of nondenaturing electrophoretic gels with the percentage of polyacrylamide indicated. The logarithm of the retention coefficients (R_F) for a number of nondenatured proteins was plotted against the logarithm of the relative molecular mass to generate a series of gradients. The relative molecular mass for the nuclear proteins binding the oligonucleotide was approximated after correction was made for the contribution to mass by the oligonucleotide (18,400 M_r).

ability to bind to recombinant NF- κ B. One oligonucleotide (P-SSRE) did not compete with ³²P-P-SSRE for binding to shear-induced p50-p65 or recombinant p50-p50 even at 200 \times molar excess (Fig. 4 A). The same block mutation was introduced into the intact PDGF-B promoter-reporter construct, d26-CAT (Fig. 4 B), whose expression in endothelial cells is activated by shear stress (12). BAEC transiently transfected with the mutant plasmid (d26mSSRE-CAT) failed to respond to 10 dyn/cm² laminar shear stress, unlike cells transfected with the wild-type counterpart (Fig. 4 B). When a shorter promoter fragment was placed upstream of an SV40 promoter-CAT reporter, the intact SSRE (SSRE.SV40-CAT), or its complementary sequence (SSRE(C).SV40-CAT), conferred shear responsiveness on the heterologous construct (Fig. 4 B). Mutation of the SSRE itself (SSRE(5'm).SV40-CAT) abolished the response to laminar shear stress (Fig. 4 B). Similarly, no response to shear stress was observed when the CAT reporter gene was placed under the transcriptional control of two viral enhancers (CMV-CAT, SV40-CAT), despite different amounts of expression in transfected endothelial cells (Fig. 4 B). To demonstrate that the SSRE could serve as a functional NF- κ B site, the effects of recombinant NF- κ B and cytokine on reporter expression driven by this region of the PDGF B-chain promoter were as-

essed. CAT activity increased when BAEC were cotransfected with SSRE.SV40-CAT and expression constructs of NF- κ B (p50 and p65) (Fig. 5), whereas levels of the reporter did not change when the vector alone was used (Fig. 5). Expression driven by a fragment of the human VCAM-1 promoter that contained a single NF- κ B binding site (VCAM-1.SV40-CAT) was similarly induced by p50-p65 (Fig. 5). CAT activity also increased when BAEC were transfected with SSRE.SV40-CAT or VCAM-1.SV40-CAT and subsequently exposed to TNF- α (Khachigian, L. M., and T. Collins, unpublished data). These data provide evidence, independently of shear stress per se, that NF- κ B can activate reporter gene expression through the SSRE. The ability of the SSRE to function in isolation with a heterologous promoter is consistent with the functional properties of NF- κ B binding elements reported elsewhere (25).

Discussion

In this paper, we demonstrate that heterodimeric (p50-p65) NF- κ B interacts functionally with the SSRE in the promoter of the human PDGF B-chain gene in vascular endothelial cells exposed to fluid shear stress. Gel retardation assays indicate that both p50 and p65 accumulate in endothelial nuclei and bind to

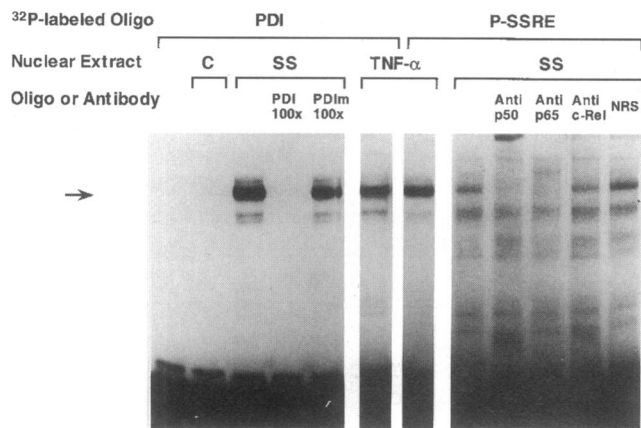


Figure 2. NF- κ B interacts with the PDGF-B SSRE in endothelial cells exposed to fluid shear stress. *C* and *SS* denote nuclear extracts from static and shear-stressed BAEC, respectively. The oligonucleotide sequence of 32 P-labeled PDI is 5'-GGATGCCATTGGGGATTTCCCTTTACTGGATGT-3'. Competitor oligonucleotides were PDI (5'-AGCTTAGAGGGGATTTCCGAGAGGA-3') and PDIIm (5'-AGCTTAGATtttATTTCCGAGAGGA-3'). The NF- κ B consensus site is underlined and mutations are indicated by lower case lettering. *NRS* denotes normal rabbit serum.

the PDGF-B SSRE within 1 h of exposure to shear stress. This is consistent with a recent report that indicates increased amounts of nuclear p65 using an oligonucleotide probe bearing a consensus κ B site derived from an immunoglobulin promoter sequence (14). Mutation of the PDGF-B SSRE abrogated both recombinant and shear-induced p50-p65 binding and abolished activation of PDGF-B SSRE-driven gene expression in the context of hybrid promoter and promoter-reporter constructs in endothelial cells exposed to shear stress. In transient cotransfection experiments, recombinant p50-p65 activated PDGF-B SSRE-dependent reporter gene expression. These findings suggest, using the PDGF-B promoter as a model, that the interaction of NF- κ B with an SSRE may be an important regulator of gene expression in cultured vascular endothelial cells exposed to physiologically relevant biomechanical forces.

Although nuclear translocation of NF- κ B clearly occurs when endothelial cells are exposed to biomechanical forces, this does not appear to be sufficient to modulate the expression of all genes that contain NF- κ B nucleotide recognition sequences. For example, it has been established that cytokine stimulation of E-selectin (23, 24, 26) and VCAM-1 (15) expression is mediated in part by multiple NF- κ B binding sites in their respective promoters. Yet, these genes are not induced by laminar shear stress in cultured human umbilical vein endothelial cells (10). Consistent with this, nuclear run-off assays do not indicate increased rates of transcription of these genes (Khachigian, L. M., and T. Collins, unpublished data). Similarly, reporter expression driven by minimal promoters from E-selectin and VCAM-1 in transfected BAEC is not responsive to physiological levels of fluid shear stress (Resnick, N., and M. A. Gimbrone, Jr., unpublished data). Nonetheless, the E-selectin-derived PDI probe (which contains a consensus NF- κ B-binding site) can interact with shear-stressed endothelial nuclear extracts (Fig. 2) and a reporter construct driven by a fragment of the VCAM-1 promoter bearing an NF- κ B site can be transactivated by p50-p65 cotransfection in a manner similar to that observed using PDGF-B SSRE (Fig. 5).

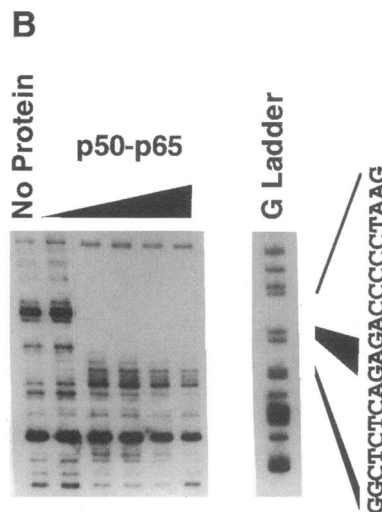
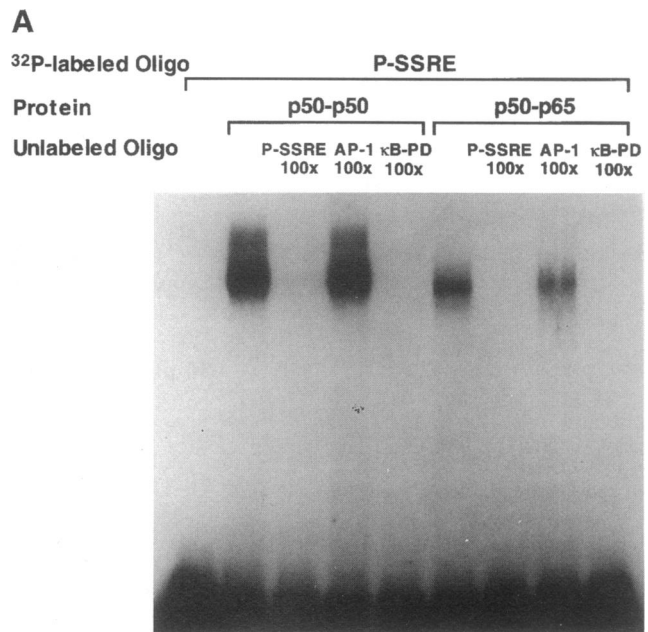


Figure 3. Recombinant NF- κ B interacts with the PDGF-B promoter SSRE. (A) EMSA using bacterially expressed NF- κ B. The sequence of κ B-PD is 5'-CAACGGCAGGGGAATTTCCCTCTCCTT-3'. The palindromic NF- κ B binding site is underlined. The sequence of oligonucleotides P-SSRE and AP-1 appears under Fig. 1 A. (The preparation of p65 was truncated, leaving the DNA-binding domain intact, to enable efficient purification of this bacterially expressed protein. The p50 subunit was full length. Accordingly, the p50-p65 heterodimer has a molecular mass lower than p50-p50, thus accounting for the faster electrophoretic mobility of nucleoprotein complexes involving p50-p65). (B) In vitro DNase I footprinting using a PDGF-B promoter fragment spanning the SSRE.

Conceivably, selectivity in the shear-induced transactivation of certain endothelial genes that bear NF- κ B-binding sites could be explained by one or more mechanisms. First, differences in the primary sequence of the NF- κ B-binding site in different endothelial genes might modulate the binding and functional properties of NF- κ B (20, 25, 27, 28). Second, NF- κ B could be functionally cooperating with other transcription factors; indeed, its activation alone is not sufficient to stimulate

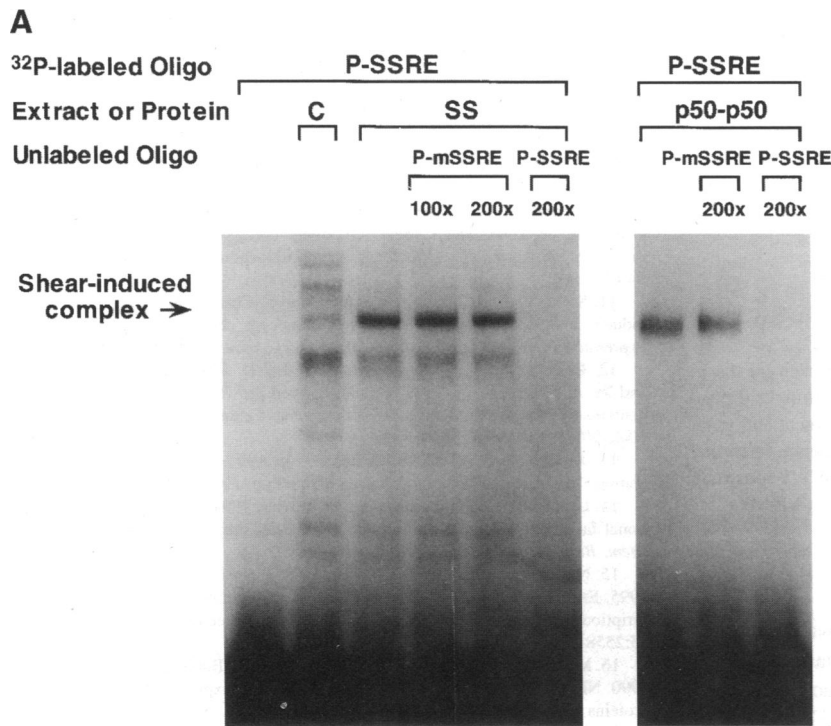


Figure 4. Effect of fluid shear stress on cultured BAEC transiently transfected with wild-type and mutant PDGF-B promoter-CAT constructs. (A) EMSA using wild-type (P-SSRE) and mutant (P-mSSRE) PDGF-B promoter oligonucleotides. C and SS denote nuclear extracts from static and shear-stressed BAEC, respectively. The sequence of P-mSSRE is 5'-CTCGGCTCTacactgtagcaTAAGCGCC-3' (mutation indicated by lower case lettering). (B) Fold induction in growth hormone-normalized CAT activity by shear stress in BAEC transiently transfected with PDGF-B promoter-CAT constructs (17). SSRE(C) denotes the SSRE complementary sequence. Statistical analyses using Student's two-tailed *t* test indicated a significant difference in the normalized CAT activities between static and shear-stressed BAEC transfected with d26-CAT ($P = 0.0093$, $n = 3$), SSRE.SV40-CAT ($P = 0.0012$, $n = 3$), and SSRE(C).SV40-CAT ($P = 0.0017$, $n = 3$). In contrast, differences in CAT activities between static and shear-stressed BAEC transfected with d26mSSRE ($P = 0.980$, $n = 3$), SSRE(5'm).SV40-CAT ($P = 0.864$, $n = 3$), SV40-CAT ($P = 0.990$, $n = 3$), and CMV-CAT ($P = 0.962$, $n = 3$) were not significant.

B

Construct	Sequence	Fold Induction
PDGF-B		
d26-CAT	11bp TCGGCTCTCAGAGACCCCT 122bp TATA	3.3
d26mSSRE-CAT	11bp TCGGCTCTacactgtagcaT 122bp TATA	1.1
Heterologous		
SSRE.SV40-CAT	TCGGCTCTCAGAGACCCCT	3.6
SSRE(C).SV40-CAT	TCGGCTCTCAGGCTCTCCCT	3.8
SSRE(5'm).SV40-CAT	TCGGCTCTCAccctaaCCCT	1.3
SV40-CAT		0.9
CMV-CAT		1.0

E-selectin (23, 26, 29, 30) or VCAM-1 (15) reporter expression in endothelium. Third, shear stress may act to modify the activity of NF- κ B or alter the expression of transcription factors that bind to NF- κ B. NF- κ B is known to interact with Sp1 (31), c-Fos (32), c-Jun (32), HMG1(Y) (33), C/EBP (34), and ATF-2 (35, 36), and at least one of these transcription factors, Sp1, plays a functional role in the PDGF-B core promoter (17). Finally, shear stress may generate negative regulatory signals that attenuate the induction of gene expression by NF- κ B (37, 38). For example, the constitutively high level of VCAM-1 expression observed in certain cultured mouse lymph node endothelial cells is actually down-regulated by shear stress (39). Thus, the precise basis for the selective activation by shear stress of certain endothelial genes bearing NF- κ B sites is not yet clear, and even in the case of the PDGF-B gene, may involve transcription factors other than NF- κ B.

The mechanism with which NF- κ B is itself activated by shear stress may involve signal transduction events which result

in phosphorylation and subsequent degradation of its cytoplasmic inhibitor, I κ B- α (40-42). Protein kinases, that have been implicated in the phosphorylation of I κ B- α (43-45), are activated in endothelial cells exposed to shear stress (5). The recent demonstration that depolymerization of microtubules can lead to the activation of NF- κ B (46) is consistent with the hypothesis that fluid biomechanical forces may influence gene expression via changes in cytoskeletal architecture (1, 47).

Endothelial cells appear to respond to hemodynamically generated forces by elaborating a variety of autocrine and paracrine mediators through poorly understood signal transduction pathways (1, 13, 48). The data presented here provide a link between a pluripotent transcription factor, NF- κ B (49), and a functionally defined laminar SSRE in the context of a physiologically relevant gene expressed by vascular endothelial cells, PDGF-B. Modulation of PDGF-B gene expression in endothelial cells by changes in the local fluid-mechanical environment may contribute to altered vascular reactivity and struc-

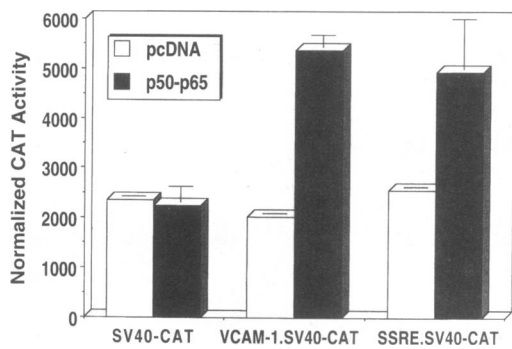


Figure 5. Effect of recombinant NF- κ B on expression driven by the PDGF-B SSRE. BAEC were transiently cotransfected with hybrid promoter-reporter constructs and p50-p65 expression constructs and assessed for normalized CAT activity. The nucleotide sequence from the human VCAM-1 promoter present in the VCAM-1.SV40-CAT construct was 5'-TTGAAGGGATTTCCTCCGCCTCTGCAA-3' (NF- κ B-binding site underlined).

tural remodeling that typically occurs in hypertensive and atherosclerotic vascular disease (50, 51). Investigation of the biochemical pathways responsible for NF- κ B activation by shear stress and elucidation of other SSREs (11, 52) may provide further basic insights into the intracellular signal transduction mechanisms involved in biomechanical coupling.

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