

Fluid Shear Stress Activation of Focal Adhesion Kinase

LINKING TO MITOGEN-ACTIVATED PROTEIN KINASES*

(Received for publication, April 15, 1997, and in revised form, August 26, 1997)

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Shear stress, the tangential component of hemodynamic forces, activates the extracellular signal-regulated kinase (ERK) and c-Jun NH₂-terminal kinase (JNK) signal transduction pathways in cultured vascular endothelial cells to induce the transcriptional activation of many immediate early genes. It appears that integrins, protein-tyrosine kinases, and the structural integrity of actin are important factors involved in these shear stress-induced responses. The underlying molecular events were investigated by the application of a shear stress of 12 dyn/cm² on bovine aortic endothelial cells (BAEC). We found that such a shear stress increased the tyrosine phosphorylation and the kinase activity of focal adhesion kinase (FAK) and its association with growth factor receptor binding protein 2 (Grb2) in a rapid and transient manner, suggesting that FAK may be linked to these mitogen-activated protein kinase signaling pathways through a Grb2-Son of sevenless (Sos) complex. FAK(F397Y), which encodes a dominant negative mutant of FAK, attenuated the shear stress-induced kinase activity of Myc epitope-tagged ERK2 and hemagglutinin epitope-tagged JNK1. ΔSos1, encoding a dominant negative mutant of Sos in which the guanine nucleotide exchange domain has been deleted, also attenuated shear stress activation of Myc-ERK2 and hemagglutinin-JNK1. Pretreating the confluent BAEC monolayers with a blocking type anti-vitronectin receptor monoclonal antibody had similar inhibitory effects in these shear stress-activated ERKs and JNKs. Confocal microscopic observation further demonstrated that FAK tended to cluster with vitronectin receptor near the abluminal side of the sheared BAEC. These results demonstrate that FAK signaling is critical in the shear stress-induced dual activation of ERK and JNK.

Hemodynamic forces play important roles in maintaining cardiovascular homeostasis, but they can also be pathophysiological factors in conditions such as atherosclerosis. The involvement of hemodynamic forces in atherogenesis is mani-

festated by the focal distribution of atherosclerotic lesions in the bifurcations and curved regions of the arterial tree where blood flow is disturbed with flow separation. Fluid shear stress, the tangential component of the hemodynamic forces, is low and unsteady in these lesion-prone areas (1, 2). Vascular endothelial cells (ECs),¹ serving as a barrier between the vessel and blood, are exposed to shear stress under physiological and pathophysiological conditions. To unravel the roles played by hemodynamic forces in atherogenesis, flow channels have been used as *in vitro* systems to study functional changes of ECs in response to shear stress (for a review, see Ref. 3). These studies indicate that fluid shear stress induces a rapid induction of immediate early genes. c-Src and Ras are important mediators of shear stress activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases, to activate these immediate early genes (4–8). Although many mechanisms have been proposed to explain the EC response to shear stress, including those via G-proteins, cytoskeletal structure, membrane-associated K⁺ channels, and integrins (for a review, see Ref. 9), there is little experimental evidence indicating where and how the mechanotransduction occurs to activate the downstream signaling events, *e.g.* the activation of the MAPKs.

In response to shear stress, concomitant with the elongation of ECs and the alignment of stress fibers with the direction of flow, the focal adhesions on the abluminal side of ECs undergo dynamic, local reorientation without a noticeable change in the total attachment area (10). At the molecular level, such a dynamic rearrangement of focal adhesions may be related to spatial and temporal responses of the associated proteins, *e.g.* focal adhesion kinase (FAK), paxillin, tensin, and Src family protein-tyrosine kinases (PTKs) (for a review, see Ref. 11). Indeed, it was recently shown that shear stress causes a rapid and transient activation of c-Src in ECs (6). Both chemical stimulation, *e.g.* the treatment of monocytes with monocyte/macrophage colony-stimulating factor, and the adhesion of NIH3T3 fibroblasts on fibronectin promote the interaction of growth factor receptor-binding protein 2 (Grb2) with FAK (12–14). During cell attachment to the substratum, the association of integrins with the extracellular matrix (ECM) induces FAK autophosphorylation on Tyr-397, which leads to Src recruit-

* This study was supported in part by NHLBI, National Institutes of Health, Grants HL19454, HL43026 (to S. C.), and HL56707 (to J. S.) and by a biomedical engineering development award from the Whitaker Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: EC, vascular endothelial cell; BAEC, bovine aortic endothelial cell(s); ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase(s); FITC, fluorescein isothiocyanate; Grb2, growth factor receptor-2; GST, glutathione S-transferase; JNK, c-Jun NH₂-terminal kinase; MAPKs, mitogen-activated protein kinases; MBP, myelin basic protein; PTKs, protein-tyrosine kinases; SH2 and SH3 domain, Src homology-2 and -3 domain, respectively; Sos, Son of sevenless; VNR, vitronectin receptor(s); mAb, monoclonal antibody; HA, hemagglutinin.

ment. The association of FAK with Src family PTKs at focal adhesions further increases the phosphorylation of FAK at Tyr-925, creating a Grb2 binding site (14–17). Grb2 has a relatively simple structure that contains one Src homology-2 (SH2) domain flanked by two SH3 domains (18). Grb2 is constitutively bound to Son of sevenless (Sos), a guanine nucleotide exchange factor, via its SH3 domain. The Grb2-Sos complex activates Ras by converting the GDP-bound inactive state to the GTP-bound active state (19, 20). Grb2 also associates with activated receptor tyrosine kinases such as the epidermal growth factor receptor and monocyte/macrophage colony-stimulating factor receptor, through the interaction of its SH2 with the phosphotyrosines of these receptor tyrosine kinases (21–23). The association of Grb2-Sos with activated receptor tyrosine kinases brings Sos to the cytoplasmic face of the plasma membrane, where it can interact with membrane-associated Ras to catalyze GDP-GTP exchange (24).

Results from the current study demonstrate that shear stress induces the activation of FAK and the formation of FAK-Grb2-Sos ternary complex in ECs. This signaling pathway leads to the activation of both ERK and JNK. On the upstream side, integrins (*e.g.* VNR), by interacting with FAK, may be involved in the mechanotransduction that transfers fluid shear stress into biochemical signals.

EXPERIMENTAL PROCEDURES

Cell Cultures and Cell Treatment—Cell culture reagents were purchased from Life Technologies, Inc. Bovine aortic endothelial cells (BAEC) were isolated from bovine aorta either with collagenase or by gently scraping with a rubber policeman. BAEC were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1 mM each of penicillin-streptomycin and sodium pyruvate. Cell cultures were maintained in a humidified 95% air, 5% CO₂ incubator at 37 °C. All experiments were conducted with cultures prior to passage 10. Genistein (100 μM, from Sigma), cytochalasin B (1 μM, Sigma), and anti-α₅β₃ integrin (*i.e.* the VNR) mAb (clone LM609, 10 μg/ml, from Chemicon, Temecula, CA) were used to treat BAEC in various experiments prior to the application of shear stress.

Shear Stress Experiments—A flow system was used to impose fluid shear stress on cultured ECs as described previously (25). In brief, a 75 × 38-mm glass slide was seeded with a confluent monolayer of BAEC. A silicone gasket was sandwiched between the glass slide and an acrylic plate to create a rectangular flow channel (0.025 cm in height, 2.5 cm in width, and 5.0 cm in length) with inlet and outlet for exposing the cultured BAEC to fluid shear stress. Steady, laminar flow across the channel was generated by using a peristaltic pump and a damping reservoir. During the flow experiments, the system was kept at 37 °C and equilibrated with 95% humidified air with 5% CO₂.

DNA Plasmids and Transient Transfection—Plasmids hemagglutinin (HA)-JNK1, HA-FAK(wt), HA-FAK(F397Y), Myc-ERK2, and ΔmSos1 were described previously (13, 26, 27). The various DNA plasmids were transfected into BAEC at 80% confluence using the lipofectamine method (Life Technologies, Inc.). After incubation for 6 h, the transfected cells were washed with Dulbecco's modified Eagle's medium and incubated in fresh complete Dulbecco's modified Eagle's medium medium to reach confluence. Within 48 h after transfection, the BAEC monolayer was serum-starved for 12 h and then either subjected to fluid shear stress or kept as static controls.

Immunoprecipitation and Immunoblotting—The antibodies used in immunoprecipitation and immunoblotting were polyclonal anti-FAK (14), PY20 anti-phosphotyrosine mAb (Transduction Laboratories, Lexington, KY), polyclonal anti-Grb2/Sem5 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-JNK1 antibody (Santa Cruz Biotechnology), anti-ERK2 mAb (Upstate, Lake Placid, NY), anti-HA mAb (Boehringer Mannheim), and anti-c-Myc mAb (Santa Cruz Biotechnology). For immunoprecipitation, cells were scraped into lysis buffer; the lysates were centrifuged, and the supernatants were immunoprecipitated with appropriate antibodies and protein A-Sepharose beads (Pharmacia Biotech Inc.) at 4 °C overnight. The immunoprecipitated complexes were washed and used for either kinase activity assay or immunoblotting. For SDS-polyacrylamide gel electrophoresis, the samples, in 2% SDS sample buffer, were separated on an SDS-polyacrylamide gel. For immunoblotting, proteins in the gel were transferred to a nitrocellulose membrane. The membrane was blocked with 3% nonfat

milk followed by incubation with the primary antibody in TTBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) containing 0.1% bovine serum albumin. The bound primary antibodies were detected by using a goat anti-mouse or a goat anti-rabbit IgG-horse radish peroxidase conjugate (Santa Cruz Biotechnology) and the ECL detection system (Amersham Corp.).

Kinase Activity Assays—To assay endogenous ERK activity, BAEC were lysed in a kinase lysis buffer (25 mM HEPES, pH 7.4, 0.5 M NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 50 mM NaF, 10 mM Na₃VO₄, and 2 mM β-glycerophosphate). ERK2 was immunoprecipitated with anti-ERK2 mAb and protein A-Sepharose beads. To perform immunocomplex kinase assays, the immunoprecipitates were washed twice in the lysis buffer and twice in a kinase assay buffer (25 mM HEPES, pH 7.4, 20 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, and 2 mM dithiothreitol). Two micrograms of myelin basic protein (MBP) and 10 μCi of [³²P]ATP (ICN, Irvine, CA) in 30 μl of kinase assay buffer with 25 μM ATP were added to each immunocomplex pellet for kinase reaction at 30 °C for 20 min. The phosphoproteins were separated by SDS-polyacrylamide gel electrophoresis, and the gels were dried for autoradiography. In the inhibition experiments, epitope-tagged Myc-ERK2 plasmids (3 μg) were co-transfected with plasmids encoding the various negative mutants (9 μg) into BAEC. After shear stress experiments, the cells were lysed in the kinase lysis buffer. Myc-ERK2 was immunoprecipitated with anti-c-Myc mAb and protein A-Sepharose beads, followed by Myc-ERK2 kinase activity assays using the same procedures as described above. The kinase activities of the endogenous JNK1 and epitope-tagged HA-JNK1 were assessed by using essentially the same method as those for ERK, except that glutathione S-transferase (GST)-c-Jun-(1–79) fusion protein was used as the substrate. For FAK kinase activity assays, cells were lysed in a lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 1 mM EGTA, 1.5 mM MgCl₂, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM Na₃VO₄. The cell lysates were immunoprecipitated with polyclonal anti-FAK followed by kinase activity assays in a kinase assay buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM Na₃VO₄, 2 mM dithiothreitol, 25 μCi of [³²P]ATP, and 25 μM ATP.

Immunostaining and Confocal Microscopy—Confluent monolayers of BAEC were fixed in acetone at room temperature for 3 min. The cells were then incubated in phosphate-buffered saline containing a polyclonal anti-FAK (SC-903, Santa Cruz Biotechnology) at a concentration of 1:200 (v/v) and 20 μg/ml anti-VNR mAb (LM609, Chemicon) for 3 h at room temperature. The specimens were washed in phosphate-buffered saline and incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and a rhodamine-conjugated goat anti-mouse IgG (Molecular Probes). Double immunostaining of FAK and VNR was observed under a confocal microscopy system (MRC-1000, Bio-Rad) equipped with an argon/krypton laser line, a scan head, and a Nikon Diaplot 300 inverted microscope. FITC was excited at a wavelength of 488 nm and detected within a band between 506 and 538 nm. Rhodamine was excited at 568 nm and detected within a band between 589 and 621 nm.

RESULTS

Tyrosine Kinases and Actin Structure Integrity Are Critical in the Shear Stress Activation of ERK and JNK—We and others have previously shown that shear stress activates many transcriptional factors in ECs, including c-Jun and NF-κB, which lead to the induction of several inflammation-related genes such as the monocyte chemotactic protein-1 gene (28–30). Some of this transcriptional activation is due, at least in part, to the activation of MAPKs, including ERK and JNK (4–7). To examine the functional roles of PTKs and actin organization involved in shear stress activation of ERK and JNK, confluent monolayers of BAEC cultured on glass slides were pretreated with genistein (PTKs inhibitor) or cytochalasin B (actin microfilament disrupting reagent) followed by an exposure to fluid shear stress for 10 min (for ERK2 assay) or 30 min (for JNK1 assay); these are the time periods at which the shear-induced activities are highest for the two kinases (5). Densitometry analysis indicates that shear stress drastically increased the

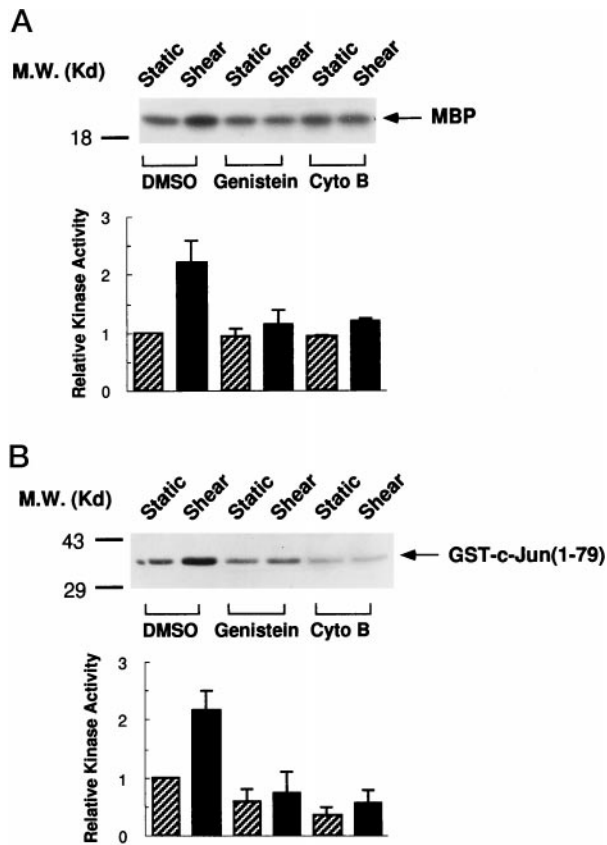


FIG. 1. Genistein and cytochalasin B attenuate the shear stress activation of ERK and JNK. *A*, confluent monolayers of BAEC were treated with 0.1% Me₂SO (DMSO), 100 μM genistein, or 1 μM cytochalasin B for 1.5 h before being subjected to a shear stress of 12 dyn/cm² for 10 min or a static incubation for the same length of time. The cells were then lysed, and ERK2 was immunoprecipitated from the cell lysates with an anti-ERK2 mAb for immunocomplex kinase assays using MBP and [γ-³²P]ATP as substrates. The bands indicated by the arrow represent the phosphorylated MBP after SDS-polyacrylamide gel electrophoresis and autoradiography. *B*, BAEC were treated with genistein or cytochalasin B under similar conditions as those in panel *A*. The cells were then either kept as static controls or sheared for 30 min. The cell lysates were immunoprecipitated with a polyclonal anti-JNK1 antibody, and GST-c-Jun-(1-79) and [γ-³²P]ATP were added to the immunoprecipitates for immunocomplex kinase assays. The bands indicated by the arrow represent the phosphorylated GST-c-Jun-(1-79). Bar graphs, representing mean ± S.E. from three separate experiments, show the kinase activities of the various samples relative to those in the Me₂SO-treated, static controls.

kinase activities of ERK and JNK in cells that were treated with Me₂SO as solvent control (compare *first* and *second* lanes in Fig. 1, *A* and *B*). Pretreatment of BAEC with either genistein or cytochalasin B had little effect on the ERK2 activity but significantly decreased JNK1 activity in the static state as compared with Me₂SO control. However, both the pretreatment abolished the shear stress activation of ERK2 and JNK1. These results suggest that the shear stress activation of MAPKs depends upon the upstream PTK signaling as well as the integrity of the actin cytoskeleton.

Shear Stress Induces FAK Phosphorylation and the Association of FAK and Grb2—To investigate tyrosine phosphorylation in ECs in response to shear stress, confluent monolayers of BAEC were subjected to a shear stress of 12 dyn/cm² for various lengths of time, and cell lysates from either static or sheared BAEC were immunoblotted with PY20 anti-phosphotyrosine mAb. Proteins with molecular masses of 45, 60, and 120 kDa were inducibly phosphorylated on tyrosines in the sheared cells (data not shown). To investigate whether the 120-kDa tyrosine-phosphorylated protein corresponds to FAK,

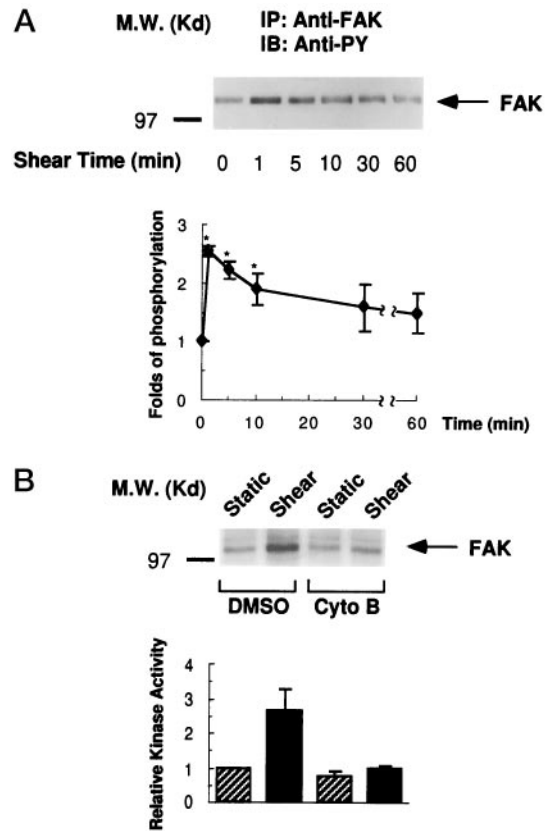


FIG. 2. Shear stress increases tyrosine phosphorylation of FAK and the kinase activity of FAK. After serum-starvation for 15 h, BAEC monolayers were either kept as static controls (represented by time 0) or subjected to a shear stress of 12 dyn/cm². *A*, 500 μg of the cell lysate from each sample following different durations of shearing was subjected to immunoprecipitation (IP) with a polyclonal anti-FAK antibody and immunoblotting (IB) with PY20 anti-phosphotyrosine mAb. The bound antibodies were detected by the ECL system. Shown in the *bottom part* is densitometry analysis representing the mean ± S.E. from three separate experiments. The asterisks indicate significant difference ($p < 0.05$) between sheared samples and static controls (time 0). *B*, BAEC monolayers were either pretreated with 0.1% Me₂SO (DMSO) or 1 μM cytochalasin B for 1.5 h. The cells were then kept as static controls or subjected to a shear stress of 12 dyn/cm² for 5 min. FAK was immunoprecipitated with a polyclonal anti-FAK antibody for kinase activity assays. Shear stress activation of FAK is indicated by the increased autophosphorylation of FAK in the Me₂SO samples, but not following cytochalasin B treatment. The bar graph at the *bottom*, representing mean ± S.E. from three separate experiments, shows the kinase activities of the various samples relative to those in the Me₂SO-treated, static controls.

FAK was immunoprecipitated from these cell lysates and detected by immunoblotting with PY20 mAb. As shown in Fig. 2*A*, shear stress induced a rapid and transient tyrosine phosphorylation of FAK with a molecular mass of 120 kDa. A maximum level was reached within 1 min, which was then decreased gradually. Fig. 2*B* indicates that the kinase activity of FAK was also increased by shear stress. After a 5-min shearing treatment, the kinase activity of FAK increased by 3-fold (assessed by FAK autophosphorylation). The shear stress activation of FAK was also dependent upon actin structure integrity, since pretreatment of the cells with cytochalasin B attenuated such an activation.

The activation of ERK and JNK in response to shear stress is mediated by Ras (5, 7), which is activated, in many other instances (*e.g.* epidermal growth factor stimulation), by the Grb2·Sos complex. Thus, we examined next whether shear stress induced the association of Grb2 with FAK in BAEC by immunoblotting the nitrocellulose membrane used in Fig. 2*A*

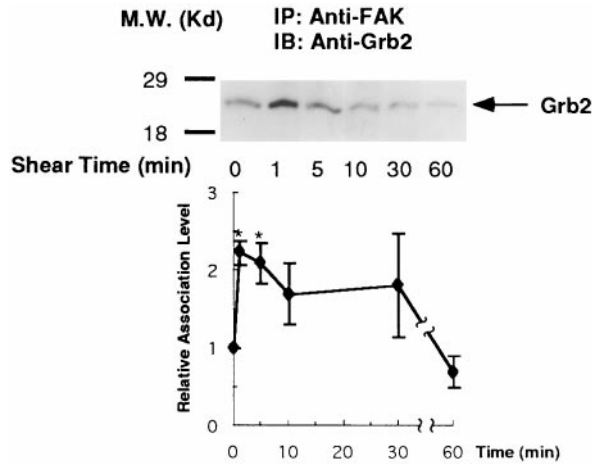


FIG. 3. Shear stress increases FAK/Grb2 association in BAEC. The nitrocellulose membrane used in Fig. 2A was immunoblotted with polyclonal anti-Grb2. The bands were detected by the ECL system. Shown in the *bottom part* is densitometry analysis representing the mean \pm S.E. from three experiments. The *asterisks* indicate significant difference ($p < 0.05$) between sheared samples and static controls (time 0).

with a polyclonal anti-Grb2. As shown in Fig. 3, there was an increase in the amount of Grb2 co-immunoprecipitated with FAK in ECs subjected to shear stress for 1 min. The increased association of Grb2 with FAK lasted for at least 5 min and then decreased to a level similar to that in the static controls. In a separate experiment, cell lysates immunoprecipitated with a polyclonal anti-Sos and immunoblotted with the polyclonal anti-Grb2 revealed a constant level of Grb2/Sos association in both static and sheared cells (data not shown), confirming that Grb2 is constitutively associated with Sos. Together, the results presented in Figs. 2 and 3 suggest that the FAK-Grb2-Sos pathway is activated by shear stress, which may mediate the upstream PTK signaling to activate the downstream ERK and JNK pathways in ECs.

FAK-Grb2-Sos Pathway Regulates the Activity of ERK and JNK—We used dominant negative mutants of FAK and Sos to test the above hypothesis that the FAK-Grb2-Sos pathway is critical for shear stress activation of ERK and JNK. Tyr-397 of FAK is the major autophosphorylation site, which binds Src family PTK (13, 17). FAK(wt) encodes a wild-type HA-tagged FAK. FAK(F397Y) encodes a mutated HA-FAK in which Tyr-397 has been replaced by Phe that blocks the binding of both Src family and Grb2 to FAK (31). We co-transfected an empty plasmid pcDNA3, HA-FAK(wt), or HA-FAK(F397Y) together with either the epitope-tagged Myc-ERK2 or the epitope-tagged HA-JNK1 to assess the functional roles of the Tyr-397 of FAK in shear stress activation of ERK and JNK. The transfected cells were either kept as static controls or subjected to a treatment of shear stress for 10 min (for Myc-ERK2 assay) or 30 min (for HA-JNK1 assay) followed by immunocomplex kinase assays using MBP or GST-c-Jun-(1-79) followed by immunocomplex kinase assays using MBP or GST-c-Jun-(1-79) as the respective substrate. As shown in the *top part* of Fig. 4A, in cells transfected with the pcDNA3 empty vector or HA-FAK(wt), shear stress significantly increased the kinase activity of Myc-ERK2, as demonstrated by the enhanced MBP phosphorylation. In contrast, co-transfection of FAK(F397Y) blocked the shear stress activation of Myc-ERK2. Immunoblotting with anti-Myc mAb (*bottom part* of Fig. 4A) revealed that the Myc-ERK2 expression was not affected by the co-transfection of the various plasmids. While shear stress also enhanced the phosphorylation of GST-c-Jun-(1-79) in cells transfected with the pcDNA3 empty vector or HA-FAK(wt), co-transfection with FAK(F397Y) reduced the phosphorylation by 50% (Fig.

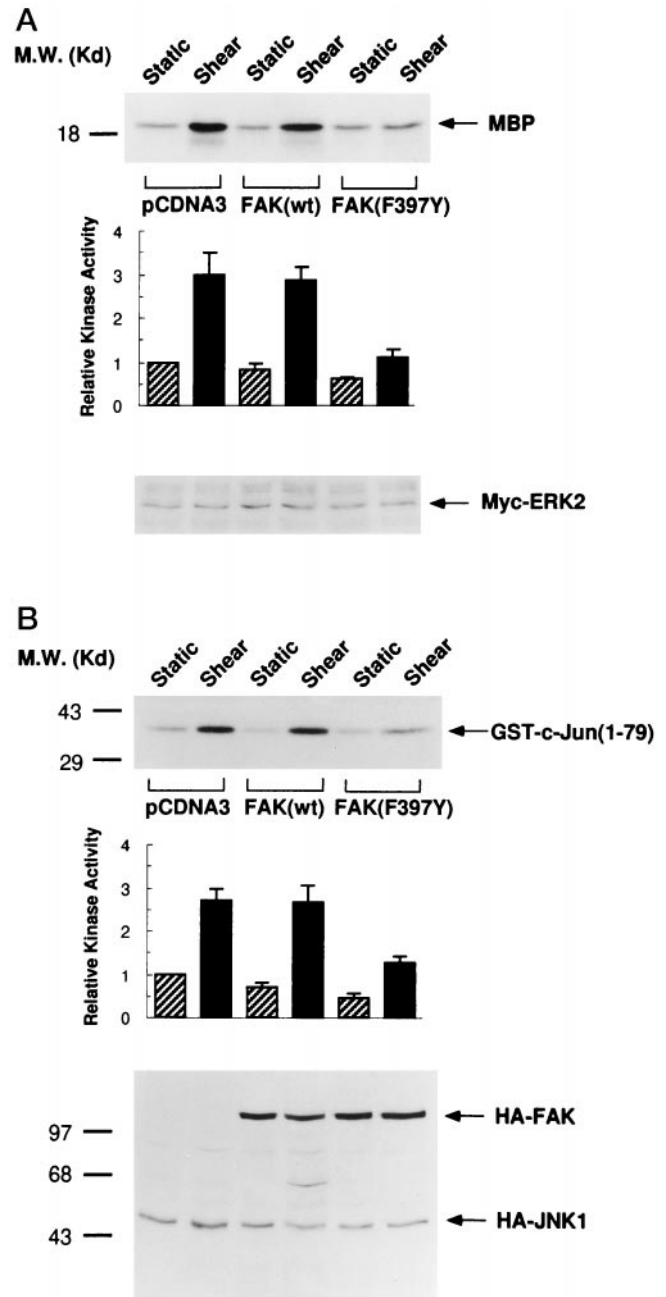


FIG. 4. Dominant negative mutant of FAK attenuates shear stress activation of Myc-ERK2 and HA-JNK1. A, 3 μ g of epitope-tagged Myc-ERK2 was co-transfected with 9 μ g of pcDNA3 empty vector, HA-FAK(wt), or HA-FAK(F397Y) into BAEC in 75-cm² tissue culture flasks. The transfected cells were passed onto slides and serum-starved for 12 h before being subjected to a shear stress of 12 dyn/cm² for 10 min. The cell lysates were immunoprecipitated with anti-Myc mAb for immunocomplex kinase assays using MBP and [γ -³²P]ATP as substrates. The bands indicated by the *arrow* represent the phosphorylated MBP after SDS-polyacrylamide gel electrophoresis and autoradiography. Shown in the *bottom panel* is immunoblotting with anti-Myc mAb, indicating that comparable amounts of Myc-ERK2 were expressed in the various cells. B, the result of a parallel set of experiments in which 3 μ g of HA-JNK1 were co-transfected with 9 μ g of pcDNA3, HA-FAK(wt), or HA-FAK(F397Y) into BAEC, followed by the application of shear stress for 30 min. HA-JNK1 was immunoprecipitated for immunocomplex kinase assays using GST-c-Jun-(1-79) and [γ -³²P]ATP as substrates. Shown in the *bottom part* is an immunoblot with anti-HA mAb. *Bar graphs*, representing mean \pm S.E. from three separate experiments, show the kinase activities of the various samples relative to those in the pcDNA3-transfected, static controls.

4B). The phosphorylation of GST-c-Jun-(1-79) was attributed to the HA-JNK1 activity rather than that of the co-immunoprecipitated HA-FAK, since immunocomplex kinase assays indicated that the FAK immunoprecipitated from the same sample by polyclonal anti-FAK could not phosphorylate GST-c-Jun-(1-79) (data not shown). The *bottom part* of Fig. 4B also shows that the HA-JNK1 expression was not affected by the co-transfection of the various plasmids.

Similar experiments were performed by using Δ mSos1 to assess the effects of functional blocking of Grb2·Sos on the shear stress activation of ERK and JNK. In Δ mSos1, the segment between nucleotides 618 and 1036 that corresponds to the guanine nucleotide exchange domain in the wild-type Sos cDNA has been deleted. Hence, the encoded protein acts as a dominant negative mutant of Sos (27). As shown in Fig. 5, A and B, co-transfection of Δ mSos1 caused decreased phosphorylation of both MBP and GST-c-Jun-(1-79) in response to shear stress, indicating a suppression of Myc-ERK2 and HA-JNK1 activities in these cells. The results presented in Figs. 4 and 5 indicate that shear stress activates FAK-Grb2·Sos in ECs, which is a functional signaling pathway upstream of ERK and JNK.

VNR Is Involved in the Shear Stress Activation of ERK and JNK—The adhesion of fibroblasts to fibronectin-coated dishes promotes the interaction of Grb2 with FAK (14). Activation of $\alpha_2\beta_1$ integrin in T lymphocytes results in an increase in the GTP-bound Ras (32). Furthermore, cyclic stretch-induced mitogenic response of smooth muscle cells is dependent upon fibronectin and vitronectin receptors (33). These previous studies, together with the finding that actin organization is important for the shear stress activation of ERK and JNK (Fig. 1), prompted us to investigate the roles of integrins in the shear stress activation of ERK and JNK. We chose VNR (*i.e.* $\alpha_v\beta_3$ integrin) in this study due to its abundance in EC and its presence in the atherosclerotic plaques (34). Confluent monolayers of BAEC were preincubated for 2 h with antibody LM609, an anti- $\alpha_v\beta_3$ mAb that has been shown to inhibit endothelial spreading (35) and to decrease angiogenesis in tumors (36). After a 2-h incubation, the LM609 mAb was detected at focal adhesion sites, cell junctions, and cytoplasm by immunostaining with rhodamine-conjugated goat anti-mouse IgG (data not shown), indicating that such an incubation allowed access of the antibody to the abluminal side of cells. A shear stress of 12 dyn/cm² was applied to these LM609-incubated cells for 10 min (for the ERK2 activity assay) or 30 min (for the JNK1 activity assay). As shown in Fig. 6, preincubation BAEC with LM609 attenuated the shear stress activation of ERK2 and JNK1, as indicated by the decreased phosphorylation of MBP and GST-c-Jun-(1-79). These results suggest that VNR is involved in the mechanotransduction that mediates the shear stress activation of ERK and JNK pathways.

Dynamic Responses of FAK and VNR to Shear Stress—We also investigated microscopically the distribution of FAK in EC, its spatial relation to VNR, and their dynamic interactions in response to shear stress. Confluent BAEC were kept static or subjected to a shear stress of 12 dyn/cm². Double immunostaining with a polyclonal anti-FAK and an anti-VNR mAb LM609 was performed for confocal microscopic observation. The focal pattern of staining for both FAK and VNR were found at the abluminal side of the cells (Fig. 7, A and B) but not at the luminal side of the cells (Fig. 7, C and D). Shearing BAEC for 3 min caused an enhanced staining of FAK and VNR at the peripheral region of the abluminal side of the cells (Fig. 7, E and F). Superimposing Fig. 7A with Fig. 7B and Fig. 7E with Fig. 7F shows that there was an increase in the clustering of FAK and VNR. However, not all VNR nor FAK were co-local-

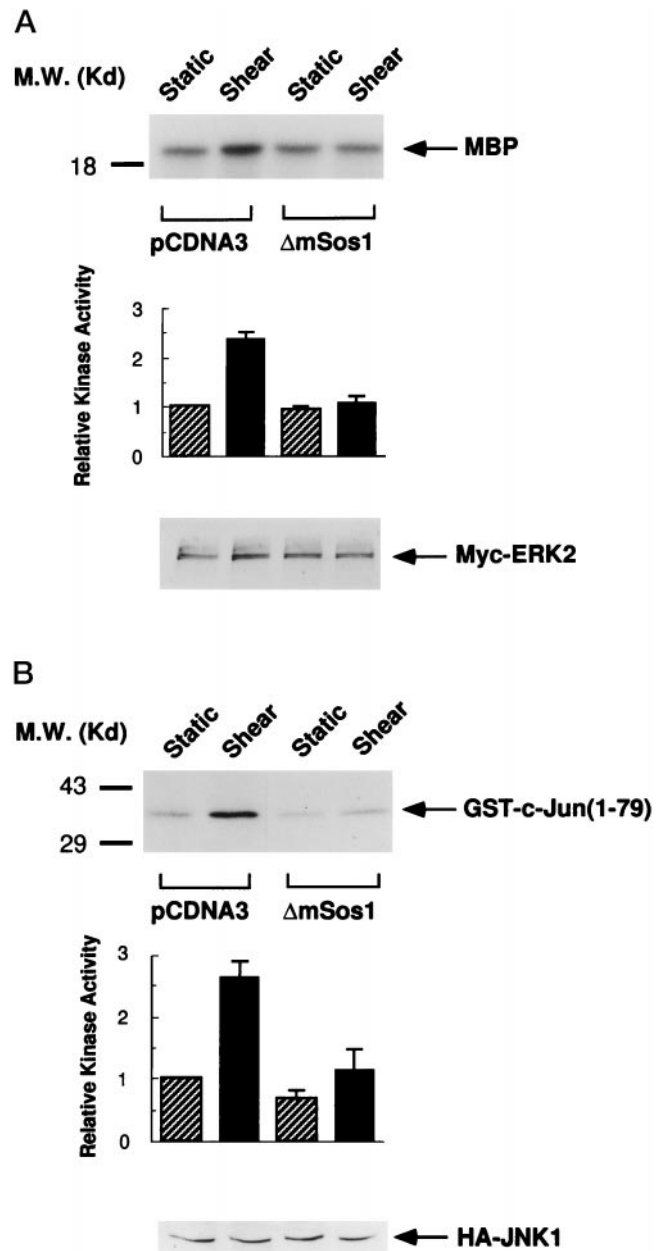


FIG. 5. Dominant negative mutant of Sos attenuates shear stress activation of Myc-ERK2 and HA-JNK1. Three micrograms of Myc-ERK2 (A) or HA-JNK1 (B) was co-transfected with either 12 μ g of pCDNA3 or Δ mSos1 into BAEC. The conditions for shear stress experiments and kinase assays for Myc-ERK2 and HA-JNK1 were the same as those described in the legend to Fig. 4. Shown in the *bottom panels* of A and B are immunoblots with anti-Myc mAb or with anti-HA mAb. *Bar graphs*, representing mean \pm S.E. from three separate experiments, show the kinase activities of the various samples relative to those in the pCDNA3-transfected, static controls.

ized in these sites. Thus, VNR may be one of the molecules linked to the shear stress-activated FAK to initiate the FAK-Grb2·Sos-Ras-ERK/JNK signaling pathways.

DISCUSSION

Fluid shear stress is a physiological form of mechanical forces, and it causes a dual activation of ERK and JNK in ECs. This may have significant impact on vascular biology since ERK is involved in the mediation of cell growth, and JNK may be engaged in the stress responses and programmed cell death (for a review, see Ref. 37). The inhibitory effects of genistein (Fig. 1) show that one or more PTKs are essential in the

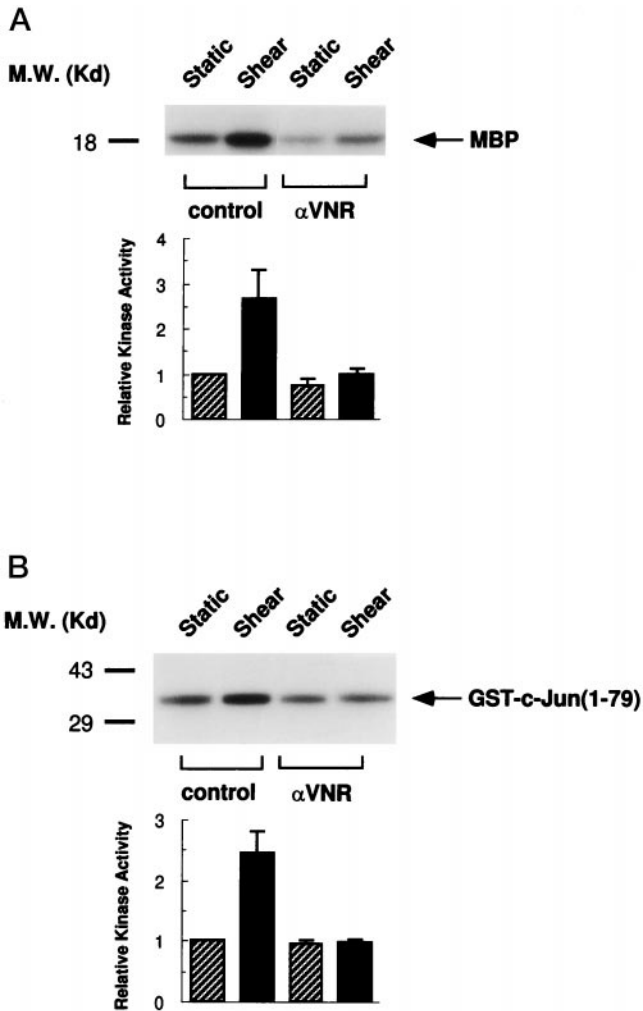


FIG. 6. Anti-VNR down-regulates the shear stress activation of ERK and JNK. Confluent monolayers of BAEC were treated with an anti- $\alpha_v\beta_3$ mAb (clone LM609, 10 $\mu\text{g}/\text{ml}$) for 2 h. The treated cells were then subjected to a shear stress of 12 dyn/cm^2 for 10 min for ERK2 kinase activity assays (A) or for 30 min for JNK1 kinase activity assays (B). The procedures for immunocomplex kinase assays were the same as those described in Fig. 1. The bands indicated by the arrows represent the phosphorylated MBP and GST-c-Jun-(1-79). Bar graphs, representing mean \pm S.E. from three separate experiments, show the kinase activities of the various samples relative to those in the non-treated, static controls.

signaling process that leads to this dual activation. Interestingly, PTKs are also critical in the shear stress regulation of endothelial cell shape and stress fibers (38). Cellular PTKs can be generally divided into two major categories, receptor tyrosine kinases and nonreceptor PTKs. Nonreceptor PTKs (e.g. FAK and c-Src) represent cellular enzymes that have intrinsic kinase activities but do not have extracellular domains. Originally identified through its association with v-Src, FAK is present in focal adhesion sites and is tyrosine-phosphorylated in response to cell adhesion as well as the stimulation by a number of growth factors (e.g. platelet-derived growth factor) and peptide hormones (e.g. angiotensin II, thrombin, bombesin, and endothelin) (for a review, see Ref. 39). Although FAK has previously been shown to be tyrosine-phosphorylated in ECs in response to shear stress (40), the functional analyses in Figs. 4 and 5 provide new evidence to show that FAK can be upstream of the activation of ERK and JNK, which is at least in part mediated by Grb2-Sos. The phosphorylation of Tyr-397 is critical in these deduced signaling pathways, since the dominant negative FAK(F397Y) mutant attenuated the shear stress ac-

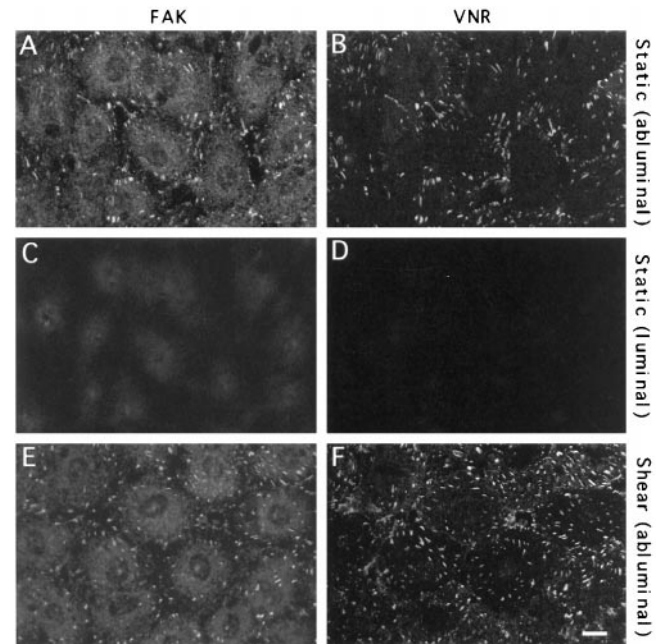


FIG. 7. Confocal microscopic views of the immunostaining of FAK and VNR in static and sheared BAEC. Confluent monolayers of BAEC were kept as static controls (A-D) or subjected to a shear stress of 12 dyn/cm^2 for 3 min with the direction of flow from the left to the right (E-F). Cells were then fixed and double immunostained with a polyclonal anti-FAK (A, C, and E) and anti-VNR mAb (B, D, and F) antibody. These primary antibodies were detected by a FITC-conjugated goat anti-rabbit antibody and a rhodamine-conjugated goat anti-mouse antibody, respectively. Detection of the FITC staining (A, C, and E), and rhodamine staining (B, D, and F) along the height of the cells was achieved by confocal microscopic scanning. Panels A, B, E, and F are the sections at the bottom of the cells, whereas panels C and D are combined projections of three sections (0.3 μm for each section) near the luminal membrane. The results for the luminal projections after shearing are similar to those for the static controls (C and D) and are not shown here. The bar in panel F represents 10 μm .

tivation of both ERK and JNK. The complete blockade of ERK but partial attenuation of JNK by FAK(F397Y) implies that the shear stress-activated ERK is FAK-dependent, but there are also FAK-independent pathways leading to the activation of JNK. PTK signaling should be involved in such unidentified pathways, since genistein blocked the shear stress activation of JNK (Fig. 1). A recent study showed that PYK2, the second PTK in the FAK family, regulates JNK in neuronal cells (41). However, we could not detect the presence of PYK2 in BAEC and in human umbilical vein endothelial cells. Other PTKs that interact functionally with FAK such as c-Src may also be involved in the shear stress activation of MAPKs. Cell adhesion on ECM activates c-Src and its association with FAK (for a review, see Ref. 42). Tyr-397 of FAK is essential in such assembly by serving as a binding site (YpAEI motif) for the SH2 domain of Src family PTKs (13, 16, 17). The subsequent phosphorylation of Tyr-925 of FAK promotes the recruitment of Grb2 to FAK (13). Takahashi and Berk have recently shown that shear stress activates c-Src in ECs (6). Thus, c-Src and other Src family members may work in concert with FAK to regulate the downstream MAPKs.

The dual activation of ERK and JNK in response to shear stress is regulated by Ras (5, 7). We have previously shown that expression of RasN17 and MEKK(K-M), the respective negative mutants of Ras and MEKK1, in BAEC inhibits the shear stress activation of JNK and the c-Jun transcriptional activity (5). In addition to Ras, other small GTPases such as Cdc42 and Rac have also been shown to be upstream of JNK in HeLa and NIH3T3 cells (43). We found that the shear stress activation of

ERK and JNK, as well as transcription factor activator protein 1/TPA-responsive element, was attenuated by negative mutants of Cdc42 and Rac.² Thus, Cdc42 and Rac may function together with Ras to regulate shear stress-induced JNK activation. Graf (GTPase regulator associated with FAK), a new member in the GTPase-activating protein family, was recently cloned and shown to preferentially stimulate the GTPase activities of RhoA and Cdc42 (44). Although Graf is not expressed in ECs,³ this finding suggests that alternative signaling pathways may also link FAK to MAPKs in response to shear stress.

Each individual molecule in the FAK-Grb2-Sos-Ras-ERK/JNK pathways has distinct structure-functional requisites, and their induction by shear stress is highly orchestrated. For those molecules located close to the plasma membrane, *i.e.* FAK, Grb2, Sos, and Ras, the activation by fluid shear stress is within a time frame of 1–5 min. The FAK-Grb2-Sos-Ras-ERK pathway is kinetically well correlated, since 5–10 min are required for the activation of ERK (5, 7), which is a cytoplasmic kinase involved in transnuclear activation. In contrast, maximum activation of JNK occurs at 30 min after the application of shear stress (5, 7). The discrepancy between the temporal activation of ERK and JNK is consistent with the hypothesis that FAK-independent pathways are involved in the shear stress activation of JNK. The kinetics of enzymatic reactions of the various kinases and their cellular locations may be important factors in determining the temporal sequence of the activation. A highly coordinated mechanism of MAPK deactivation must result in the transient nature of the activation. This could involve MAPKs or FAK phosphatases to “switch off” the signaling relay. Alternatively, regulation may occur upstream of FAK. In the shear stress-induced signaling system, it seems that multiple receptors and pathways can be activated. Thus, the different time course of ERK and JNK activation could also be attributed to a fine tuning among different receptors and pathways. This hypothesis is supported by a recent report from Jo *et al.* (7) that shear stress activation of ERK is $G\alpha_{i2}$ -dependent, whereas that of JNK is $G\beta/\gamma$ dependent.

Previous studies have shown that fluid shear stress regulates the focal adhesion complexes. Concomitant with cell elongation and the alignment of stress fibers with the direction of flow, there was a prominent localization of vinculin and $\alpha_v\beta_3$ integrin at the leading end of the sheared ECs (10, 45). We found that FAK and VNR, located in focal adhesion sites at the abluminal side of BAEC, enhanced their association in the sheared cells (Fig. 7). In addition to FAK, c-Src is also activated by shear stress (6) and presumably can be recruited to these focal adhesion sites. Cytochalasin B inhibits the shear stress activation of ERK, JNK, and FAK (Figs. 1 and 2B), suggesting that the activation of FAK-Grb2-Sos-Ras-ERK/JNK pathways in response to shear stress is dependent upon the integrity of the actin network, which is necessary for the integrin-mediated signaling pathways. Integrin aggregation caused by beads coated with ligands (*e.g.* fibronectin, GRGDSPC peptide, and anti-integrin antibody) triggers the accumulation of FAK and Src family PTKs, together with signaling molecules such as Grb2, Sos, and MAPKs, *i.e.* JNK and ERK (46–48). The focal accumulation of these molecules at the integrin aggregation sites and their tyrosine phosphorylation are abolished by genistein or cytochalasin D (48). The similarity between these previous findings and data presented in the current study suggests that integrin-mediated signaling is involved in shear stress activation of ERK/JNK. In addition to the actions of shear stress on ECs and cellular adhesion on ECM, stretching

vascular smooth muscle cells also involves integrin-mediated signaling (33). One possible working model is that FAK colocalizes with surface integrins at the abluminal side of the cells where they attach to ECM, and the FAK/integrin-mediated mechanotransduction occurs at least in part through cytoskeletal proteins. The tensegrity-based theory proposed by Ingber and colleagues also suggests that integrin can act as a mechanoreceptor and that mechanical signals can be transduced through the cytoskeleton (49). Recent study by Wary *et al.* demonstrated that integrins can be involved in signaling elicited by Shc, which is also an SH2-containing adaptor protein that links various tyrosine-phosphorylated signal transducers to Ras (50). Shc is recruited to a subset of β_1 integrins (*e.g.* $\alpha_1\beta_1$ and $\alpha_5\beta_1$) and $\alpha_v\beta_3$ by the extracellular or transmembrane domain of the integrin α subunit in A431 cells. Bypassing FAK, this process provides an alternative route to activate the Ras-ERK pathway (51). The roles of Shc/integrin in the shear stress induction of MAPKs in ECs remain to be determined.

Acknowledgments—We thank Dr. M. Sakaue for providing Δ mSos1 expression plasmids and Dr. J. D. Lee and Suli Yuan for excellent technical assistance.

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