Fluid shear stress activation of egr-1 transcription in cultured human endothelial and epithelial cells is mediated via the extracellular signal-related kinase 1/2 mitogen-activated protein kinase pathway.

J L Schwachtgen, … , V Sukhatme, M Braddock

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Research Article

The primary response transcription factor, early growth response-1 (Egr-1), is rapidly activated by a variety of extracellular stimuli. Egr-1 binds to a sequence found in the promoters of genes involved in vascular injury, such as PDGF-A and tissue factor, and trans-activates their expression in endothelial cells in response to fluid shear stress. Here we show that egr-1 mRNA is increased after 30 min of flow in human aortic endothelial cell and HeLa cell cultures. Transient transfection of HeLa cells with reporter gene constructs driven by the murine or human egr-1 5' flanking sequence revealed a five- and ninefold induction, respectively, in transcriptional activity after exposure to a shear stress of 5 dynes/cm2 for 3 h. Deletion of sequences in the murine promoter containing two AP1 sites and an inhibitory Egr-1 binding sequence, did not reduce shear stress inducibility. However, progressive deletion of five serum response elements, reduced both the basal promoter activity and its capacity to be activated by shear stress. Further examination indicated that the three upstream serum response elements are predominantly responsible for shear stress activation of the egr-1 promoter. Treatment of cells with PD98059, a specific inhibitor of mitogen-activated protein kinase-1 inhibited shear stress activation of egr-1. We suggest that egr-1 activation by shear stress involves activation of Elk-1 but not c-jun activity. These data, which are […]

Fluid Shear Stress Activation of *egr-1* Transcription in Cultured Human Endothelial and Epithelial Cells Is Mediated via the Extracellular Signal-related Kinase 1/2 Mitogen-activated Protein Kinase Pathway

Jean-Luc Schwachtgen, Parul Houston, Callum Campbell, Vikas Sukhatme,* and Martin Braddock

*Endothelial Cell Gene Expression Group, Vascular Diseases Unit, Glaxo-Wellcome Medicines Research Centre, Stevenage, Herts SG1 2NY England; and *Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215*

Abstract

The primary response transcription factor, early growth response-1 (Egr-1), is rapidly activated by a variety of extracellular stimuli. Egr-1 binds to a sequence found in the promoters of genes involved in vascular injury, such as PDGF-A and tissue factor, and *trans*-activates their expression in endothelial cells in response to fluid shear stress. Here we show that *egr-1* mRNA is increased after 30 min of flow in human aortic endothelial cell and HeLa cell cultures. Transient transfection of HeLa cells with reporter gene constructs driven by the murine or human *egr-1* 5′ flanking sequence revealed a five- and ninefold induction, respectively, in transcriptional activity after exposure to a shear stress of 5 dynes/cm^2 for 3 h. Deletion of sequences in the murine promoter containing two AP1 sites and an inhibitory Egr-1 binding sequence, did not reduce shear stress inducibility. However, progressive deletion of five serum response elements, reduced both the basal promoter activity and its capacity to be activated by shear stress. Further examination indicated that the three upstream serum response elements are predominantly responsible for shear stress activation of the *egr-1* promoter. Treatment of cells with PD98059, a specific inhibitor of mitogen-activated protein kinase-1 inhibited shear stress activation of *egr-1*. We suggest that *egr-1* activation by shear stress involves activation of Elk-1 but not c-jun activity. These data, which are consistent with previous findings for shear mediated signaling via the mitogen-activated protein kinase cascade, now implicate shear modulation of the Egr-1 transcription factor in this pathway. (*J. Clin. Invest.* 1998. 101:2540–2549.) Key words: fluid shear stress • *egr-1* promoter • autoregulation • extracellular signal–related kinase 1/2 mitogen-activated protein kinase • vascular endothelial cells

Introduction

Vascular endothelial cells (ECs)[1] line the vessel wall and are in intimate contact with the flowing blood, which establishes a viscous drag over the EC monolayer. Thus, frictional forces are established between the blood and this cellular interface. This physical barrier serves to regulate the transport of blood borne substances and to transduce mechanical forces to the underlying vasculature. Thus ECs are constantly exposed to mechanical forces that modulate both their morphology (1–4) and expression of genes within the endothelium (for review see references 5 and 6). Recently it has become clear that fluid shear stress, one of the most important mechanical stimulators, mediates transcription factor activation by specific intracellular kinase signaling cascades (7). Very early events after the onset of flow are the activation of K^+ membrane channels (8), G proteins (9), and signaling pathways (10–12) followed by the regulation of integrin and focal adhesion kinase function (10, 13). Structural changes and changes in cellular conformation are observed later in the sequence of mechanotransduction. (2, 3).

Fluid shear stress activates the expression of many genes predominantly at the level of transcription. These include several proadhesive, proinflammatory, or prothrombotic genes such as intercellular adhesion molecule-1 (14), monocyte chemoattractant protein-1 (15), and tissue factor (TF) (16). Transcriptional activation of these genes has been shown to proceed via *cis*-acting sequences that bind transcription factors Sp1 (16), NF-κB (17), AP1 (18, 15), and early growth response-1 (Egr-1) (12, 13). Transcription factors AP1 and NF-κB are themselves upregulated by shear stress (19). Transcriptional activation of *egr-1* is responsible for the coordinated expression of endothelial proteins such as PDGF-A, PDGF-B, TGFβ, uPA, and TF that are involved in vascular remodelling after injury (20). All of these genes contain Egr-1 binding sites (EBSs) in their 5′-flanking sequence and they have been shown to be transcriptionally responsive to biochemical stimulation such as alteration in fluid shear stress (for

Address correspondence to Dr. Martin Braddock, Endothelial Cell Gene Expression Group, Vascular Diseases Unit, Glaxo-Wellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts SG1 2NY England. Phone: 01144-1438-764149; FAX: 01144-1438-764488; E-mail: mb4554@ggr.co.uk

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1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cells; CAT, chloramphenicol acetyl transferase; CMV, cytomegalovirus; dbd, DNA binding domain; EBS, Egr-1 binding site; EC, endothelial cell; Egr, early growth response; ERK, extracellular signal–related kinase; HAEC, human aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; MEK1, mitogen-activated protein kinase kinase-1; nt, nucleotide(s); SRE, serum response element; SRF, serum response factor; SSREs, shear stress responsive elements; TCF, ternary complex factor; TF, tissue factor; WT, Wilm's tumor suppressor protein.

review see references 6 and 21–23). In a recent study, Khachigian et al. showed that shear stress *trans*-activation of PDGF-A expression was dependent on the binding of Egr-1 to its cognate *cis*-acting regulatory element in the promoter (24). Therefore, a model has been proposed (22, 25), suggesting that rapid shear stress mediated *trans*-activation of *egr-1* results in the displacement of Sp1 on the TF promoter by a mechanism analogous to injury-mediated induction of TF expression (20).

The *egr-1* gene codes for a zinc finger–containing DNA binding factor and is part of the family of immediate early response genes (26–28). Egr-1 is induced by signals that stimulate mitogenesis and differentiation (29–34) and also by changes in the local cellular environment such as variation in osmotic stress (35). Invariably, the transcriptional induction of *egr-1* is mediated through serum response elements (SREs) in the 5′-flanking region of the gene.

In the light of the suggested role of Egr-1 in shear stress *trans*-activation, we wanted to explore the possibility that the *egr-1* gene may itself be upregulated by shear stress and investigate the signal transduction intermediates involved in this pathway. We now report that shear stress *trans*-activation of *egr-1* proceeds via SREs in the *egr-1* promoter and that the mitogen-activated protein kinase kinase-1 (MEK1) inhibitor PD98059 inhibits shear stress activation of *egr-1*. Furthermore, we suggest that shear stress increases Elk-1, but not c-jun activity, and that the extracellular signal related kinase (ERK)-1/2 signaling pathway predominates for shear stress activation of *egr-1*.

Methods

Cell culture and shear stress experiments. Human aortic ECs (HAEC) and human umbilical vein ECs (HUVEC), frozen in liquid nitrogen, were purchased from BioWhittaker. Cells were thawed and grown in endothelial growth medium (BioWhittaker, Workingham, UK) according to the manufacturer's instructions. HeLa cells and bovine aortic ECs (BAEC) were cultured in DME (HyClone, Logan, Utah) supplemented with 10% FCS, 2 mM glutamine, and 100 U/100 μg of penicillin/streptomycin (Life Technologies, Grand Island, NY). For shear stress experiments cells were seeded into 9 cm^2 Nunc Slide-Flasks (Life Technologies). After the cells reached confluence, the base of the slide was assembled into a parallel plate flow chamber using a similar parallel plate flow chamber as described previously (36). Parallel plate flow chambers were custom made at the Glaxo-Wellcome Department of Bioengineering (Stevanage, UK). The temperature of the flow system was kept at a constant 37°C and the pH was kept constant by gassing with 95:5% air/CO_2. A peristaltic pump (Masterflex, Barrington, IL) was calibrated to deliver a shear stress of 2.2, 5, 10, and 20 dynes/cm^2 for up to 3 h. Static controls were performed on cells not exposed to shear. In some experiments, cells were preincubated with either 0.1% DMSO or 50 or 75 μM PD98059 (New England Biolabs, Hitchin, UK) in 0.1% DMSO for 30 min and exposed to shear stress at 5 dynes/cm^2 for 1 h.

Western blot analysis of Egr-1 induction in HAEC subjected to shear stress. Protein extracts (20 μg) were run under reducing conditions in 8% SDS-PAGE gels and transferred onto nitrocellulose membranes according to the manufacturer's instructions (Novex, San Diego, CA). Egr-1 protein was detected with antibody Egr-1 588 (Santa Cruz Biotechnology, Santa Cruz, CA) using enhanced chemiluminescence (Amersham International, Little Chalfont, UK).

RNA isolation and ribonuclease protection assay. To obtain an antisense *egr-1* RNA probe, HeLa cells were serum-starved in DME containing 0.5% FCS for 24 h and then incubated in DME containing 20% FCS for 15 min. Total RNA was isolated by the RNeasy kit (Qiagen, Chatsworth, CA) and cDNA was synthesized from 500 ng of total RNA by reverse transcription as previously described (37). A fragment spanning from nucleotide (nt) +507 to +978 of the human *egr-1* gene was synthesized by PCR. The sequence of the forward primer was 5′-CAG CAG CAG CAG CAG CAC C-3′ and the sequence of the reverse primer was 5′-TAA TAC GAC TCA CTA TAG GCA GGG TAG GCA GGA GGC GG-3′. The reverse primer incorporated a T7 RNA polymerase promoter (underlined) into the PCR fragment, allowing the synthesis of a probe spanning 488 nt of the human *egr-1* gene by in vitro transcription in the presence of [α-^{33}P]UTP (3,000 Ci/mmol) (Amersham International) with the MaxiScript kit (Ambion, Austin, TX). The antisense human β-actin and cyclophilin RNA probes were synthesized from the T7 promoter in the plasmids pTri-β-actin-125-human and pTri-cyclophilin, respectively (Ambion). The RNA probes were hybridized to 5 μg of total RNA using the HybSpeed RPA Kit (Ambion) and digested by RNase A/T1 digestion, and samples were run on 6% denaturing polyacrylamide gels. According to the manufacturer (Ambion), the presence of several protected bands with the β-actin probe could be due to breathing of the extremities in the β-actin RNA–RNA hybrid. The transcript levels were quantitated with a Storm system with ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA).

Plasmid constructions. The mouse *egr-1* promoter chloramphenicol acetyl transferase (CAT) reporter plasmids have been described previously (38). Mouse Egr-1 expression plasmids p930 and p858 and the Wilm's tumor suppressor protein (WT)1/Egr expression plasmids have also been described previously (39). The human *egr-1* promoter fragment spanning from nt −674 to +12 (40) was synthesized by PCR in a reaction containing 0.5 μg of human placental genomic DNA as a template, 0.4 mM of dATP, dCTP, dGTP, and dTTP, 25 pmoles of the forward primer (5′-GGC CAC GCG TCG TCG GTT CGC TCT CAC GGT CCC-3′ [MluI restriction site is underlined]), 25 pmoles of the reverse primer (5′-GCA GCT CGA GGC TGG ATC TCT CGC GAC TCC-3′ [XhoI site is underlined]), and Vent DNA polymerase (New England Biolabs). The PCR fragment was cut with MluI and XhoI, agarose gel-purified, and cloned between the MluI and XhoI sites in the multiple cloning site of the vector pGL3 basic (Promega, Madison, WI). The plasmid pGLE(−409/−300)d was synthesized by inverse PCR using plasmid pGLE as template and Pfu polymerase (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The forward (5′-GGA ATTC GAA GGC GGA GGG GC-3′) and the reverse (5′-GGA ATT CCG GTC CGA GCT GGT TGC-3′) primers incorporate an EcoRI site. After amplification, the linear construct pGLE (−409/−300)d was cut by EcoRI, gel-purified, and circularized by ligation. The plasmids pGLEmSRE4, pGLEmEts, and pGLEmEBS were constructed using the QuickChange mutagenesis kit according to the instructions of the manufacturer (Stratagene) with primers spanning SRE4; 5′-CCC GCC GGA ACA ACC CCA ATC GGG GCA GCA CCT TAT TTG G-3′: the Ets sequence at nt −315; 5′-GAT ATG GCC CGG CGC TTG CGC CTC TGG GAG G-3′; and the EBS: 5′-CGA CTC GCC CTC GCT ATC GCT CTG GGT CTG G-3′ (mutated nt are underlined). To generate the pGLSV constructs, three double-stranded oligonucleotides spanning 83 nt of the *egr-1* gene 5′-flanking sequence between nt −399 and −316 were inserted in front of the SV40 minimal promoter into the NheI site of the plasmid pGL3 promoter (Promega). For plasmid pGLSV1 the sequence of the sense strand is 5′-AGC AGG AAG GAT CCC CCG CCG GAA CAA CCC TTA TTT GGG CAG CAC CTT ATT TGG AGT GGC CGG ATA TGG CCC GGC GCT TCC GC-3′. The sequence inserted into plasmid pGLSV2 is 5′-AGC AGG AAG GAT CCC CCG CCG GAA CAA CCG TAA TTT CGG CAG CAC GTA ATT TCG AGT GGC CGG ATA TGG CCC GGC GCT TCC GC-3′, where the two CC(A/T)6G boxes have been changed to a sequence (underlined) that does not permit serum response factor (SRF) binding. Plasmid pGLSV3 contains the sequence 5′-AGC ACG AAG GAT CCC CCG CCC GAA CAA CCC TTA TTT GGG CAG CAC CTT ATT TGG AGT GGC CGG ATA TGG CCC GGC GCT TCG GC-3′. In this construct the three Ets binding sites have been mutated (underlined). The orientation and boundaries of all constructs and complete sequences of fragments obtained

by PCR were confirmed by sequencing using T7 polymerase (Pharmacia Biotech, Piscataway, NJ).

Transient transfections. Plasmid DNA was prepared by alkaline lysis and column purification (Qiagen). HeLa cells were transfected using calcium phosphate–DNA coprecipitation with 5 μg of reporter vector and 0.5 μg of control vector CMVβ (Clontech, Palo Alto, CA) coding for β-galactosidase. BAEC were transfected with 0.1 μg of reporter vector, 0.07 μg of vector cytomegalovirus (CMV)β, and 4.8 μg of PBS (Stratagene) as carrier plasmid. After an 18-h incubation, the culture medium was changed to DME supplemented with either 1% FCS for HeLa cells or 10% FCS for BAEC. Cells were cultured for a further 24 h before being subjected to shear stress for up to 3 h. At the end of the experiment luciferase activity was measured and normalized by the β-galactosidase activity.

CAT, luciferase, and β-galactosidase assays. After shear stress stimulation, cells were washed twice in PBS followed by lysis in reporter lysis buffer (Promega). CAT activity was measured by a dual phase diffusion assay (41) in the presence of 1.4 μM [^{3}H]acetyl-coenzyme A (200 mCi/mmol) (Amersham) and 1 mM chloramphenicol. Luciferase activity was measured using the luciferase assay system (Promega). CAT and luciferase activities were determined on a microtiter counter (Wallac, Gaithersburg, MD) and β-galactosidase activity was assayed as described previously (42). CAT and luciferase values were normalized to the β-galactosidase levels and each data point represents the average of at least three separate experiments.

Elk-1 in vitro kinase assays. HUVEC were exposed to shear at 10 dynes/cm^2 for 0, 10, 30, 60, and 180 min, washed with ice-cold PBS, and scraped in 0.2 ml of lysis buffer (New England Biolabs). The extracts were incubated at 4°C for 30 min and centrifuged at 12,000 *g* for 10 min. Protein concentration of each sample was measured using a Micro BCA protein assay (Pierce Chemical Co., Rockford, IL). Soluble lysates (5 μg) were then used to phosphorylate 1 μg of Gst-Elk-1 (New England Biolabs) in kinase buffer (New England Biolabs) in the presence of 200 μM ATP and 5 μCi of [γ-^{33}P]ATP. Reactions were carried out at 30°C for 15 min and were stopped by boiling in Laemmli sample buffer, and followed by SDS-PAGE electrophoresis. Gels were dried and analyzed on a Storm system with ImageQuaNT software (Molecular Dynamics).

Results

Fluid shear stress activates the egr-1 promoter in HAEC and HeLa cells. Modulation of gene expression in vivo may be due to minor differences in localized levels of shear stress leading us to determine whether *egr-1* transcriptional activity could similarly be induced by a small change in shear stress. HeLa cell cultures were transfected with a plasmid comprising the human *egr-1* promoter linked to the luciferase reporter gene (pGLE). Cells were exposed to either no shear stress, or shear stress at 2.2, 5, or 20 dynes/cm^2 for 3 h. The results, shown in Fig. 1, indicate that promoter activity is increased $\sim$ 4.5-fold when exposed to 2.2 dynes/cm^2 and further increased to $>$ 6-fold when exposed to a shear stress of 5 dynes/cm^2. Exposure of cells to a shear stress of 20 dynes/cm^2 did not further increase the response of the *egr-1* promoter.

Having shown that *egr-1* promoter activity could be activated by low levels of shear stress we then asked whether this was correlated with the level of Egr-1 protein produced under shear stress. EC cultures were exposed to flow and extracts were prepared and probed in a Western blot with antibodies directed against Egr-1. The results in Fig. 2 *A* show a rapid upregulation of Egr-1 after only 15 min of exposure to shear, which was sustained over a 7-h period and declined to a low level after 18 h under flow conditions. These data suggest that the induction of Egr-1 expression in HAEC in response to shear stress is rapid but transient.

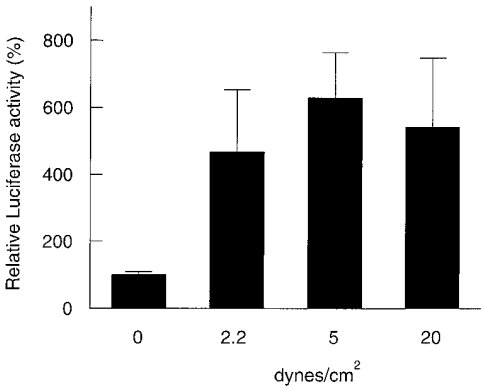

Figure 1. Low levels of fluid shear stress activate *egr-1* activity in HeLa cells. HeLa cells were transfected with 5 μg of plasmid pGLE in the presence of 0.5 μg of pCMVβ. 18 h after the transfection medium was changed to DME/1% FCS and after further incubation for 24 h, cells were exposed to zero shear stress or shear stress of 2.2, 5, or 20 dynes/cm^2 for 3 h. Extracts were prepared and luciferase activity was determined and normalized for β-galactosidase activity

We next determined whether endogenous *egr-1* transcription was also activated. Fig. 2 *B* shows the induction of *egr-1* mRNA steady-state levels in HeLa cells and in HAEC exposed to 5 dynes/cm^2. The steady-state *egr-1* mRNA levels were measured by PhosphorImager and compared to the levels of β-actin mRNA, which was not responsive to shear stress. The ratio of *egr-1* to β-actin signal was then calculated for each time point. In both HAEC and HeLa cell cultures, *egr-1* mRNA was markedly induced after 30 min of shear stress and the induction persisted after 180 min in HeLa cells.

Determination of shear stress responsive elements (SSREs) in the 5′-flanking region of the murine and human egr-1 genes. Previous studies have shown an increase in steady-state levels of *egr-1* mRNA after stimulation with serum (43), urea (44), retinoic acid (45), and PGA_2 (46) without affecting mRNA stability. Therefore, the 5′-flanking regions of the murine and human *egr-1* genes were next used to study the mechanism of shear stress induction of *egr-1*. The two promoters depicted in Fig. 3 are 80% conserved (40). Both 5′-flanking regions contain several consensus transcription factor binding sites as shown. The most striking of these regulatory elements are the multiple SREs, organized into a downstream and an upstream cluster. At least one Ets sequence is found in each of these clusters. In the same manner as for the single *c-fos* gene SRE, this organization allows ternary complex formation between the SRF and ternary complex factor (TCF) transcription factors and their SRE and Ets DNA binding sites (29, 47). The murine and the human *egr-1* gene promoters both contain five SREs. Other functional *cis*-acting elements are EBS and cAMP response elements. The two putative AP1 sites in the murine promoter have not yet been shown to be functional and do not seem to be conserved in the human promoter (40).

The Egr-1 mRNA steady-state level was enhanced by shear stress in human ECs and also in epithelial carcinoma cells (HeLa). This observation is unremarkable given that we (Dickson, M., and M. Braddock, unpublished data) and others

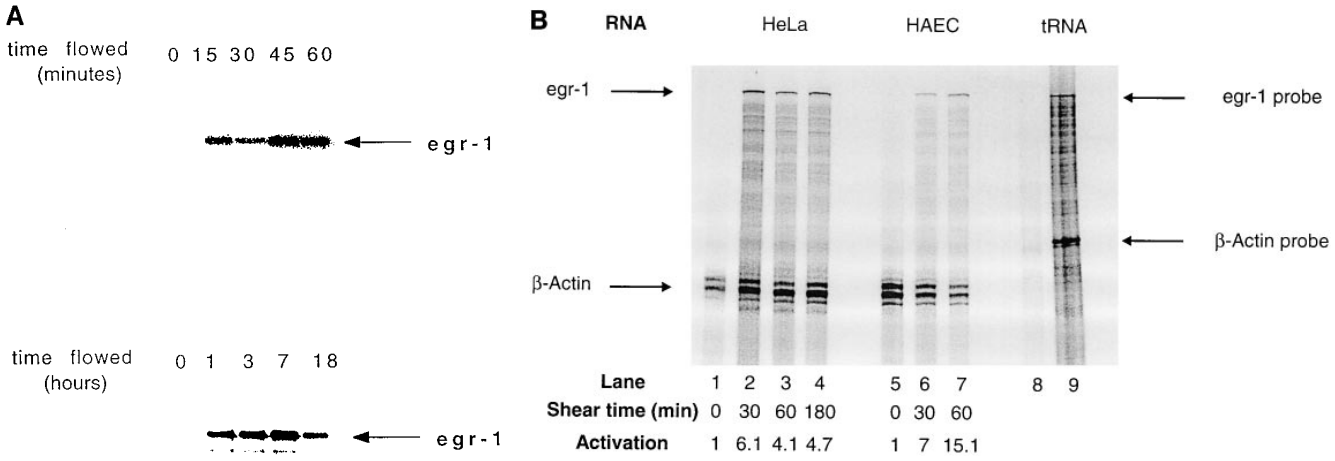

Figure 2. Fluid shear stress induces a rapid upregulation of the Egr-1 transcription factor in HAEC and enhances the *egr-1* mRNA levels in HAEC and HeLa cells. (*A*) HAEC cultures were subjected to flow for the times indicated. Cell extracts were prepared and egr-1 protein levels were determined by Western blot analysis. Duplicate SDS gels were run to ensure equality of protein loading (data not shown). (*B*) Ribonuclease protection assay. Total RNA was isolated from HAEC or HeLa cells. RNA (5 μg) was hybridized with ^{33}P-labeled antisense *egr-1* and human β-actin antisense RNA probes and digested by Rnases A and T1. Protected fragments were analyzed on a 6% acrylamide-7M urea sequencing gel and quantitated by densitometry. The position of the protected fragments is indicated in the figure. Lane *8* shows the unprotected Egr-1 and β-actin probes digested by Rnase. Lane *9* shows the undigested probes.

(15) have shown that HeLa cells are shear stress responsive. Therefore, we decided to use HeLa cells as an initial model system to identify potential *cis*-acting regulatory elements implicated in shear stress–inducible transcription of the murine *egr-1* gene. Plasmid constructs containing progressive deletions of the 5′-flanking sequence linked to the CAT reporter gene were transfected into HeLa cells and the results are shown in Fig. 4. Cells were subjected to shear stress for 3 h at 5 dynes/cm^2. Plasmid p1.2 (nt −903 to +248) was activated more than fivefold as compared to the static control. When the cells were transfected with p1.2d, which contains no SREs, the basal and *trans*-activated promoter activity was reduced to background levels. This suggests that the putative upstream AP1 site cannot, in isolation, confer shear stress transcriptional induction on this promoter. Additionally, deletion of both the AP1 site and the EBS in plasmid p425 did not prevent shear stress activation. The remaining constructs (p425, p395, p342, p125, and pE50) have one or more of the SREs removed and the results reveal a progressive loss of basal activity and shear stress inducibility. In construct p425d, the two proximal SREs have been deleted but the promoter is still activated fourfold by shear stress. Removal of the upstream SREs in p342 resulted in shear stress–induced activity being only 1.7-fold higher than the static control. Additional deletion of 5′-flanking sequence to nt 125 (p125) and −50 (pE50) abolished the observed shear stress response, although it remains a formal possibility that the basal and induced CAT levels are below experimental background. These data indicate that shear stress, in common with other stimulatory factors, may activate the *egr-1* promoter principally through the upstream SREs.

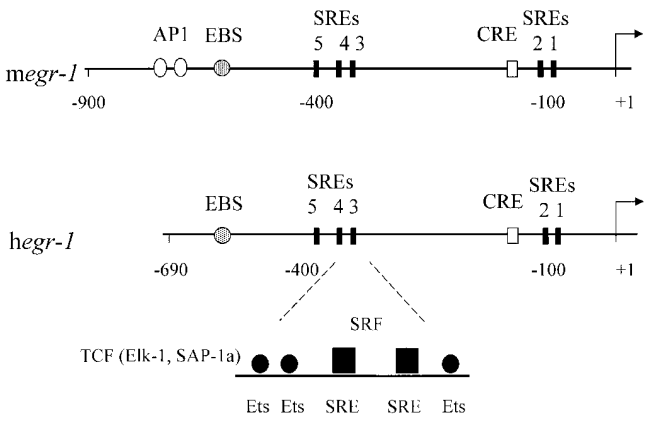

Figure 3. 5′-flanking sequence of the mouse and human egr-1 promoters. Sequences are numbered relative to the transcription start site (*arrow*). The SREs (*closed squares*), the cAMP-response sites (*CRE*, *open squares*), the EBSs (*shaded circle*), and the AP1 binding sites (AP1, *open circles*) are shown. The SRE is composed of the SRF binding site (CC[A/T]6G box) and flanking Ets binding site(s).

To extend the data obtained with the murine *egr-1* promoter, the human promoter was derived from a previously published sequence (40) by PCR amplification and linked to the luciferase gene in the reporter plasmid pGL3. Deletions and mutations of this promoter were constructed and HeLa cells were transiently transfected using the same experimental conditions as described in Fig. 4. Construct pGLE, which contains the full-length human *egr-1* promoter (nt −697 to +12), was activated ∼ 9-fold by shear stress as compared to the static control (Fig. 5 *A*). Point mutations in SRE 4 alone (pGLEmSRE4) or in the Ets binding site situated at nt −315 (pGLEmETS) had no dramatic impact on shear stress activation. Both of these sites had been previously identified as crucial for the activation of the murine *egr-1* promoter after antigen cross-linking in B cells (29). However, the deletion of nt −409 to −300, containing the upstream SREs 3, 4, and 5 with their flanking Ets binding sites, reduced shear stress promoter activation of construct pGLE(−409/−300)d to 2.2-fold. Transient transfection analysis was then used to determine whether

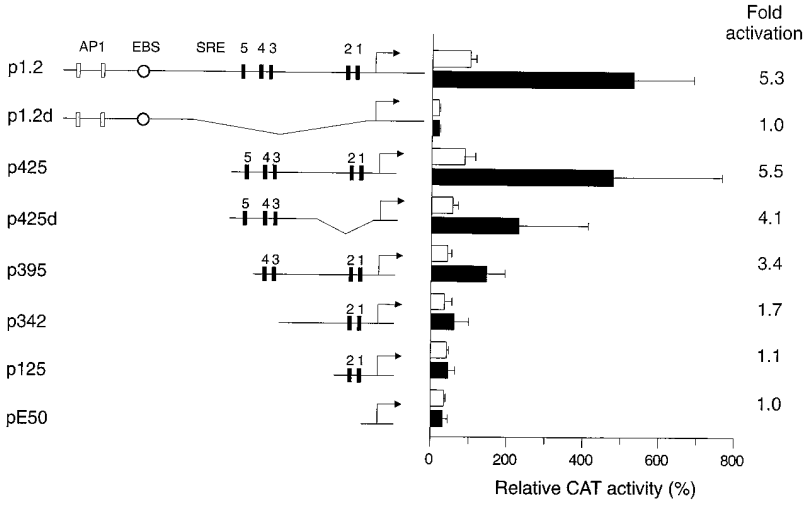

Figure 4. Activation of CAT reporter gene constructs containing deletions of the −930 to +237 mouse egr-1 promoter. Maps of the deletion constructs of the mouse *egr-1* promoter used in transient transfection experiments are shown on the left. Construct numbers are shown; arrows indicate the transcription start site. The plasmids (5 μg) were transfected into HeLa cells in the presence of 0.5 μg of pCMVβ plasmid. 18 h after transfection, medium was changed to DME (1% FCS) and after further incubation for 24 h, cells were exposed to either zero shear stress or to a shear stress of 5 dynes/cm^2 for 3 h. Cells were lysed and extracts were prepared. The level of CAT activity was measured and normalized for β-galactosidase activity. CAT activity is expressed relative to the activity of the −930 to +237 promoter from cells not exposed to shear stress. CAT activity from static control cells is represented by open bars while CAT activity from cells exposed to 5 dynes/cm^2 of shear stress for 3 h is represented by solid bars. The results shown are the mean±SD of three to five experiments.

the region between nt −409 to −300 of the *egr-1* promoter is also mediating increased promoter activity in ECs. BAEC were transfected with the full-length human *egr-1* promoter construct and the vector pGLE(−409/−300)d and were exposed to shear stress of 10 dynes/cm^2 for 3 h. Construct pGLE was activated 2.5-fold by shear stress as compared to the static control (Fig. 5 *B*). Deletion of the distal SREs in plasmid pGLE(−409/−300)d resulted in the loss of shear stress activation. These observations with the human promoter, further implicate the distal SREs as being primarily responsible for shear stress *egr-1* activation in HeLa cells and in ECs.

To determine whether SREs 3 and 4 together with their adjacent Ets binding sites represent a novel SSRE, we investigated whether they could confer shear stress responsiveness

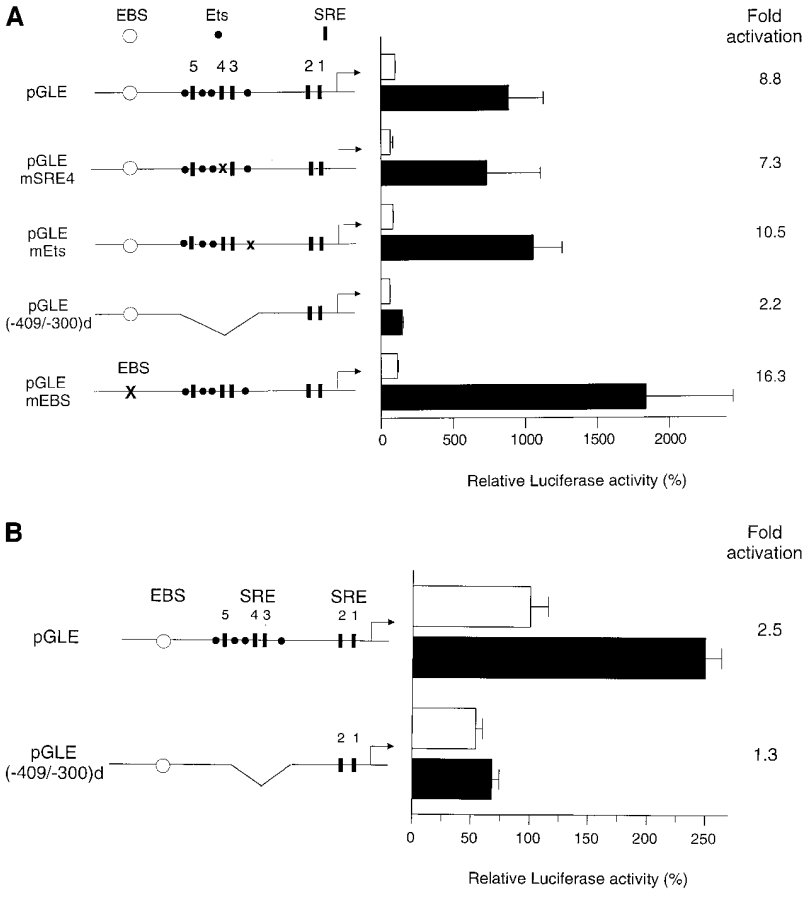

Figure 5. Activation of luciferase reporter gene constructs containing point mutations and deletions of the −697 to +12 human *Egr-1* promoter. Deletion constructs of the human *egr-1* promoter were used in transient transfection experiments and are drawn on the left. Construct numbers are indicated; arrows indicate the transcription start site. Luciferase activity is expressed relative to the activity of the −697 to +12 human *egr-1* promoter from cells not exposed to shear stress. (*A*) HeLa cells were transfected as indicated in Fig. 4. BAEC were transfected with 0.1 μg of plasmid in the presence of 0.07 μg of pCMVβ plasmid. (*B*) 18 h after transfection, medium was changed to DME (10% FCS) and after further incubation for 24 h, cells were exposed either to zero shear stress or to a shear stress of 10 dynes/cm^2 for 3 h. Cells were lysed and extracts were prepared. Activity from static controls is represented by open bars and activity from cells exposed to 5 or 10 dynes/cm^2 of shear stress for 3 h is represented by solid bars. The EBS is shown as an open circle, SREs as solid squares, and Ets sites as closed circles.

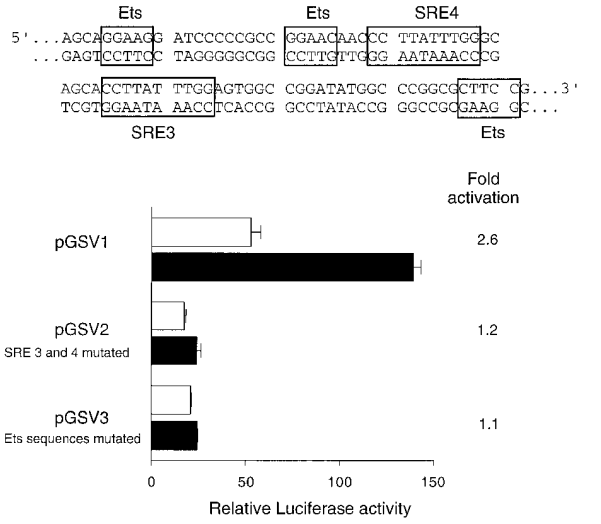

Figure 6. SREs 3 and 4 in the human *Egr-1* promoter define a novel SSRE. (*A*) The sequence of SREs 3 and 4 and their neighboring Ets binding sites are shown. Plasmids pGSV1 (wild-type SREs 3 and 4 and wild-type Ets binding sites), pGSV2 (mutant SREs 3 and 4 and wild-type Ets binding sites), and pGSV3 (wild-type SREs 3 and 4 and mutant Ets binding sites) were transfected into HeLa cells and exposed to shear stress as described in Fig. 5. Luciferase activity from static control cells is represented by open bars while activity from cells exposed to 5 dynes/cm^2 of shear stress for 3 h is represented by closed bars. Error bars shown represent data from three independent experiments.

on a heterologous promoter in a manner similar to that undertaken with the GAGACC sequence first identified in the PDGF-B promoter (17, 48). Sequences comprising SREs 3 and 4 were inserted upstream of a SV40 minimal promoter vector (pGL3 promoter) resulting in plasmid pGLSV1. Two further reporter plasmids harboring mutations in the two SREs (pGLSV2) or three Ets binding sites (pGLSV3) were constructed and transfected into HeLa cells. The results, shown in Fig. 6, demonstrate that the 83 nt sequence, spanning nt −399 to −316 in the human *egr-1* promoter, constitutes a novel SSRE. Mutations of the SREs, or of their cognate Ets binding sites, which abolish binding of SRF and Ets respectively, abrogated the capacity of the SSRE to confer shear responsiveness on the SV40 promoter.

EBS negatively regulates egr-1 promoter activity. Cotransfection of ECs with a titrated quantity of the TF promoter construct spanning nt −111 to +14, together with a limiting amount of pCMV–Egr-1 (p930), results in a clear superactivation of the TF promoter under flow conditions (Houston, P., M.C. Dickson, V. Ludbrook, B.P. White, J.-L. Schwachtgen, J.H. McVey, N. Mackman, J.M. Reese, D.G. Gorman, C.J. Campbell, et al., manuscript submitted for publication). However, the level of superactivation is progressively reduced with an increasing amount of p930 *trans*-activator DNA. In addition, it has been reported that Egr-1 downregulates its own promoter by binding through an upstream EBS (49). We reasoned that deletion of the EBS from the human *egr-1* promoter may relieve an inhibitory effect of Egr-1 binding and result in an enhanced activation of this promoter by shear stress. Plasmids pGLE (wild-type promoter) and pGLEmEBS (harboring a deletion of 108 nt at the EBS) were transfected into HeLa cells and subjected to shear stress. The results, shown with the data obtained from the human *egr-1* promoter deletion constructs in Fig. 5, clearly show that the EBS is exerting a repressive effect on the capacity of the full-length promoter to be activated by shear stress (compare 8.8-fold activation for pGLE and 16.3-fold activation for pGLEmEBS), while leading to a small (50%) induction of the basal promoter. To confirm that overproduction of Egr-1 acts in an inhibitory fashion on its own promoter activity, HeLa cells were transfected with equal amounts of the murine *egr-1* promoter construct p1.2 and plasmids expressing Egr-1 as wild type (p930), mutant (1.5 zinc fingers deleted; p858), or as a chimera between the WT1 protein and the Egr-1 DNA binding domain (dbd) (p1130). Results, shown in Fig. 7, confirm that wild-type Egr-1 inhibits basal reporter gene activity compared to the mutant protein, which cannot bind to the EBS in the promoter. Interestingly, the WT1–Egr-1 fusion protein is an even more potent inhibitor of the *egr-1* promoter.

Fluid shear stress increases Elk-1 transcriptional activity. Our data so far are consistent with the notion that shear stress activates the *egr-1* promoter via an SRE–Ets–dependent interaction. The composite SRE–Ets *cis*-acting site has been shown to be inducible by growth factors through the phosphorylation and activation of the TCF proteins Elk-1 and SAP-1 by the ERK1/2 mitogen-activated protein kinase (MAPK) subfamily (50).

To test the hypothesis that shear stress would activate Elk-1, we cotransfected plasmid pFR-Luc, a construct containing the

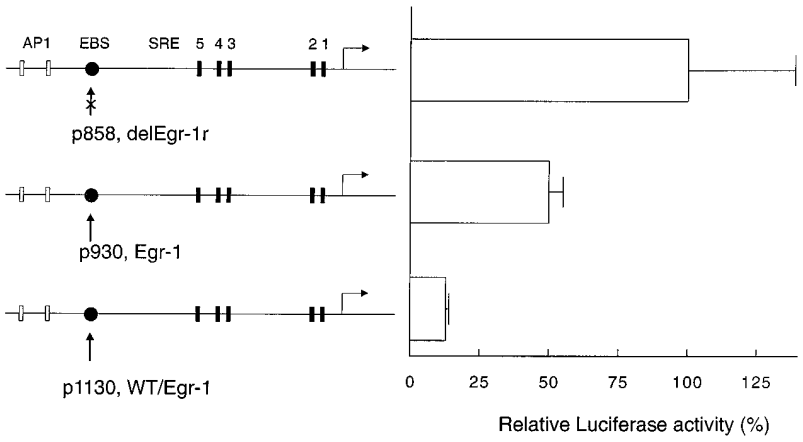

Figure 7. The EBS negatively regulates the *egr-1* promoter. Transient transfection experiments in HeLa cells were carried out as described in Fig. 4 using plasmids p1.2 (5 μg), together with plasmid p930 (5 μg), p858 (5 μg), or p1130 (5 μg) in the presence of 0.5 μg of pCMVβ. 18 h after transfection, extracts were prepared and CAT activity was determined and normalized for β-galactosidase activity. Error bars shown represent data from three independent experiments. The repressive effects after expression of p930 and p1130 are shown relative to p858.

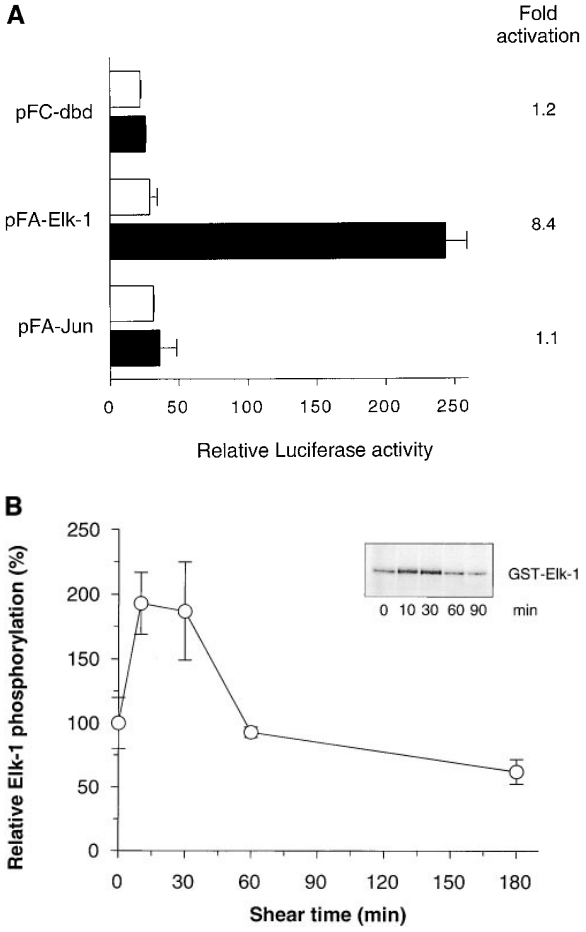

Figure 8. Shear stress activates Elk-1 in HeLa cells and in HUVEC. The Gal4 dbd, Elk-1–Gal4–dbd, and c-jun–Gal4–dbd expression plasmids together with Gal4 luciferase reporter plasmid, pFR-Luc (Stratagene), were transfected into HeLa cells. The cells were either kept static (*open bars*) or subjected to shear stress at 5 dynes/cm^2 for 3 h (*closed bars*). Bars represent the fold-activation of luciferase expression of shear-stressed cells versus static controls. (*A*) Error bars shown represent data from three independent experiments. (*B*) Total cell lysates prepared from HUVEC exposed to shear stress (10 dynes/cm^2 for 0, 10, 30, 60, or 180 min) were used to phosphorylate Gst-Elk-1 for 15 min. The reactions were resolved by SDS-PAGE and the extent of Gst-Elk-1 phosphorylation was revealed by PhosphorImager. Representative autoradiograms are shown in the inset. The radioactivity of the phosphorylated bands was quantitated by densitometry. The line graph shows the mean±standard error of results obtained from three independent studies.

Gal4 binding sequence upstream of the luciferase reporter gene, and plasmid pFA–Elk-1, a construct encoding a fusion protein of the Gal4 dbd and the Elk-1 activation domain into HeLa cells. This assay system allows for the direct assessment of Elk-1 activation in response to a physiological stimulus such as shear stress. As shown in Fig. 8 *A*, shear stress induced Elk-1 activation by $\sim$ 9-fold as compared to static controls. There was no response to shear stress when cells were transfected with plasmid pFR-Luc and the control vector pFC-dbd, indicating that activation was specific for the Elk-1 interaction. In addition, we transfected a construct encoding a Gal4 c-Jun fusion protein (pFA–c-Jun) together with pFR-Luc, the reporter plasmid. In contrast to Elk-1 activation, we find no evidence for c-jun activation by shear stress in HeLa cells (Fig. 8 *A*).

To assess directly the level of Elk-1 phosphorylation in ECs exposed to a fluid shear stress of 10 dynes/cm^2, HUVEC lysates were used in an in vitro kinase assay using GST–Elk-1 as a substrate (Fig. 8 *B*). Shear-induced phosphorylation was monitored as a function of shear exposure time. Extracts from static controls already exhibited a high phosphorylation activity, as is to be expected from cells grown in growth factor–containing medium. However, Elk-1 phosphorylation activity was further increased in cell extracts from HUVEC subjected to shear stress for up to 30 min.

The MEK1 inhibitor PD98059 inhibits activation of the egr-1 promoter in HeLa cells and inhibits induction of egr-1 mRNA steady-state levels in HAEC by shear stress. The compound PD98059 is a specific inhibitor of the protein kinase MEK1, which is a key intermediate enzyme in the Ras-MEK-ERK signal transduction cascade. MEK1 activates the ERK1 and ERK2 MAPK family members whose downstream targets include Elk-1 and, therefore, Elk-1 induces SRE-mediated transcription. The contribution of ERK1/2 activation to the shear stress–mediated transcriptional activity of the *egr-1* gene promoter was assessed in cells transfected with the human *egr-1* promoter construct pGLE. Cells were pretreated for 30 min with PD98059 (50 or 75 μM in 0.1% DMSO) or with 0.1% DMSO alone before being kept as static control or subjected to 60 min of flow (5 dynes/cm^2). The data in Fig. 9 *A* show that under these experimental conditions, shear stress increased luciferase activity by threefold. PD98059 inhibited this effect in a dose-dependant manner suggesting that the shear stress signal transduction pathway in HeLa cells requires activated (e.g., phosphorylated) MEK1.

The involvement of the Ras-MEK-ERK signal transduction cascade in the shear stress–mediated activation of *egr-1* transcription was also assessed in HAEC. Cells were preincubated for 30 min with PD98059 (50 μM in 0.1% DMSO) or with 0.1% DMSO alone. Levels of egr-1 and cyclophilin mRNA from cells subjected to shear stress for 60 min and from static controls were analyzed by ribonuclease protection assay and quantitated by densitometry (Fig. 9 *B*). HAEC in these experiments were grown in medium containing bFGF, bovine brain extract, and 2% FCS. Cells not subjected to flow showed a low level of *egr-1* transcription that was reduced by treatment with PD98059. The induction of *egr-1* mRNA level by exposure to 5 dynes/cm^2 shear stress for 30 (data not shown) or 60 min was notably reduced in the presence of 50 μM of PD98059 but not totally abolished. These results suggest that signal transduction via ERK1/2 is predominantly involved in the shear stress activation of *egr-1* gene activity but that additional pathways, distinct from the Ras-MEK-ERK cascade may play a role in shear stress–mediated *egr-1* activation in HAEC.

Discussion

In this study we demonstrate that the expression of the immediate-early gene *egr-1* is induced by changes in the level of fluid shear stress in HAEC. The induction of Egr-1 is rapid after the onset of flow, persists for 7 h, and declines after 18 h. These data support the findings that the transcription factor Egr-1, induced by shear stress, may be responsible for activa-

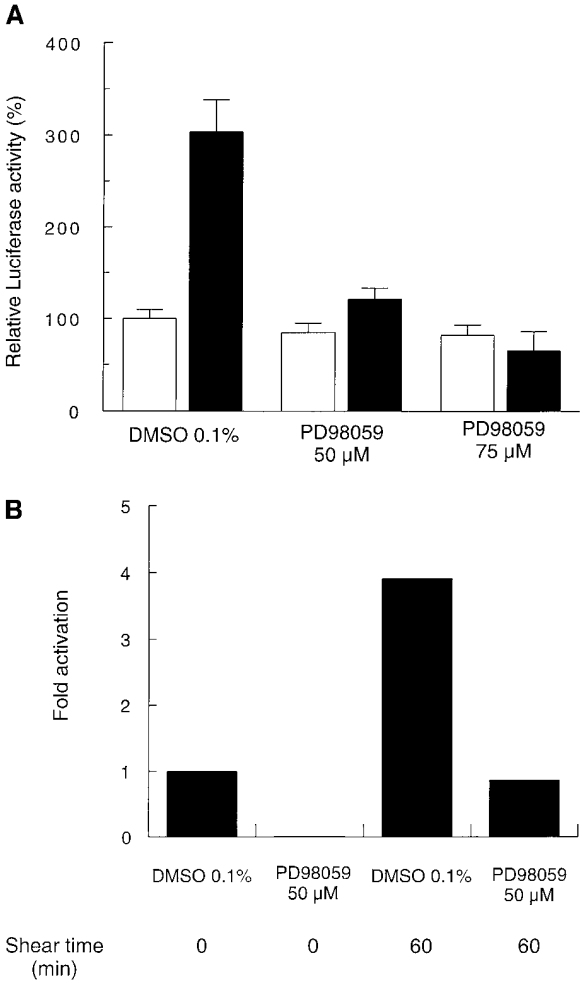

Figure 9. The MEK1 inhibitor PD98059 inhibits the activation of the *egr-1* promoter in HeLa cells and the induction of *egr-1* mRNA steady-state levels in HAEC by shear stress. (*A*) HeLa cells, transfected with the −697 to +12 human *egr-1* promoter, were preincubated with 0.1% DMSO, 50 μM PD98059 in 0.1% DMSO, or 75 μM PD98059 in 0.1% DMSO for 30 min and exposed to shear stress at 5 dynes/cm^2 for 1 h. Luciferase activity of cells subjected to shear stress was measured and is represented as solid bars. Luciferase activity of static controls is represented as open bars. Total RNA (5 μg) was isolated from HAEC and hybridized with ^{32}P-labeled antisense *egr-1* and cyclophilin antisense RNA probes followed by ribonuclease protection analysis using the HybSpeed RPA kit. Protected fragments were analyzed on a 6% acrylamide-7M urea sequencing gel and quantitated by densitometry on a Molecular Dynamics PhosphorImager. (*B*) Results are expressed as a bar chart relative to cyclophilin transcription.

tion of target genes through Egr-1 motifs in cognate promoters like PDGF-A24.

By deletion analysis of the 5′-flanking sequence of the mouse *egr-1* gene we show that sequences containing the three upstream SREs (SREs 3, 4, and 5) are primarily responsible for transcriptional activation by shear stress. Similarly, deletion of the region including the upstream SREs 3, 4, and 5 in the human promoter demonstrates that this region of the promoter is crucial to shear stress induction of *egr-1* transcriptional activity in both HeLa and ECs (compare data obtained with pGLE and pGLE[−409/−300]d in Fig. 5, *A* and *B*). The region containing SREs 3 and 4 is capable of conferring shear stress inducibility to the minimal SV40 promoter when taken out of sequence context and, thus, constitutes a novel SSRE.

This SSRE has a more complex structure than previously defined SSREs (15, 16, 48) in that both functional SRE and Ets binding sites are required for shear stress activation to proceed. Interestingly, although SREs 3 and 4 will confer shear response on the SV40 promoter, the 2.6-fold activation typically observed falls below that observed for shear stress induction of the *egr-1* promoter, which is typically activated 8.8-fold. The simplest interpretation of this finding is that our new SSRE is functioning out of sequence context and that full shear stress response relies on additional sequence determinants outside of those that reside within SREs 3 and 4 and their associated Ets binding sites. Indeed, SRE 5 is functional and contributes significantly to the shear stress response in the mouse *egr-1* promoter (Fig. 4). Control of *egr-1* promoter activity via the upstream SREs is not solely confined to shear stress activation. Recent studies have shown that the maximal inducible expression of the *egr-1* gene in response to B cell activation (29, 51) and urea (35) is controlled by the promoter regions containing the distal SREs. However, the proximal SREs are necessary for maximal induction by GM-CSF (34, 52). The SRE has been extensively studied in the context of the immediate early gene *c-fos* as a model transcriptional unit. The *c-fos* promoter contains a single SRE (CC[A/T]6G) in close proximity to a single Ets (GGAA) site. These motifs are bound by the SRF and TCF (Elk-1 or SAP1) transcription factors, respectively. By contrast, the *egr-1* promoter contains five potential SREs resulting in serum induction of *egr-1* that is five- to tenfold greater than that of *c-fos.*

A previous report has shown that Egr-1 may downregulate transcription from the *egr-1* promoter (49). Given this observation, we reasoned that shear stress activation of Egr-1 synthesis may be subject to a negative regulatory circuit given that the *egr-1* promoter harbors an EBS. Deletion of the EBS from construct pGLE resulted in a further twofold activation of the *egr-1* promoter (Fig. 5, compare 8.8-fold with 16.3-fold). Data obtained in *trans*-activation experiments using wild-type and mutant Egr-1-expressing plasmids and the wild-type *egr-1* promoter support this finding. Binding of wild-type Egr-1 repressed the level of basal pGLE activity, whereas expression of a mutant Egr-1, having 1.5 zinc fingers deleted and being unable to bind to the EBS (39), did not repress *egr-1* activity to the same extent (Fig. 7). Intriguingly, expression of a hybrid WT/Egr-1 protein, which consists of the dbd of Egr-1 and the protein interaction domain of WT1 (39), further repressed *egr-1* promoter activity when compared with the wild-type Egr-1-expressing plasmid. The WT1 protein is a member of the *egr* gene family and functioned as a repressor of transcription when bound to the Egr-1 site. This may suggest that the repressive function is a general property common to the Egr family of proteins. These data and the fact that shear-induced activation of the human *egr-1* promoter is potentiated by Egr-1 suggests an important role for Egr-1 in self-limiting its own expression after shear stress stimulation.

Data in this paper draw from shear stress experiments performed in both ECs and in HeLa cells. Evidence in the literature supports our finding that HeLa cells are highly responsive to shear stress. Monocyte chemoattractant protein-1 was activated 2–3-fold in HUVEC exposed to 16 dynes/cm^2 and 6.1-

fold in HeLa cells exposed to 12 dynes/cm^2 (15). HeLa cells are epithelial-like and the epithelium is constantly exposed to transient changes in stretch and torsional strain. Nevertheless, laminar shear stress and torsional deformation are two distinct mechanical stimuli. Interestingly, some aspects of mechanical stimulation can therefore be sensed by ECs, which are exposed to fluid shear stress, and HeLa cells, which normally are not. Thus, it appears that HeLa cells and ECs share common features in transducing shear stress stimulation from the cell surface to the nucleus. However, HeLa cells are responsive to shear stress and their ease of maintenance and culture makes them a valid model system to assist in understanding the shear stress induction pathway.

We have shown in both HeLa cells and ECs that *egr-1* activation may result from a small increase in the level of shear stress. In HeLa cells, *egr-1* promoter activation was observed with a shear stress as slight as 2.2 dynes/cm^2, although maximal response was observed at 5 dynes/cm^2 (Fig. 1). In HAEC shear stress of 5 dynes/cm^2 also caused a large increase in *egr-1* transcription. These findings may, in part, be due to previous observations that both ERK1/2 and c-jun NH_3-terminal kinase signaling pathways are activated by shear stress levels as low as 0.5 or 1 dynes/cm^2 and that ERK activity is transiently induced by a shear stress of 5 dynes/cm^2 (53). Our findings that Elk-1 activity (ERK1/2 signaling pathway) but not c-jun (c-jun NH_3-terminal kinase signaling pathway) was activated by shear stress in HeLa cells and HUVEC, and that Elk-1 activation paralleled the activation of *egr-1* activity, leads us to conclude that the ERK1/2 pathway predominates for shear stress signaling to the *egr-1* gene. This conclusion is further strengthened by data obtained with the MEK1-specific inhibitor PD98059, which abolishes the response of the *egr-1* promoter to shear stress in a reporter construct (Fig. 9 *A*), but that does not completely inhibit *egr-1* mRNA transcription. The ERK1/2 signaling pathway has been previously implicated in the induction of *egr-1* activity by urea (54), and our data imply that shear stress activation of *egr-1* is also mediated via this signaling cascade.

Our observations that slight increases in the levels of shear stress may have profound effects on the levels of gene expression, at least in vitro, prompted us to speculate on the implications this may have in vivo. Several laboratories including our own (55–57) (Dickson, M.C., C.J. Campbell, and M. Braddock, unpublished data), have established differential display programs with the aim of identifying genes whose activity is induced by shear stress and that may be atheroprotective. There is evidence that genes with EBSs in the promoter region are responsive to shear stress (22, 24, 58). However, these genes generally seem to be promoting atherosclerosis. Many studies have suggested that atherogenesis is initiated and atherosclerosis develops at areas of low shear stress, implying that low shear stress is atherogenic (59–63). Here we show that shear stress induces Egr-1 protein in HAEC for up to 7 h and that the response declines after 18 h. This result suggests that cells using the MEK–ERK–Elk-1–Egr-1 pathway to transmit shear stress signals negatively autoregulate this signal transduction cascade. One level of negative feedback exists at the level of the *egr-1* promoter itself, through the inhibitory EBS. It is conceivable that continuous normal levels of shear stress such as 15–30 dynes/cm^2 (36) may exert a protective effect on the endothelium by desensitizing ECs to further stimuli and that low shear stress may result in EC sensitization to injury. Supporting this hypothesis several groups have shown that shear stress protects against EC apoptosis (64–66) and EC response to cytokines is diminished when cells are exposed to shear stress (67, 68). If this theory is correct, the implications for EC biology are profound. We would predict that slight increases in shear stress in areas of the endothelium that have become sensitized (i.e., areas of low shear stress) would have significant effects on gene expression within this cell population. Further work in our laboratory is aimed at qualifying this hypothesis.

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