

Shear Stress Activates p60src-Ras-MAPK Signaling Pathways in Vascular Endothelial Cells

Shila Jalali, Yi-Shuan Li, Mohammad Sotoudeh, Suli Yuan, Song Li, Shu Chien, John Y-J. Shyy

Abstract—The aim of this study was to elucidate the upstream signaling mechanism that mediates the fluid shear stress activation of mitogen-activated protein kinases (MAPKs), including c-Jun NH₂-terminal kinase (JNK) and extracellular signal-regulated kinases (ERKs), in vascular endothelial cells (ECs). Our results indicate that p60src is rapidly activated by fluid shear stress in bovine aortic endothelial cells (BAECs). Shear stress induction of the hemagglutinin (HA) epitope-tagged HA-JNK1 and the Myc epitope-tagged Myc-ERK2 was significantly attenuated by v-src(K295R) and c-src(K295R), the kinase-defective mutants of v-src and c-src, respectively. HA-JNK1 and Myc-ERK2 were activated by c-src(F527), a constitutively activated form of p60src, and the activation was abolished by RasN17, a dominant-negative mutant of p21ras. In contrast, although HA-JNK1 and Myc-ERK2 were also activated by RasL61, an activated form of p21ras, the activation was not affected by v-src(K295R). These results indicate that p60src is upstream to the Ras-JNK and Ras-ERK pathways in response to shear stress. The shear stress inductions of the promoters of monocyte chemoattractant protein-1 (MCP-1) and *c-fos*, driven by TPA-responsive element (TRE) and serum-responsive element (SRE), respectively, were attenuated by v-src(K295R). This attenuation is associated with decreased transcriptional activities of c-Jun and Elk-1, the transcription factors targeting TRE and SRE, respectively. Thus, p60src plays a critical role in the shear stress activation of MAPK pathways and induction of Activating Protein-1 (AP-1)/TRE and Elk-1/SRE-mediated transcription in ECs. (*Arterioscler Thromb Vasc Biol.* 1998;18:227-234.)

Key Words: shear stress ■ src ■ MAPK ■ mechanotransduction ■ endothelial cells

Shear stress is the tangential component of hemodynamic forces acting on the vessel wall and constitutes a risk factor of cardiovascular diseases.¹ Application of shear stress to ECs cultured in flow channels induces a number of morphological and functional changes. Previous studies have shown that shear stress induces several genes that encode proteins implicated in atherosclerosis and other vascular diseases, including MCP-1, intercellular adhesion molecule-1, heparin-binding EGF-like growth factor, PDGF, nitric oxide synthase, and *c-fos* proto-oncogene.²⁻⁸ In the cytoplasm of ECs, MAPKs, including JNK and ERK, are activated by shear stress.⁹⁻¹³ Studies by Berk and colleagues^{11,14} demonstrated that the activation of ERK by shear stress requires PKC and a herbimycin-sensitive tyrosine kinase. It was also shown that shear stress differentially regulates JNK and ERK by signaling that involves PTx-insensitive G protein-dependent and G₁₂-dependent pathways, respectively.¹² Genistein, an inhibitor of tyrosine kinases, inhibits shear stress activation of ERK and JNK.¹² Transcription factors that contain the ETS domain, MADS box, zinc finger, HMG box, or bZIP domain have been implicated in the MAPK-mediated gene regulation (see References 15 and 16 for review). For example, the activation of ERK leads to the phosphorylation of ternary complex factor TCF/Elk-1,¹⁷ an important transcription factor involved in the regulation of *c-fos* gene expression.¹⁸ On the other hand, JNK binds to c-Jun to

specifically phosphorylate Ser-63 and -73 at the N-terminal of c-Jun,¹⁹ a major component of AP-1 transcriptional complex.²⁰ Despite many investigations on MAPKs in response to shear stress, the upstream molecules involved in the mechanotransduction to activate MAPKs are still unclear. Furthermore, how these signaling events regulate the downstream genes (eg, MCP-1 and *c-fos*) via MAPKs remains to be elucidated.

Recent reports indicate that the activities of PTKs in cardiac myocytes, platelets, and ECs are increased by mechanical stimuli such as cyclic stretch and shear stress.²¹⁻²³ Thus, PTKs seem to play important roles in the signaling events elicited by mechanical forces. Cellular PTKs, in general, can be divided into two major categories, RTKs and nonreceptor PTKs (see Reference 24 for review). The extracellular domains of RTKs can bind polypeptide growth factors and initiate signal transmission by phosphorylating the tyrosine residues in the intracellular domains. Nonreceptor PTKs such as p60src represent cellular enzymes that have intrinsic kinase activities without extracellular domains. p60src is the cellular counterpart of the product of the *v-src* gene of Rous sarcoma virus. Many growth stimuli, such as PDGF,²⁵ MCSF,²⁶ serum, and environmental stress (eg, UV),²⁷ activate p60src in different cell types. We hypothesized that p60src, like PKC and G proteins, play critical roles in the shear stress induction of JNK and ERK in ECs. One approach to delineate the functions of PTKs in the

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Selected Abbreviations and Acronyms

BAEC	= bovine aortic endothelial cell
ECs	= vascular endothelial cells
EGF	= epidermal growth factor
EGFR	= EGF receptor
ERK	= extracellular signal-regulated kinase
FBS	= fetal bovine serum
HA	= hemagglutinin
IP	= immunoprecipitate
JNK	= c-Jun NH ₂ -terminal kinase
mAb	= monoclonal antibody
MAPKs	= mitogen-activated protein kinases
MBP	= myelin basic protein
MCP-1	= monocyte chemoattractant protein-1
MCSF	= monocyte colony-stimulating factor
PBS	= phosphate-buffered saline
PDGF	= platelet-derived growth factor
PDGFR	= PDGF receptor
PMSF	= phenylmethylsulfonyl fluoride
PTK	= protein tyrosine kinase
PTx	= pertussis toxin
RTK	= receptor tyrosine kinase
SRE	= serum-responsive element
TRE	= phorbol ester TPA-responsive element
UV	= ultraviolet light

signal transduction pathways in response to extracellular stimuli is to overexpress negative mutants of the various PTKs in the target cells to block the upstream pathways. Alternatively, their wild-type or activated mutants may be overexpressed to activate the downstream pathways. In the present study, by using the dominant-negative mutants and the constitutively activated forms of p60src and p21ras, we found that p60src is a common upstream mediator for both the Ras-JNK and the Ras-ERK pathways in ECs in response to shear stress. Consequently, transcription factors c-Jun and Elk-1 are activated, which act on the *cis*-elements TRE and SRE to induce target genes, such as those encoding MCP-1 and *c-fos*.

Methods

Cell Culture and Shear Stress Experiments

BAECs were used in all experiments. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. All cell cultures were kept in a humidified 5% CO₂/95% air incubator at 37°C. BAECs cultured on 38×76-mm slides to confluence were either kept as static controls or subjected to a laminar shear stress in a rectangular flow channel. The flow experiments were performed by using the system designed by Frangos et al.²⁸ with minor modifications, so that multiple slides could be sheared simultaneously. A surface area of 14 cm² on the BAEC-seeded slide, confined by a gasket, was exposed to fluid shear stress, which was generated by perfusing the conditioned culture medium (10% FBS) over the cells. The pH of the system was kept constant by gassing with 5% CO₂/95% air, and the temperature was maintained at 37°C by maintaining the flow system in a temperature-controlled box. The shear stress was 12 dyne/cm², which is relevant to the physiological range in the human major arteries and has been found to induce the expression of several immediate early genes *in vitro*.^{2,6}

Kinase Activity Assays

The IP kinase assays of p60src, HA epitope-tagged HA-JNK1, and Myc-tagged Myc-ERK2 were performed according to the procedures described by Gould and Hunter.²⁹ After they were subjected to shear

stress, BAECs were rinsed with ice-cold PBS and lysed in a buffer containing 25 mmol/L HEPES (pH 7.5), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.5 mol/L NaCl, 50 mmol/L NaF, 1 mmol/L Na₃VO₄, 5 mmol/L EDTA, 10 μg/mL leupeptin, and 1 mmol/L PMSF. The cell lysate was centrifuged at 13 000g for 10 minutes at 4°C. After quantification using Bio-Rad protein reagent assay, 300 μg cell lysate was incubated with 2 μg anti-p60src mAb (Upstate Biotechnology) for p60src, 2 μg anti-HA mAb (Boehringer Mannheim) for HA-JNK1, or 2 μg anti-Myc mAb (Santa Cruz) for Myc-ERK2, together with 20 μL 10% protein A-Sepharose beads for 12 hours at 4°C. The immunocomplexes were washed four times with TPBS containing 0.2% Triton X-100 and twice with a kinase buffer (25 mmol/L HEPES pH 7.4, 20 mmol/L MgCl₂, 20 mmol/L β-glycerol-phosphate, 0.1 mmol/L Na₃VO₄, 1 mmol/L PMSF, 10 μg/mL leupeptin, and 2 mmol/L dithiothreitol). The kinase reaction was initiated by suspending the immunoprecipitates in 20 μL kinase buffer containing 1 μCi [³²P]ATP, 1 μL of 25 μmol/L ATP, 0.2 μg enolase (Sigma) for p60src, 2 μg GST-c-Jun(1-79)¹⁰ fusion protein for HA-JNK1, or 5 μg MBP (Sigma) for Myc-ERK2. The reaction mixture was incubated for 20 minutes at 30°C and terminated by the addition of 6× SDS sample buffer. The proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis followed by autoradiography.

DNA Plasmids, Transfection, and Luciferase Assays

DNA plasmids were transfected into BAECs at 70% confluence using the lipofectamine method (GIBCO-BRL). Gal4-c-Jun(1-223) and Gal4-ElkC(307-428) encode the fusion proteins of the Gal4 DNA-binding domain fused to the activation domains of c-Jun or Elk-1.^{30,31} 5XGal-Luc is a chimeric construct consisting of five copies of the Gal4-binding sequence and the luciferase reporter. MCP1-Luc and *c-fos*-Luc are promoter constructs in which the luciferase reporters are driven by the 540-bp MCP-1 and the 800-bp *c-fos* 5' promoters, respectively.^{9,32} v-src(K295R) and c-src(K295R) are the respective kinase-defective mutants of v-src and c-src in which Lys-295 has been replaced by Arg, whereas c-src(wt) encodes the wild-type c-src.^{33,34} RasN17 is a dominant-negative mutant of p21ras in which the Ser-17 in the wild type has been replaced by an Asn.³⁵ c-src(F527) encodes a constitutively activated p60src in which the Tyr-527 in the wild type has been replaced by a Phe.³⁶ RasL61 is a constitutively activated form of p21ras, in which the Glu-61 in the wild type has been replaced by a Leu.³⁷ The pSV-β-gal plasmid, which contains a β-galactosidase gene driven by the SV40 promoter and enhancer, was also included in the transient transfection assays to monitor transfection efficiency. After the cells had been incubated with the plasmids for 6 hours, DMEM containing 10% FBS was added until the cells reached confluence. In general, 10% to 15% of the cells were plasmid transfected, as determined by X-gal staining. The cells in the tissue culture flasks were then subcultured on glass slides and either used for shear stress experiments or maintained as static controls. The luciferase reporter activities of the various experiments were normalized to the levels of the expressed β-galactosidase for transfection efficiency.

Statistics

The various kinase and luciferase activities assays were performed at least three times for each set of experiments. The results were analyzed using a computer statistical package (Excel). Difference between sets of experiments was statistically significant if *P* < .05 by Student's *t* test.

Results

Shear Stress Increases the Kinase Activities of p60src in ECs

To investigate whether shear stress increases the kinase activity of p60src in ECs, confluent monolayers of BAECs in flow channels were subjected to a steady shear stress of 12 dyne/cm² for different durations ranging from 1 to 20 minutes. Equal amounts of cell lysates were immunoprecipitated with anti-p60src, and kinase assays were performed using enolase and

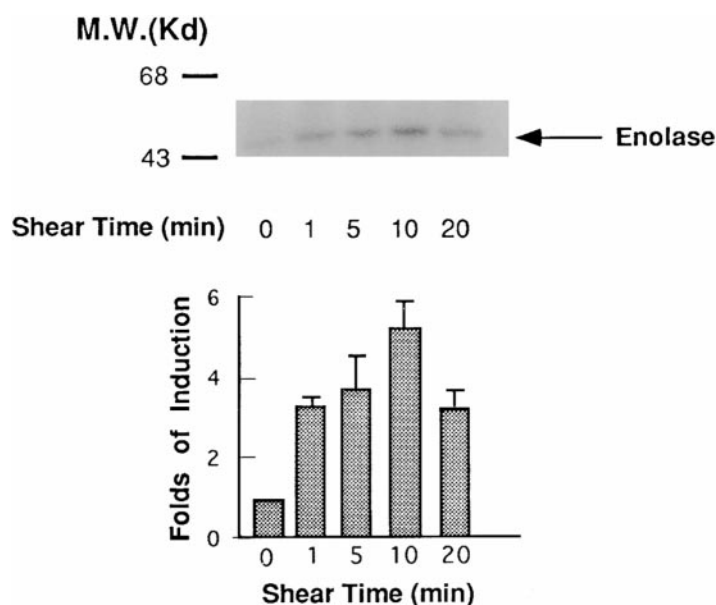


Figure 1. Shear stress increases the kinase activity of p60src in BAEC. Confluent monolayers of BAEC were subjected to a shear stress of 12 dyne/cm² in flow channels for periods of time as indicated or kept as static controls (shown as shear time 0). The kinase activities of p60src from the various samples were assessed by IP kinase assays using enolase and [γ -³²P]ATP as substrates. The phosphorylated enolase in the various samples is indicated by the arrow and the intensity was determined by densitometry. Bar graph, representing mean \pm SD from three sets of experiments, shows the increases in induction relative to that in the static controls.

[γ -³²P]ATP as the substrates. As shown in the top panel of Fig 1, the kinase activity of p60src was increased by shear stress as early as 1 minute, peaked at 10 minutes, and decreased afterward as indicated by the enolase phosphorylation. There was a 3.3 ± 0.2 -fold increase in p60src activity determined by densitometry in cells that had been subjected to shear stress for 1 minute compared with the static controls. Induction of kinase activity increased to 3.9 ± 0.8 -fold and 5.3 ± 0.6 -fold for cells subjected to shear stress for 5 and 10 minutes, respectively. However, the activity in cells subjected to shear stress for 20 minutes decreased to 3.2 ± 0.4 -fold. These results demonstrate that p60src is rapidly activated by shear stress in ECs in a transient manner.

Negative Mutants of src Attenuate the Shear Stress Activation of JNK and ERK

We and others have previously shown that shear stress activates JNK and ERK pathways in ECs.⁹⁻¹³ To investigate whether the shear-induced p60src can be upstream to JNK, BAECs were cotransfected with the epitope-tagged HA-JNK1 together with either an expression plasmid encoding v-src(K295R), a kinase-defective mutant of v-src, or an empty vector as a control. As shown in Fig 2A, subjecting the control cells (transfected with empty plasmid) to 30 minutes' shear stress increased the kinase activity of HA-JNK1 as demonstrated by the phosphorylation of GST-c-Jun(1-79) (lane 2 versus lane 1 in Fig 2A). In contrast, cotransfection of v-src(K295R) markedly attenuated the shear-induced JNK activity (lane 4 versus lane 2 in Fig 2A). Densitometry analysis showed that shear stress caused a 6.0-fold increase in the kinase activity of HA-JNK1 in control cells and that this was reduced to a 1.7-fold increase in cells cotransfected with v-src(K295R). In a separate set of experiments (Fig 2B), shear stress increased the kinase activity of HA-JNK1 in c-src-overexpressed cells (transfected with c-src[wt] encoding wild-type c-src). Cotransfection of c-src(K295R), a kinase-defective mutant of p60src, attenuated the shear stress activation of HA-JNK1 (lane 4 versus lane 2 in Fig 2B). The levels of the expressed HA-JNK1 in these BAECs, examined by immunoblotting, were essen-

tially the same (data not shown), indicating that the expression of HA-JNK1 was not affected by cotransfection of the various plasmids. To further demonstrate the requirement of p21ras in the induction of JNK by p60src, we tested whether a constitutively activated p60src, ie, c-src(F527), can activate JNK, and if it does, whether the activation can be blocked by RasN17, a dominant-negative mutant of p21ras. As shown in Fig 2C, cotransfection of c-src(F527) with HA-JNK1 indeed increased the kinase activity of HA-JNK1 (lane 2). Cotransfection of RasN17 markedly reduced the c-src(F527)-induced HA-JNK1 kinase activity (lane 3). Cotransfection of RasL61 (activated form of p21ras) also increased the HA-JNK1 activity (lane 4), but this could not be decreased by cotransfection of v-src(K295R) (lane 5). These results, in conjunction with those in Fig 2A, suggest that p60src is upstream to p21ras in the shear stress activation of JNK in BAECs.

To investigate whether p60src can also regulate the shear-induced ERK, BAECs were cotransfected with the epitope-tagged Myc-ERK2 together with either an empty vector as a control or plasmid v-src(K295R). As shown in lane 2 in Fig 3A, subjecting the control cells (transfected with empty plasmid) to shear stress induced the kinase activity of Myc-ERK2 by 5.3-fold. Cotransfection of v-src(K295R) reduced the shear stress-induced Myc-ERK2 activity to 2.3-fold (lane 4 versus lane 2 in Fig 3A). Expression plasmids encoding the wild-type c-src and c-src(K295R) were used in similar experiments as those in Fig 3A. Shear stress increased the kinase activity of Myc-ERK2 in cells transfected with c-src(wt) (lane 2 in Fig 3B). Cotransfection of c-src(K295R) attenuated the shear stress activation of Myc-ERK2 (lane 4 in Fig 3B). The expression of Myc-ERK2 was not affected by the cotransfection of the various plasmids (data not shown). Experiments were also performed using c-src(F527) (activated form of p60src) together with RasN17 (dominant-negative mutant of p21ras) or RasL61 (activated form of p21ras) together with v-src(K295R) (dominant-negative mutant of p60src) to examine the role of p21ras in its linkage between src and ERK. Cotransfection of RasN17 (lane 3 in the top panel of Fig 3C) abolished the

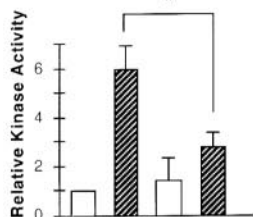
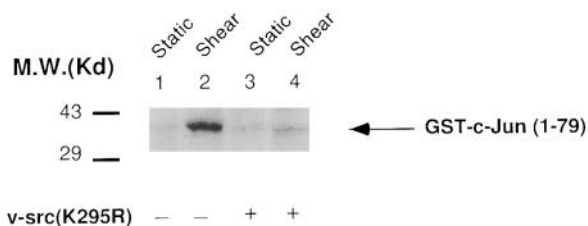
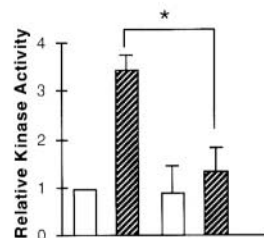
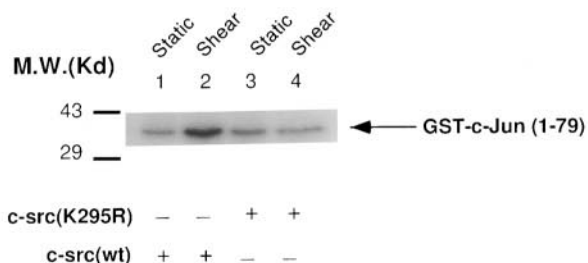
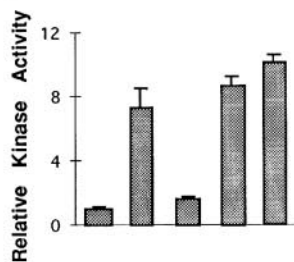
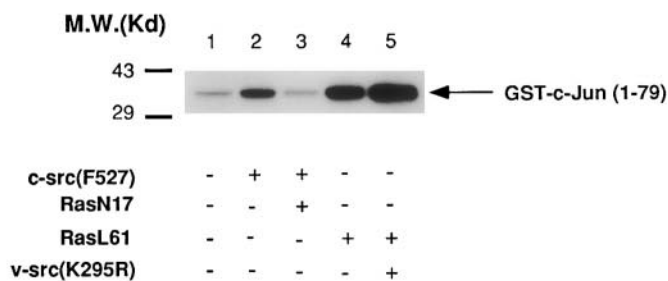
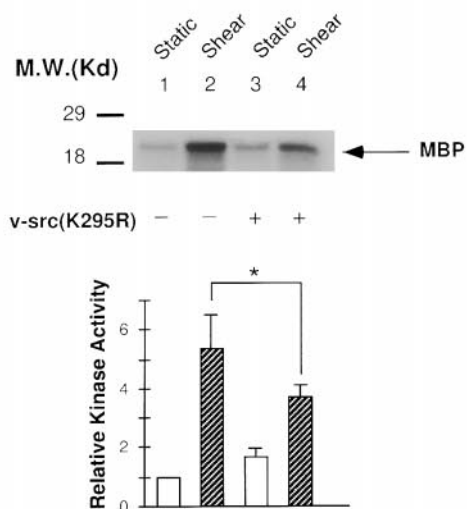
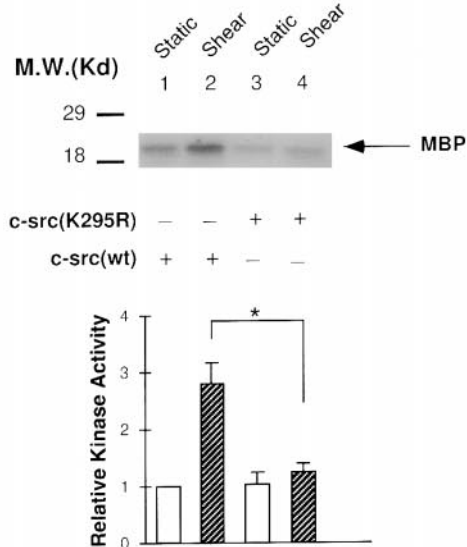
A.**B.****C.**

Figure 2. p60src is upstream to the Ras-JNK pathway in ECs in response to shear stress. In (A), 3 μ g of an expression plasmid encoding HA-JNK1 was cotransfected with either 6 μ g of pGL2 as an empty plasmid (lanes 1 and 2) or 6 μ g of v-src(K295R) (lanes 3 and 4) into BAEC in a T-75 tissue culture flask. The transfected cells were subcultured on glass slides and were either subjected to a shear stress of 12 dyne/cm² for 30 minutes or kept as static control. After the shear stress, HA-JNK1 was immunoprecipitated with anti-HA mAb and subjected to kinase assays using GST-c-Jun(1-79) and [γ -³²P]ATP as substrates. The phosphorylated GST-c-Jun(1-79) is indicated by the arrow. Intensities of phosphorylated bands were determined by densitometry. Bar graph shows the relative kinase activity (mean \pm SD) from three sets of experiments. The kinase activities of lanes 2, 3, and 4 have been normalized to that of lane 1. Asterisk indicates that the difference is significant ($P < .05$) between lanes 2 and 4. In (B), 3 μ g of HA-JNK1 was cotransfected with 6 μ g of the c-src(wt) (lanes 1 and 2) or 6 μ g of c-src(K295R) (lanes 3 and 4). The experimental conditions and data analysis were the same as those in (A). In (C), 3 μ g of HA-JNK1 was cotransfected with 6 μ g of pGL2 as an empty plasmid (lane 1), 6 μ g of c-src(F527) (lane 2), 6 μ g of c-src(F527) and 12 μ g of RasN17 (lane 3), 6 μ g of RasL61 (lane 4), or 6 μ g of RasL61 and 12 μ g of v-src(K295R) (lane 5). IP kinase assays for activities of HA-JNK1 in the various samples were performed with the same procedures as described above. Shown in the bottom is the relative activity representing mean \pm SD from three separate experiments. The results indicate that the c-src(F527)-activated HA-JNK1 (lane 2) is blocked by RasN17 (lane 3). In contrast, v-src(K295R) (lane 5) had little effect on the RasL61-activated HA-JNK1 (lane 4).

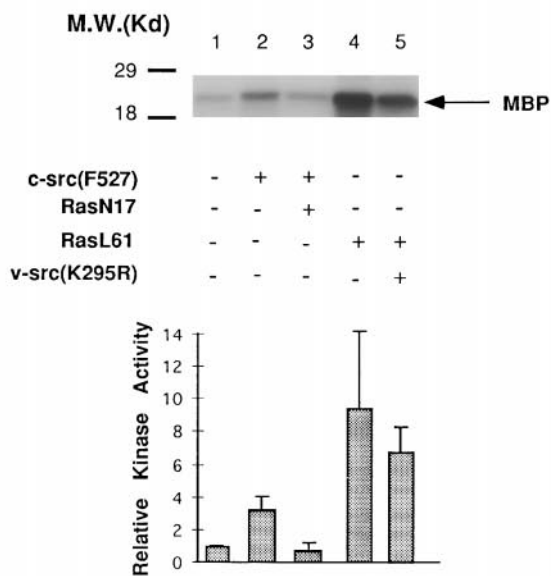
A.



B.



C.



c-src(F527)-induced Myc-ERK2 activity. In contrast, the activation of Myc-ERK2 by RasL61 was not attenuated by v-src(K295R) (lane 5 versus lane 4 in the top panel of Fig 3C). These results suggest that p60src is upstream to p21ras in the shear stress activation of ERK, as in the case of JNK activation.

src Regulates the Shear Stress-Induced c-Jun/TRE and Elk-1/SRE

Shear stress-elicited p60src signaling diverges at p21ras to activate JNK and ERK (ie, src-Ras-JNK and src-Ras-ERK). This suggests that transcriptional factors activated by either JNK or ERK pathway in response to shear stress could be regulated by p60src. JNK increases the transcriptional activity of c-Jun by phosphorylating its Ser-63 and Ser-73.³⁰ Similarly, ERK1 and ERK2 increase the transcriptional activity of Elk-1 by phosphorylating its Ser-383 and Ser-389.³⁸ We thus tested whether p60src regulates the shear stress-elicited transcriptional activities of c-Jun and Elk-1. Gal4-c-Jun(1-223), which encodes the fusion protein of the Gal4 DNA-binding domain and the c-Jun activation domain,³⁰ and 5XGal-Luc, which is a chimeric construct consisting of the Gal4-binding sequence and the luciferase reporter, were cotransfected into BAECs, which were then subjected to shear stress experiments. As shown in Fig 4A, shear stress caused a 3.7-fold induction of the luciferase activity, indicating an increased c-Jun transcriptional activity. When these plasmids, ie, Gal4-c-Jun(1-223) and 5XGal-Luc, were cotransfected with v-src(K295R), shear stress did not cause a significant increase in the luciferase activity (1.1-fold). Plasmid Gal4-ElkC(307-428), which encodes the Gal4 DNA-binding domain fused to the C-terminal activation domain of Elk-1,³¹ was cotransfected with 5XGal-Luc into BAECs to test whether shear stress increases the transcriptional activity of Elk-1. As shown in Fig 4B, shear stress caused a 2.7-fold increase in the luciferase activity. However, this induction was abolished when Gal4-ElkC(307-428) and 5XGal-Luc were cotransfected with v-src(K295R). These results demonstrate that shear stress increases the tran-

Figure 3. p60src is upstream to the Ras-ERK pathway in BAEC in response to shear stress. In (A), 3 μg of an expression plasmid encoding Myc-ERK2 was cotransfected with either 6 μg of pGL2 as an empty plasmid (lanes 1 and 2) or 6 μg of v-src(K295R) (lanes 3 and 4). The shear stress experiments were the same as those described in Fig 2A. Myc-ERK2 in the various samples were immunoprecipitated with anti-Myc mAb for IP kinase assays using MBP and [γ-³²P]ATP as substrates. The phosphorylated MBP is indicated by the arrow. Bar graph is the mean ± SD from three sets of experiments, showing the relative kinase activities normalized to that of lane 1. Asterisk indicates that the difference is significant (P < .05) between lanes 2 and 4. In (B), 3 μg of Myc-ERK2 was cotransfected with 6 μg of the c-src(wt) (lanes 1 and 2) or 6 μg of c-src(K295R) (lanes 3 and 4). The experimental conditions and data analysis were the same as those in (A). In (C), 3 μg of Myc-ERK2 was cotransfected with 6 μg of pGL2 as an empty plasmid (lane 1), 6 μg of c-src(F527) (lane 2), 6 μg of c-src(F527) and 12 μg of RasN17 (lane 3), 6 μg of RasL61 (lane 4), or 6 μg of RasL61 and 12 μg of v-src(K295R) (lane 5). Myc-ERK2 was immunoprecipitated for IP kinase assays. The relative activity shown in the bottom panel indicates that the c-src(F527)-activated Myc-ERK2 (lane 2) is blocked by RasN17 (lane 3). In contrast, cotransfection of v-src(K295R) (lane 5) did not significantly decrease the RasL61-activated Myc-ERK2 (lane 4).

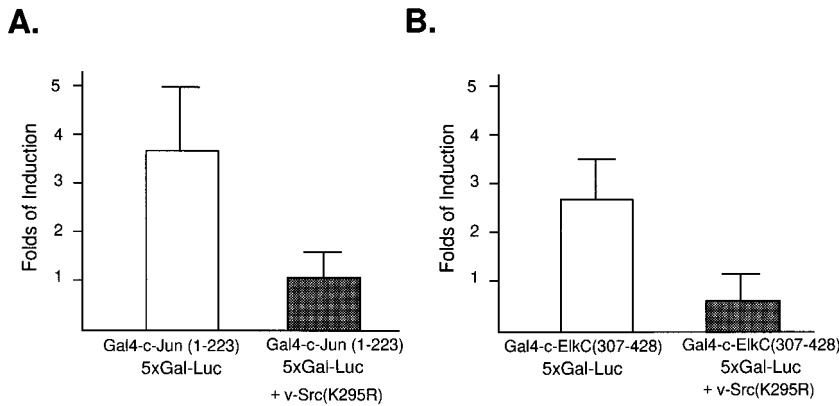


Figure 4. The shear stress activation of transcriptional activities of c-Jun and Elk-C are attenuated by negative mutant of v-src. (A) Plasmid Gal4-c-Jun(1-223) was cotransfected with 5xGal4-Luc, pSV- β -gal, and either with pGL2 as an empty vector or with v-src(K295R) into BAEC in a tissue culture flask. The transfected cells were subcultured on glass slides and either subjected to a shear stress of 12 dyne/cm² for 8 hours or kept as static controls, followed by luciferase activity assays. The normalized luciferase activities are the luminometer readings of the luciferase activity normalized for transfection efficiency based on the β -galactosidase activity. The increase in induction is defined as the ratio of the normalized

luciferase activity in samples subjected to shear stress to that in the static controls. The results represent mean \pm SD from three separate experiments. Shown in (B) are similar experiments using Gal4-ElkC(307-428) 5xGal4-Luc, and v-src(K295R).

scriptional activities of c-Jun and Elk-1 and that the induction is mediated, at least in part, by p60src.

Using the promoter construct MCP1-Luc, we have previously demonstrated that shear stress upregulates the MCP-1 gene through the activation of c-Jun/TRE.⁹ To investigate the involvement of p60src in the shear stress-induced MCP-1 gene, MCP1-Luc and v-src(K295R) were cotransfected into BAECs, and the transfected cells were then subjected to shear stress followed by luciferase assays. As shown in Fig 5A, the increments in induction decreased from 3.6-fold in the control group (transfected with empty plasmid) to 1.3-fold in the experimental group (transfected with v-src[K295R]). It has been shown that shear stress activates the *c-fos* gene in ECs.⁶ SRE is an essential *cis*-element in the *c-fos* promoter in response to many extracellular stimuli (see Reference 39 for review), and there is ample evidence indicating that JNK and ERK pathways converge on SRE to mediate gene expression.⁴⁰ The ETS motif of SRE is recognized by (TCF)/Elk-1.⁴¹ Thus, we tested whether p60src regulates the shear stress induction of *c-fos* promoter. Plasmid *c-fos*-Luc containing the *c-fos* promoter region (bp -750 to +45) fused to the luciferase reporter was transfected into BAECs to test the shear stress inducibility. Compared with the static controls, the luciferase activity in the sheared BAECs was increased by 16.1-fold (Fig 5B). Cotransfection of v-src(K295R) attenuated the shear stress induction of the *c-fos* promoter to 1.6-fold. These results, together with those in Fig 4B, demonstrate that p60src is upstream to the shear stress-activated Elk-1 in regulating the *c-fos* promoter.

Discussion

The major finding of this study is the identification of a shear stress-induced p60src signaling pathway that regulates the transcriptional activation of TRE and SRE via MAPKs, including both JNK and ERK pathways. The src-family PTKs possess several structural features that include (1) an approximately 60-amino acid src homology 3 (SH3) domain that is involved in the interaction with signaling molecules that have proline-rich regions, (2) an approximately 100-amino acid src homology 2 (SH2) domain that can recognize the phosphorylated tyrosine of many RTKs, and (3) a kinase catalytic domain (see References 42 and 43 for review). The activation of p60src by shear stress suggests that SH2- and SH3-containing molecules may be involved in the shear stress-induced signal transduction pathways and that RTKs may be upstream to such pathways. We have recently found that the Shc·Grb2·Sos ternary complex is involved in the endothelial gene expression in response to shear stress.⁴⁴ Shc and Grb2 are SH2-containing adaptor proteins that can associate with Sos, which is a guanine nucleotide exchange factor specific to p21ras.⁴⁵ The assembly of such a complex enables the activation of the Ras pathway by converting p21ras·GDP to p21ras·GTP. In NIH3T3 cells, the v-src transformation requires Ras activity.⁴⁶ In Rat-2 cells transformed by the v-src oncogene, Shc gene product becomes highly tyrosine phosphorylated.⁴⁷ In response to shear stress, the endothelial p21ras may therefore be activated by p60src via an Shc·Grb2·Sos-dependent pathway. Consistent with this hypothesis, we recently found that the dominant-negative mutant of Sos is able to attenuate the shear stress-induced c-Jun transcriptional activity.¹⁰

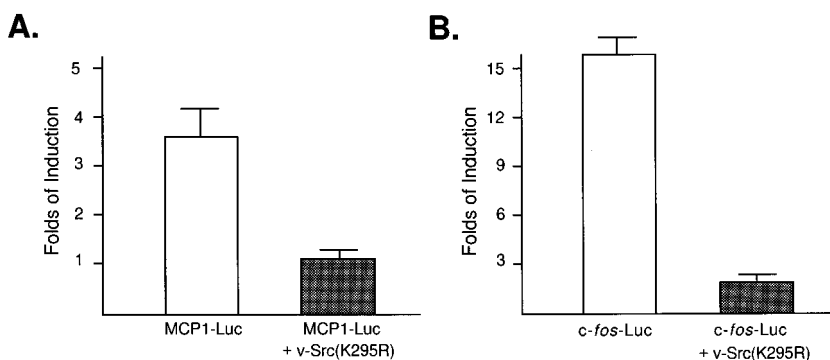


Figure 5. Negative mutant of v-src attenuates the shear stress-activated MCP1-Luc and *c-fos*-Luc. Plasmid MCP1-Luc (A) or *c-fos*-Luc (B) was cotransfected with pSV- β -gal, and with either pGL2 as an empty vector or an expression plasmid encoding v-src(K295R) into BAEC. The experimental conditions for shear stress followed by luciferase assays were the same as those described in Fig 4. The results representing mean \pm SD from three separate experiments indicate that the shear stress-increased promoter activities of MCP1-Luc and *c-fos*-Luc were attenuated by the cotransfection of v-src(K295R).

On stimulation of cells with growth factors such as EGF or PDGF, the cognate receptors (ie, EGFR and PDGFR, respectively) oligomerize while binding to their substrates. Subsequently, the kinase domains autophosphorylate the tyrosine residues at the regulatory domains to induce an affinity for the binding of proteins containing SH2 domain, including p60src, Grb2, and Shc (see Reference 48 for review). To examine the shear stress-activated molecules on the EC membrane that activate p60src, we have attempted to detect the phosphorylation of EGFR or PDGFR and their association with p60src, Shc, or Grb2 in BAECs in response to shear stress. However, these results were negative, which could be caused by the low expression of EGFR and PDGFR in large-vessel endothelium.^{49,50} It is possible that shear stress activates other endothelium-abundant RTKs or G proteins. In line with this notion, Jo et al¹² recently showed that shear stress activation of JNK involves a PTx-insensitive G protein-dependent pathway, whereas activation of ERK is G₁₂ dependent.

In addition to RTKs and G proteins, integrins may also be involved in the activation of p60src in response to shear stress. There is ample evidence that focal adhesion kinase (FAK) and p60src are involved in the integrin-mediated signal transduction (see Reference 51 for review). For example, an enhanced interaction between these two cytoplasmic nonreceptor PTKs was found when fibronectin bound to the integrins of NIH3T3 cells.⁵² Scanning confocal microscopy demonstrated the remodeling of focal adhesion sites, at which FAK and p60src aggregate, in the direction of flow.⁵³ Application of shear stress on ECs and the adhesion of ECs on fibronectin induce many similar biochemical responses, which include but are not limited to p60src activation, FAK tyrosine phosphorylation, and ERK activation.¹⁴ It was further shown that the endothelial responses elicited by a β 1-activating antibody involve some of those induced by shear stress.²³ Thus, integrins, in addition to G proteins, may be alternative mechanotransducers to activate p60src in ECs in response to shear stress. This hypothesis is supported by the studies demonstrating that the responses of rat smooth muscle cells to mechanical strain is mediated via both α v β 5 and β 3 integrins.⁵⁴

Our work reinforces the previous study by Takahashi and Berk¹⁴ that shear stress activates p60src in ECs and that the shear stress-activated ERK requires a herbimycin-sensitive kinase. Src family tyrosine kinases are inhibited by herbimycin A. The data in Figs 2 through 4 provide new, direct evidence that the shear-activated p60src is upstream to the Ras-JNK and Ras-ERK pathways, which in turn regulate the transcriptional activities of c-Jun and Elk-1, respectively. The induction of many genes requires the activation of these transcription factors to act on the corresponding *cis*-elements in the 5' promoter regions of the various genes. For example, the shear stress induction of the MCP-1 gene in ECs requires the TRE site² and the induction of the PDGF-B gene in the mechanically injured arterial ECs depends on the Egr-1 site.⁵⁵ ERK has also been suggested to regulate Egr-1.⁵⁶ It is likely that shear stress activates these genes through the Src-Ras-JNK or Src-Ras-ERK pathways. In addition, shear stress is able to regulate the morphology of ECs to cause their orientation in the direction of flow, and stress fibers are formed in these aligned cells. PTKs and possibly integrins are involved in those remodeling pro-

cesses.^{57,58} p60src may also be a key molecule regulating these endothelial events because of its functional role in the regulation of cytoskeletal rearrangement and shape change.⁵⁹⁻⁶¹ This is supported by the observation that p60src is activated and associated with the actin filament-associated protein, AFAP-110, in mechanically strained fetal rat lung cells.⁶²

Results from many research groups in this field suggest that shear stress activates multiple signal transduction pathways involving G proteins, integrins, RTKs, and so forth in ECs. The current and previous studies indicate that p60src plays a critical role in mediating these shear stress-elicited pathways.

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References

- Davies PF. Flow-mediated endothelial mechanotransduction. *Physiol Rev.* 1995;75:519-560.
- Shyy Y-J, Hsieh H-J, Usami S, Chien S. Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium. *Proc Natl Acad Sci U S A.* 1994;91:4678-4682.
- Nagel T, Reswick N, Atkinson WJ, Dewey CF Jr, Gimbrone MA Jr. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. *J Clin Invest.* 1994;94:885-891.
- Morita T, Yoshizumi M, Kurihara H, Maemura K, Nagai R, Yazaki Y. Shear stress increases heparin-binding epidermal growth factor-like growth factor mRNA levels in human vascular endothelial cells. *Biochem Biophys Res Commun.* 1993;197:256-262.
- Hsieh HJ, Li NQ, Frangos JA. Shear-induced platelet-derived growth factor gene expression in human endothelial cells is mediated by protein kinase C. *J Cell Physiol.* 1992;150:552-558.
- Hsieh HJ, Li NQ, Frangos JA. Pulsatile and steady flow induces c-fos expression in human endothelial cells. *J Cell Physiol.* 1993;154:143-151.
- Ranjan V, Xiao Z, Diamond SL. Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress. *Am J Physiol.* 1995;269:H550-H555.
- Uematsu M, Ohara Y, Navas JP, Nishida K, Murphy TJ, Alexander RW, Nerem RM, Harrison DG. Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *Am J Physiol.* 1995;269:C1371-C1378.
- Shyy J, Lin MC, Han J, Lu Y, Petrim M, Chien S. The *cis*-acting phorbol ester "12-O-tetradecanoylphorbol 13-acetate"-responsive element is involved in shear stress-induced monocyte chemotactic protein 1 gene expression. *Proc Natl Acad Sci U S A.* 1995;92:8069-8073.
- Li Y-S, Shyy Y-J, Li S, Lee JD, Su B, Karin M, Chien S. The Ras/JNK pathway is involved in the shear-induced gene expression. *Mol Cell Biol.* 1996;16:5947-5954.
- Tseng H, Peterson TE, Berk BC. Fluid shear stress stimulates mitogen-activated protein kinase in endothelial cells. *Circ Res.* 1995;77:869-878.
- Jo H, Sipos K, Go Y-M, Law R, Rong J, McDonald JM. Differential effect of shear stress on extracellular signal-regulated kinase and N-terminal Jun kinase in endothelial cells. *J Biol Chem.* 1997;272:1395-1401.
- Pearce MJ, McIntyre TM, Prescott SM, Zimmerman GA, Whately RE. Shear stress activates cytosolic phospholipase A2 (cPLA2) and MAP kinase in human endothelial cells. *Biochem Biophys Res Commun.* 1996;218:500-504.
- Takahashi M, Berk BC. Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase. *J Clin Invest.* 1996;98:2623-2631.
- Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem.* 1995;270:16483-16486.
- Hill CS, Treisman R. Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell.* 1995;80:199-211.
- Gille H, Sharrocks A, Shaw P. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at *c-fos* promoter. *Nature.* 1992;358:414-417.
- Treisman R. The serum response element. *Trends Biochem Sci.* 1992;17:423-426.

19. Dérjard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*. 1994;76:1025–1037.
20. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta*. 1991;1072:129–157.
21. Sadoshima J, Izumo S. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J*. 1993;12:1681–1692.
22. Razdan K, Hellums JD, Kroll MH. Shear-stress-induced von Willebrand factor binding to platelets causes the activation of tyrosine kinase(s). *Biochem J*. 1994;302:681–686.
23. Ishida T, Peterson TE, Kovach NL, Berk BC. MAP kinase activation by flow in endothelial cells. Role of $\beta 1$ integrins and tyrosine kinases. *Circ Res*. 1996;79:310–316.
24. Hunter T, Cooper JA. Tyrosine protein kinases and their substrates: an overview. *Adv Cyclic Nucleotide Protein Phosphorylation Res*. 1984;17:443–455.
25. Courtneidge SA, Kypta RM, Cooper JA, Kazlauskas A. Platelet-derived growth factor receptor sequences important for binding of src family tyrosine kinases. *Cell Growth Differ*. 1991;2:483–486.
26. Murata M, Kubota Y, Tanaka T, Iida-Tanaka K, Takahara J, Irino S. Effect of ubenimex on the proliferation and differentiation of U937 human histiocytic lymphoma cells. *Leukemia*. 1994;8:2188–2193.
27. Devary Y, Gottlieb RA, Smeal T, Karin M. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. *Cell*. 1992;71:1081–1091.
28. Frangos JA, Eskin SG, McIntire LV, Ives CL. Flow effects on prostacyclin production by cultured human endothelial cells. *Science*. 1985;227:1477–1479.
29. Gould KL, Hunter T. Platelet-derived growth factor induces multisite phosphorylation of pp60c-src and increases its protein-tyrosine kinase activity. *Mol Cell Biol*. 1988;8:3345–3356.
30. Minden A, Lin A, Claret F-X, Abo A, Karin M. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell*. 1995;81:1147–1157.
31. Marais R, Wynne J, Treisman R. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell*. 1993;73:381–393.
32. Büscher M, Rahmsdorf HJ, Litfin M, Karin M, Herrlich P. Activation of the c-fos gene by UV and phorbol ester: different signal transduction pathways converge to the same enhancer element. *Oncogene*. 1988;3:301–311.
33. Kamps MP, Sefton BM. Neither arginine nor histidine can carry out the function of lysine-295 in the ATP-binding site of p60src. *Mol Cell Biol*. 1986;6:751–757.
34. Broome MA, Hunter T. Requirement for c-Src catalytic activity and the SH3 domain in platelet-derived growth factor BB and epidermal growth factor mitogenic signaling. *J Biol Chem*. 1996;271:16798–16806.
35. Feig LA, Cooper GM. Inhibition of NIH3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol Cell Biol*. 1988;8:3235–3243.
36. Agbotounou WK, Levitzki A, Jacquelin-Sablon A, Pierre J. Effects of tyrphostins on the activated c-src protein in NIH/3T3 cells. *Mol Pharmacol*. 1994;45:922–931.
37. Quon MJ, Chen H, Ing BL, Liu M, Zarnowski MJ, Yonezawa K, Kasuga M, Cushman S, Taylor S. Roles of 1-phosphatidylinositol 3-kinase and ras in regulating translocation of GLUT4 in transfected rat adipose cells. *Mol Cell Biol*. 1995;15:5403–5411.
38. Gille H, Kortenjann M, Thomae O, Moomaw C, Slaughter C, Cobb MH, Shaw PE. ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J*. 1995;14:951–962.
39. Treisman R. Journey to the surface of the cell: fos regulation and the SRE. *EMBO J*. 1995;14:4905–4913.
40. Whitmarsh AJ, Shore P, Sharrocks AD, Davis RJ. Integration of MAP kinase signal transduction pathways at the serum response element. *Science*. 1995;269:403–407.
41. Hill CS, Marais R, John S, Wynne J, Dalton S, Treisman R. Functional analysis of a growth factor-responsive transcription factor complex. *Cell*. 1993;73:395–406.
42. Pawson T, Gish GD. SH2 and SH3 domains: from structure to function. *Cell*. 1992;71:359–362.
43. Carpenter G. Receptor tyrosine kinase substrates: src homology domains and signal transduction. *FASEB J*. 1992;6:3283–3289.
44. Shyy J, Li S, Kim M, Chien S. The shear stress-induced dual signal transduction pathways converged at Grb2. *Circulation*. 1996;94:1-443. Abstract.
45. Sadoshima J, Izumo S. The heterotrimeric Gq protein-coupled angiotensin II receptor activates p21 ras via the tyrosine kinase-Shc-Grb2-Sos pathway in cardiac myocytes. *EMBO J*. 1996;15:775–787.
46. Smith MR, DeGudicibus SJ, Stacey DW. Requirement for c-ras proteins during viral oncogene transformation. *Nature*. 1986;320:540–543.
47. McGlade J, Cheng A, Pelicci G, Pawson T. Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. *Proc Natl Acad Sci U S A*. 1992;89:8869–8873.
48. Li N, Schlessinger J, Margolis B. Autophosphorylation mutants of the EGF-receptor signal through auxiliary mechanisms involving SH2 domain proteins. *Oncogene*. 1994;9:3457–3465.
49. Lindner V, Reidy MA. Platelet-derived growth factor ligand and receptor expression by large vessel endothelium in vivo. *Am J Pathol*. 1995;146:1488–1497.
50. Bategay EJ, Rupp J, Iruela-Arispe L, Sage EH, Pech M. PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF β -receptors. *J Cell Biol*. 1994;125:917–928.
51. Parsons JT. Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins. *Curr Opin Cell Biol*. 1996;8:146–152.
52. Schlaepfer DD, Hanks SK, Hunter T, van der Geer P. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature*. 1994;372:786–791.
53. Davies PF, Robotewskyj A, Griem ML. Quantitative studies of endothelial cell adhesion. Directional remodeling of focal adhesion sites in response to flow forces. *J Clin Invest*. 1994;93:2031–2038.
54. Wilson E, Sudhir K, Ives HE. Mechanical strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrin interactions. *J Clin Invest*. 1995;96:2364–2372.
55. Khachigian LM, Lindner V, Williams AJ, Collins T. Egr-1-induced endothelial gene expression: a common theme in vascular injury. *Science*. 1996;271:1427–1431.
56. Cohen DM. Urea-inducible Egr-1 transcription in renal inner medullary collecting duct (mIMCD3) cells is mediated by extracellular signal-regulated kinase activation. *Proc Natl Acad Sci U S A*. 1996;93:11242–11247.
57. Girard PR, Norem RM. Shear stress modulates endothelial cell morphology and F-actin organization through the regulation of focal adhesion-associated proteins. *J Cell Physiol*. 1995;163:179–193.
58. Malek AM, Izumo S. Mechanism of endothelial cell shape change and cytoskeletal remodeling in response to fluid shear stress. *J Cell Sci*. 1996;109:713–726.
59. Chang J-H, Gill S, Settleman J, Parsons S. c-Src regulates the simultaneous rearrangement of actin cytoskeleton p190RhoGAP, and p120RasGAP following epidermal growth factor stimulation. *J Cell Biol*. 1995;130:355–368.
60. Wu H, Reynolds A, Kanner S, Vines R, Parsons T. Identification and characterization of a novel cytoskeleton-associated pp60src substrate. *Mol Cell Biol*. 1991;11:5113–5124.
61. Pawson T. Protein modules and signalling networks. *Nature*. 1995;373:573–580.
62. Liu M, Qin Y, Liu J, Tanswell AK, Post M. Mechanical strain induces pp60src activation and translocation to cytoskeleton in fetal rat lung cells. *J Biol Chem*. 1996;271:7066–7071.