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Mechanotransduction in Response to Shear Stress

ROLES OF RECEPTOR TYROSINE KINASES, INTEGRINS, AND Shc*

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Kuang-Den Chen, Yi-Shuan Li, Michael Kim, Song Li, Suli Yuan, Shu Chien, and John Y-J. Shyy‡

From the Department of Bioengineering and Institute for Biomedical Engineering, University of California, San Diego, La Jolla, California 92093-0412

Shear stress, the tangential component of hemodynamic forces, activates many signal transduction pathways in vascular endothelial cells. The conversion of mechanical stimulation into chemical signals is still unclear. We report here that shear stress (12 dynes/cm^2) induced a rapid and transient tyrosine phosphorylation of Flk-1 and its concomitant association with the adaptor protein Shc; these are accompanied by a concurrent clustering of Flk-1, as demonstrated by confocal microscopy. Our results also show that shear stress induced an association of $\alpha_v\beta_3$ and β1 integrins with Shc, and an attendant association of Shc with Grb2. These associations are sustained, in contrast to the transient Flk-1·Shc association in response to shear stress and the transient association between $\alpha_v\beta_3$ integrin and Shc caused by cell attachment to substratum. Shc-SH2, an expression plasmid encoding the SH2 domain of Shc, attenuated shear stress activation of extracellular signal-regulated kinases and c-Jun N-terminal kinases, and the gene transcription mediated by the activator protein-1/12-*O*-tetradecanoylphorbol-13-acetate-responsive element complex. Our results indicate that receptor tyrosine kinases and integrins can serve as mechanosensors to transduce mechanical stimuli into chemical signals via their association with Shc.

Cells in the cardiovascular system are exposed to hemodynamic forces as well as chemical factors. Shear stress, the tangential component of hemodynamic forces, acts mainly on vascular endothelial cells (ECs),[1] whereas circumferential stress is borne primarily by vascular smooth muscle cells. The mechanotransduction processes by which these vascular cells convert mechanical stimuli into biochemical signals have gained increasing attention. Several laboratories, including ours, have performed *in vitro* experiments using flow channels to study the responses of ECs to applied shear stress (see Refs. 1–4 for review). Mitogen-activated protein kinases, including extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase, are rapidly activated by shear stress (5–7). This results in the transcriptional activation of immediate early genes such as those encoding monocyte chemotactic protein-1 (MCP-1) and c-Fos (8–10). On the upstream side, the shear stress activation of ERK and JNK is modulated by Ras, which in turn is regulated by Son of sevenless (Sos), a guanine nucleotide exchange factor, as evidenced by the findings that negative mutants of Ras and Sos can block the shear stress induction of ERK and JNK (6, 7).

Conceptually, shear stress acts on the EC membrane to activate putative shear stress sensors or receptors which then lead to the activation of the Sos-Ras pathway. To date, several mechanism of mechanotransduction involving the EC membrane have been suggested. Shear stress activates the seven-span-receptor-coupled G-protein (11), ion channels such as K^+ channel (12), and the transforming growth factor-β receptor-related Smad6 and Smad7 (13). Several recent studies showed that tyrosine kinases, *i.e.* focal adhesion kinase (FAK) and c-Src in the focal adhesion site constitute a part of the mechanotransduction in ECs in response to shear stress (10, 14, 15). FAK, by forming a complex with growth factor receptor-binding protein 2 (Grb2), regulates the shear stress induction of ERK and JNK (15). The involvement of these signaling molecules in the focal adhesion sites may be correlated with the dynamic reorientation of focal adhesions in ECs under shear stress (16). Considering the multiplicity of the signaling molecules engaged in the EC responses to shear stress, there is a missing link to integrate the various pathways into an unified theme.

Shc is an adaptor protein containing a C-terminal Src homology domain-2 (SH2) domain and a central glycine/proline-rich sequence (17). In response to many growth factors such as platelet-derived growth factor and epidermal growth factor (EGF), Shc is tyrosine-phosphorylated and associates with phosphotyrosines of the cognate receptor tyrosine kinases (RTK) through SH2 binding (17–20). Tyrosine-phosphorylated Shc also associates with Grb2 through SH2 interaction (21, 22). The assembly of Shc·Grb2·Sos provides an alternative mechanism in addition to the Grb2·Sos pathway for the activation of Ras. Recently, it has been shown that Shc is involved in the integrin-mediated signal transduction. In A431 cells, Shc is recruited to $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ when these integrins have been conjugated to their corresponding antibodies (23). In the same study, it was also shown that Shc is necessary and suf-

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‡ To whom correspondence should be addressed: Dept. of Bioengineering, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0412. Tel.: 619-822-0785; Fax: 619-534-3658; E-mail: shyy@bioeng.ucsd.edu.

[1] The abbreviations used are: EC, vascular endothelial cell; AP-1, transcription factor activator protein 1; β-gal, β-galactosidase; BAEC, bovine aortic endothelial cell; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FITC, fluorescein isothiocyanate; Grb2, growth factor receptor-binding protein 2; GST, glutathione *S*-transferase; HA, hemagglutinin, IB, immunoblotting, IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MBP, myelin basic protein; MCP-1, monocyte chemotactic protein-1; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PLC, phospholipase C; RTK, receptor tyrosine kinase; SH2, Src homology domain-2; Sos, Son of sevenless; TRE, 12-*O*-tetradecanoylphorbol-13-acetate-responsive element; VEGF, vascular endothelium growth factor.

ficient for the activation of ERK in response to integrin ligation. These results suggest that both growth factors and integrins can regulate the ERK pathway via Shc.

In the current study, we show for the first time that fetal liver kinase 1 (Flk-1), an RTK specific for vascular endothelium growth factor (VEGF), and integrins ($\alpha_v\beta_3$, β_1, and β_5 integrins) can both function as mechanosensors in ECs, and that shear stress causes both to be associated with Shc. The interaction of Shc with Flk-1 is rapid and transient, whereas its association with the various integrin is sustained. These findings provide new insights into the roles of RTKs and integrins in the transduction of shear stress into chemical signals.

EXPERIMENTAL PROCEDURES

Cell Cultures—Bovine aortic endothelial cells (BAECs) were isolated from bovine aorta and cultured in a humidified 95% air, 5% CO_2 incubator at 37 °C. The culture medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1 mM each of penicillin-streptomycin and sodium pyruvate. All experiments were conducted with cultures prior to passage 10.

Shear Stress Experiments—A flow system was used to impose shear stress on cultured ECs as described previously (24). In brief, a 75 × 38-mm glass slide was seeded with BAECs, which were cultured until reaching a confluent monolayer. 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 BAECs to shear stress. A high reservoir, the flow channel, a low reservoir, and a peristaltic pump were connected to form a circulation loop. Steady, laminar flow across the channel was generated as a result of the height difference between the two reservoirs. During the flow experiments, the system was kept at 37 °C in a constant temperature cabinet and equilibrated with 95% humidified air plus 5% CO_2.

Immunoprecipitation and Immunoblotting—The antibodies used in immunoprecipitation and immunoblotting were PY20 anti-phosphotyrosine monoclonal antibody (mAb) (Transduction Laboratories, Lexington, KY), polyclonal anti-Shc (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-Grb2/Sem5 (Santa Cruz Biotechnology), anti-c-Myc mAb (Santa Cruz Biotechnology), polyclonal anti-Flk-1 (Santa Cruz Biotechnology), anti-$\alpha_v\beta_3$ LM609 mAb, polyclonal anti-β_5 (Chemicon, Temecula, CA), anti-β_1 CD29 mAb (PharMingen, San Diego, CA), and anti-hemagglutinin (HA) mAb (Roche Molecular Biochemicals). For immunoprecipitation, cells were scraped into a lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100); the lysate was centrifuged, and the supernatant was immunoprecipitated with the appropriate antibodies and protein A-Sepharose beads (Amersham Pharmacia Biotech) at 4 °C overnight. The immunoprecipitated complexes were washed and used for either kinase activity assays or immunoblotting. After SDS-PAGE, proteins in the gel were transferred to a nitrocellulose membrane for immunoblotting. The membrane was blocked with 5% bovine serum albumin followed by incubation with the primary antibody in 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-horseradish peroxidase conjugate (Santa Cruz Biotechnology) and the ECL detection system (Amersham Pharmacia Biotech).

Immunostaining and Confocal Microscopy—Confluent BAEC monolayers were fixed in a phosphate-buffered saline (PBS) containing 3% paraformaldehyde at room temperature for 10 min. The cells were then incubated in PBS containing the polyclonal anti-Flk-1 at a concentration of 1:200 (v/v) for 1 h at room temperature. The specimens were washed in PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). The immunostaining of Flk-1 was observed with 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. The pixel intensity of the confocal images was measured and analyzed by Lasersharp Processing software (Bio-Rad).

DNA Plasmids and Transient Transfection—To construct plasmid Shc-SH2 encoding the SH2 domain of Shc, the full-length Shc cDNA was first obtained from mRNA isolated from HeLa cells by reverse transcription-polymerase chain reaction using 5′-ATGAACAAGCTGAGTGGAGGC-3′ and 5′-GAGCGCTAGGGCAGATCA-3′ as the forward and reverse primers. The obtained Shc cDNA was then used as the template for the polymerase chain reaction synthesis of the SH2 domain (from Trp-378 to Ser-475) using 5′-TGGTTCCATGGGAAGCTG-3′ as the forward primer and 5′-GAGCGCTAGGGCAGATCA-3′ as the reverse primer. The 0.3-kilobase pair amplicons obtained after purification was ligated into plasmid pCRII (Invitrogen, San Diego, CA), and the fragments flanked by *Hin*dIII and *Eco*RI sites were then subcloned into the pcDNA3 vector (Invitrogen). Plasmids HA-JNK1, Myc-ERK2, 4XTRE-Pl-Luc, and MCP1-Luc-540 have been described previously (25, 26). The various DNA plasmids were transfected into BAECs at 80% confluence using the LipofectAMINE method (Life Technologies, Inc.). After incubation for 6 h, the transfected cells were washed with DMEM and incubated in fresh complete DMEM to reach confluence. Within 48 h after transfection, the BAEC monolayer was subjected to shear stress or kept as static controls.

Kinase Activity Assays—The epitope-tagged Myc-ERK2 was co-transfected with Shc-SH2 into BAECs. After shear stress experiments, the cells 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 Na_3VO_4, and 2 mM β-glycerophosphate). Myc-ERK2 was immunoprecipitated with the anti-c-Myc 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 $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, and 2 mM dithiothreitol). Two micrograms of myelin basic protein (MBP) and 10 μCi of [γ-^{32}P]ATP (ICN, Irvine, CA) in 30 μl of kinase assay buffer containing 25 μM ATP were added to each immunocomplex pellet for kinase reaction at 30 °C for 20 min. The phosphoproteins were separated by SDS-PAGE, and the gels were dried for autoradiography. The kinase activities of the epitope-tagged HA-JNK1 were assessed by using essentially the same method as those for ERK, except that HA-JNK1 was immunoprecipitated by anti-HA mAb and that glutathione *S*-transferase (GST)-c-Jun-(1–79) fusion protein was used as the substrate in the immunocomplex kinase assays.

AP-1/TRE Activation Assays—pcDNA3 or Shc-SH2 was co-transfected with either 4xTRE-Pl-Luc or MCP1-Luc-540 into BAECs at 70% confluence by using the transient transfection protocols. The pSVβ-gal plasmid, which contains a β-galactosidase (β-gal) gene driven by SV40 promoter and enhancer, was included in the co-transfection to monitor the transfection efficiency. The cells were then subjected to shear stress experiments or kept as static controls. The luciferase reporter activities normalized for transfection efficiency were used to assess the effects of Shc-SH2 on shear stress-induced transcription activation mediated by AP-1/TRE.

RESULTS

Shear Stress Increases the Tyrosine Phosphorylation of Flk-1 and Flk-1·Shc Association in BAECs—The binding of growth factors to their cognate RTKs induces the tyrosine phosphorylation of the cytoplasmic domains of RTKs, leading to the recruitment of the SH2-containing adaptor molecules such as Shc to the phosphorylated tyrosine. To test whether shear stress can activate endothelial RTKs as in the case of growth factor binding, confluent BAEC monolayers were subjected to a shear stress of 12 dynes/cm^2 for various lengths of time. In parallel positive control experiments, however, BAECs were stimulated with 10 nM VEGF in the absence of shear. The cell lysates from the various experiments were immunoprecipitated with a polyclonal antibody against Flk-1, a VEGF receptor (27). The immunoprecipitated protein complexes were then immunoblotted with PY20 mAb to detect the change in tyrosine phosphorylation of Flk-1 which has a molecular mass of 210 kDa. As shown in Fig. 1*A*, shear stress induced the tyrosine phosphorylation of Flk-1 as early as 1 min, reached a peak level at 5 min, decreased afterward, and returned to the basal level at 30 min. The temporal response of Flk-1 tyrosine phosphorylation induced by shear stress was similar to that found in cells stimulated by VEGF (Fig. 1*B*). Shear stress induction of Flk-1 tyrosine phosphorylation occurred both in the absence or presence of serum supplements and was not inhibited by pretreating BAEC monolayer with a polyclonal anti-VEGF antibody, indicating that the effect of shear stress was not due to back-

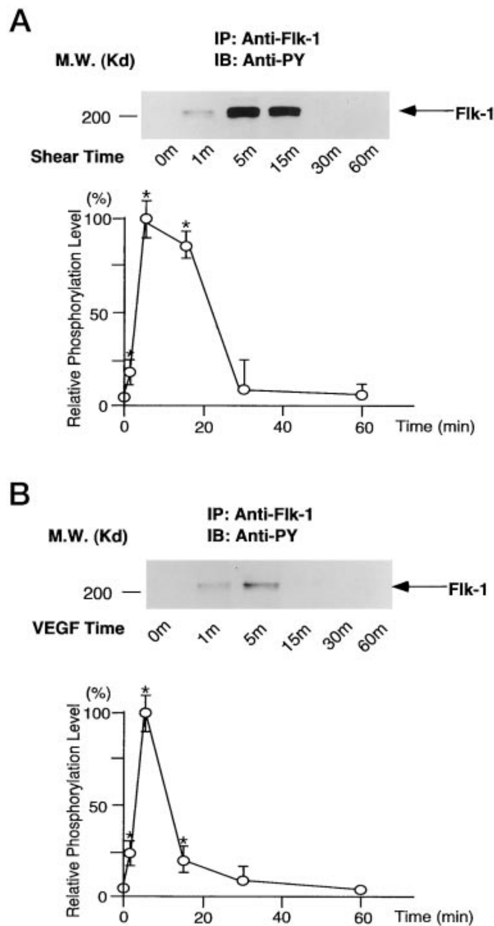

FIG. 1. **Shear stress, like VEGF, induces tyrosine phosphorylation of Flk-1 in BAECs.** BAEC monolayers were either subjected to a shear stress of 12 dynes/cm^2 (*A*) or treated with VEGF (10 nM) (*B*) for various lengths of time as indicated. Five hundred micrograms of the cell lysate from each sample were subjected to immunoprecipitation (IP) with a polyclonal anti-Flk-1 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. Relative phosphorylation level is defined as the band intensities of the various samples normalized to that in the peak induction. *Asterisks* in *A* indicate significant difference ($p < 0.05$) between sheared samples and static controls (time 0), and those in *B* indicate significant difference ($p < 0.05$) between VEGF-treated samples and untreated controls (time 0).

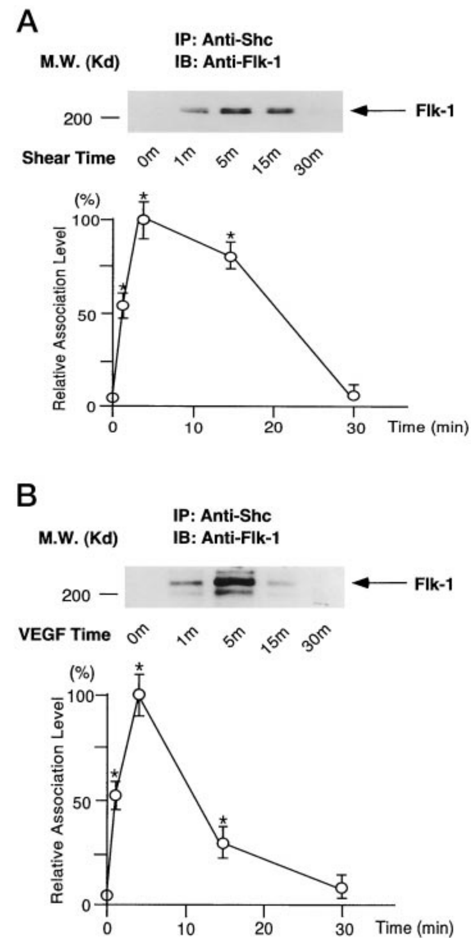

FIG. 2. **Shear stress and VEGF induce the association of Flk-1 with Shc in BAECs.** The experimental procedures were essentially the same as those described in Fig. 1, except that cell lysates from the various samples were subjected to IP with a polyclonal anti-Shc and IB with anti-Flk-1. The Flk-1·Shc association is demonstrated by the co-immunoprecipitated Flk-1 in the anti-Shc immunoprecipitates. Shown in the *bottom part* is densitometry analysis representing the mean ± S.E. from three separate experiments. Relative association level is defined as the band intensities of the various samples normalized to that in the peak induction. *Asterisks* in *A* indicate significant difference ($p < 0.05$) between sheared samples and static controls (time 0), and those in *B* indicate significant difference ($p < 0.05$) between VEGF-treated samples and untreated controls (time 0).

ground growth factors stimulation or to a paracrine or autocrine induction of VEGF.

To investigate whether shear stress induction of Flk-1 tyrosine phosphorylation was accompanied by an increased association of Flk-1 with Shc, the cell lysates were immunoprecipitated with a polyclonal antibody against Shc followed by immunoblotting with polyclonal anti-Flk-1. As shown in Fig. 2*A*, shear stress increased the association of Flk-1 and Shc in a rapid and transient manner with a time course parallel to that of tyrosine phosphorylation of Flk-1 shown in Fig. 1*A*. As a control, VEGF treatment also induced the association of Flk-1 and Shc in BAECs (Fig. 2*B*). In contrast, using anti-rabbit IgG as a negative control in the immunoblotting, the association of FlK-1 with Shc was not observed (data not shown). Neither tyrosine phosphorylation of Flk-1 nor its association with Shc was due to metabolites released from the shear stress-stimulated ECs, since these responses were not found in ECs incubated with the shearing media (data not shown).

Shear Stress Increases the Flk-1 Clustering on the Luminal Membrane—Binding of the cognate ligands induces the dimerization and thus the activation of various RTKs. To test the hypothesis that shear stress activates Flk-1 by causing its clustering, confluent monolayers of BAECs were kept static or subjected to a shear stress of 12 dynes/cm^2 followed by anti-Flk-1 immunostaining. Confocal microscopy revealed that Flk-1 was mainly distributed on the luminal side of BAECs (Fig. 3). Quantification of images from static and sheared samples showed that the application of shear stress for 1 min enhanced the clustering of Flk-1. This focal pattern of clustering peaked at 5 min and reduced to the level comparable to that in the static controls at 30 min after shearing. This time course is similar to those of Flk-1 tyrosine phosphorylation and Flk-1·Shc association.

Shear Stress Increases the Association of Shc with $\alpha_v\beta_3$ Integrin and Integrins Containing β_1 and β_5 Subunit—When ECs are exposed to shear stress, focal adhesion plaques move dynamically on the abluminal membrane (16). We have previously demonstrated that the $\alpha_v\beta_3$ integrin in focal adhesion sites is involved in the shear stress activation of ERK and JNK (15). To investigate the role of Shc in the integrin-mediated signal transduction in response to shear stress, $\alpha_v\beta_3$ was im-

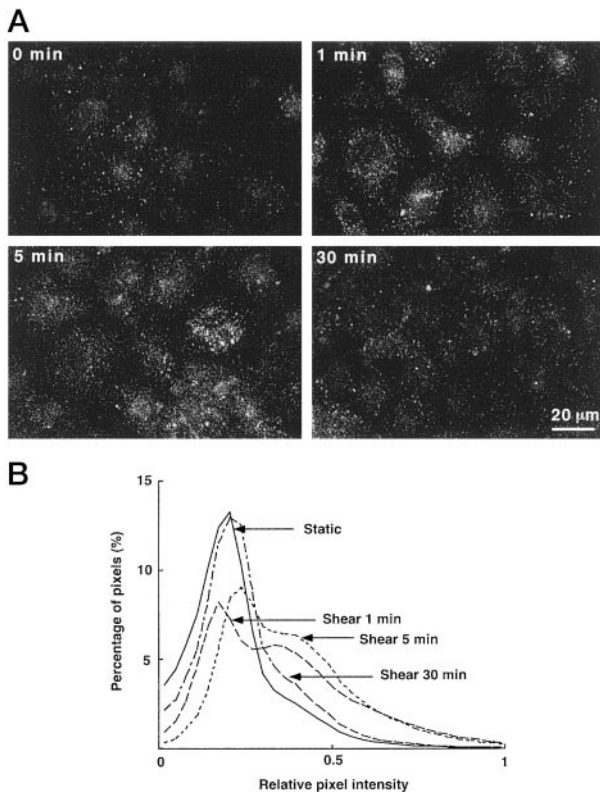

FIG. 3. **Shear stress increases the Flk-1 clustering in BAECs.** *A*, confluent monolayers of cells were either kept as static controls (represented by 0 min) or subjected to a shear stress of 12 dynes/cm^2 for 1, 15, or 30 min with the direction of flow from left to right. Cells were fixed and immunostained with a polyclonal anti-Flk-1, which was then detected by a FITC-conjugated goat anti-rabbit antibody. Detection of the FITC staining along the height of the cells was achieved by confocal microscopic scanning. Shown in each panel are combined projections of three sections (0.3 μm for each section) near the luminal membrane. *B* shows the pixel intensities of the confocal images. The relative intensity level is defined as the pixel intensities relative to the minimum represented by numerical value of 0 and the maximum with numerical value of 1. A curve was plotted for each experiment to show the percentage of pixels at various levels of intensity. The analysis shows that the number of pixels at higher intensity increases in images obtained from sheared for 1 and 5 min, indicating shear stress increased the Flk-1 clustering in these specimens.

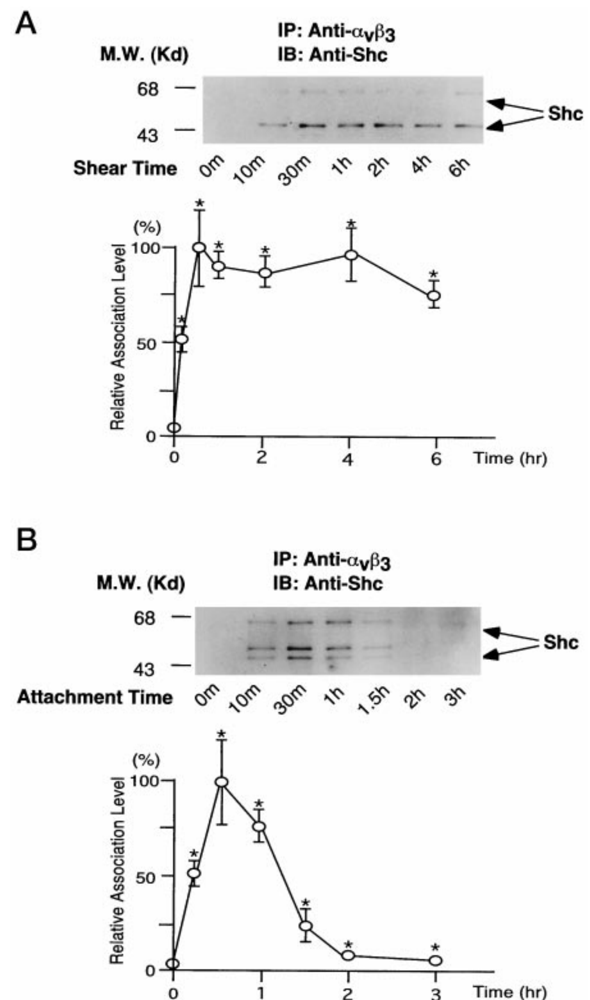

FIG. 4. **Shear stress induces a sustained association of Shc with $\alpha_v\beta_3$ integrin in BAECs, but the association is transient during BAEC adhesion.** Confluent monolayers of BAECs were subjected to a shear stress of 12 dynes/cm^2 (*A*) or BAECs in suspension were allowed to attach to fibrinogen (*B*) for the time duration as indicated. The cell lysates from the various samples were subjected to IP with anti-$\alpha_v\beta_3$ LM609 mAb, followed by IB with polyclonal anti-Shc. The Shc·$\alpha_v\beta_3$ association is demonstrated by the co-immunoprecipitation of Shc with $\alpha_v\beta_3$ in the sheared or attached BAECs. Shown in the *bottom part* is densitometry analysis representing the mean ± S.E. from three separate experiments. *Asterisks* indicate significant difference ($p < 0.05$) between sheared samples and static controls (time 0) or between attached cells and cells in suspension (time 0).

munoprecipitated from BAEC lysates by LM609 mAb, and this was followed by immunoblotting with polyclonal anti-Shc. As shown in Fig. 4*A*, Shc association with $\alpha_v\beta_3$ was not detectable in static BAECs. Application of a shear stress of 12 dynes/cm^2 rapidly augmented the formation of a complex of $\alpha_v\beta_3$·Shc, as demonstrated by their co-immunoprecipitation. This association was already detectable 10 min after the cells were exposed to shear stress, reached a peak level at 30 min, and sustained for the duration of this experiment (6 h). In two other separate experiments, the $\alpha_v\beta_3$·Shc association lasted for at least 18 h (data not shown). In contrast, neither $\alpha_v\beta_3$ integrin nor ERK was found to be associated with Shc in control experiments using anti-rat IgG or anti-ERK for the immunoprecipitation (data not shown). The $\alpha_v\beta_3$·Shc association during the endothelial attachment to fibrinogen is transient (Fig. 4*B*), becoming undetectable by 2 h. Thus, the integrin-mediated signaling in response to shear stress differs from that caused by cell adhesion in that its association with Shc is sustained.

It is possible that mechanotransduction causes the recruitment of Shc to various types of integrins in ECs. Thus, we also investigated whether shear stress increases the association of Shc with integrins containing the β_1 or β_5 subunit by immunoprecipitating the cell lysates with polyclonal anti-Shc followed by immunoblotting with anti-β_1 mAb CD29 or polyclonal anti-β_5. As shown in Fig. 5, β_1- or β_5-containing integrins were not associated with Shc in the static cells. Exposure of BAEC monolayer to shear stress increased the association of Shc with β_1 or β_5 with a time course similar to that for $\alpha_v\beta_3$. Together, the data presented in Figs. 2, 4, and 5 demonstrate that Shc associates with both RTKs and integrins in ECs in response to shear stress. In contrast to the transient Shc·Flk-1 association, the Shc·integrin association is much more sustained.

Shear Stress Induces Shc Tyrosine Phosphorylation and the Association of Shc with Grb2—When cells are stimulated by growth factors, tyrosine phosphorylation of Shc coincides with its recruitment to RTKs. To investigate whether Shc is tyrosine phosphorylated in response to shear stress, the anti-Shc immunoprecipitates were immunoblotted with PY20 mAb. As shown in Fig. 6*A*, shear stress caused a sustained increase in tyrosine phosphorylation of Shc, which lasted for at least 6 h after the exposure to shear stress.

FAK regulates Grb2·Sos-Ras pathway in the EC response to

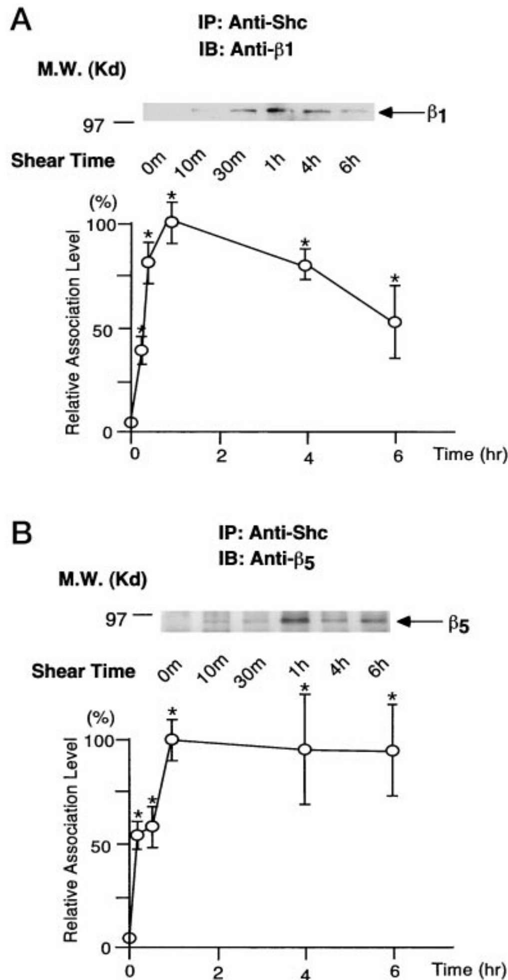

FIG. 5. **Shear stress induces a sustained association of Shc with integrins containing β_1 or β_5 subunit in BAECs.** The experimental procedures were the same as those described in Fig. 1, except that cell lysates from the various samples were subjected to IP with polyclonal anti-Shc followed by IB with anti-β_1 CD29 mAb or a polyclonal anti-β_5 antibody. The sustained association is demonstrated by the co-immunoprecipitation of Shc with β_1 in the sheared BAECs. Shown in the *bottom part* is densitometry analysis representing the mean ± S.E. from three separate experiments. *Asterisks* indicate significant difference ($p < 0.05$) between sheared samples and static controls (time 0).

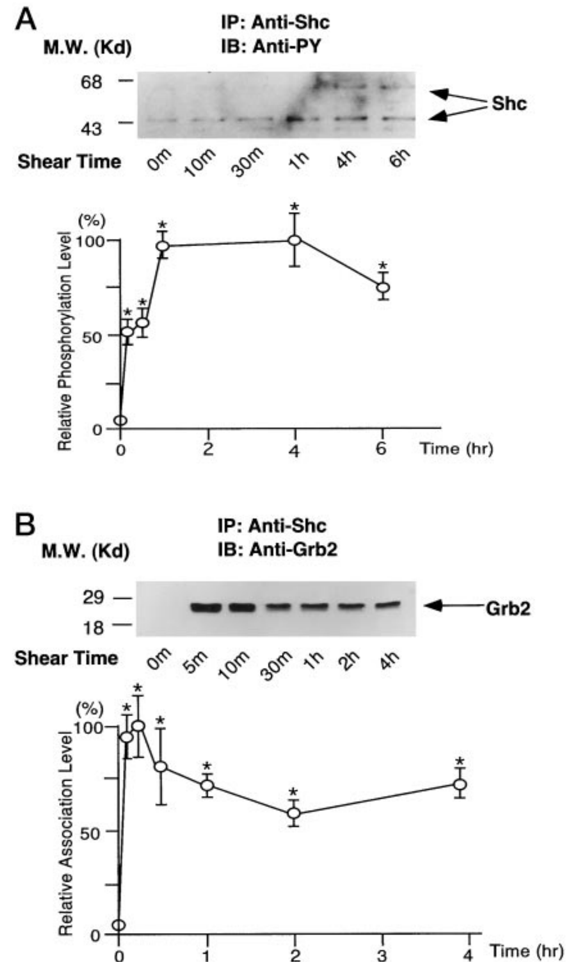

FIG. 6. **Shear stress induces a sustained tyrosine phosphorylation of Shc and its association with Grb2 in BAECs.** The experimental procedures were essentially the same as those in Fig. 1, except that cell lysates from the various samples were subjected to IP with polyclonal anti-Shc followed by IB with PY20 anti-phosphotyrosine mAb (*A*), or a polyclonal anti-Grb2/Sem5 (*B*). The sustained phosphorylation of Shc and its association with Grb2 by shear stress are demonstrated by the recognition of the tyrosine-phosphorylated Shc and the co-immunoprecipitated Grb2 by PY20 mAb and polyclonal anti-Grb2/Sem5, respectively. Shown in the *bottom panel* is densitometry analysis representing the mean ± S.E. from three separate experiments. *Asterisks* indicate significant difference ($p < 0.05$) between sheared samples and static controls (time 0).

shear stress, which was demonstrated by the association of FAK with Grb2 (15). To investigate the possible engagement of Shc in the shear stress activation of the Grb2·Sos-Ras pathway, we examined whether Shc associates with Grb2 in the sheared BAECs. As shown in Fig. 6*B*, there was an increase in the amount of Grb2 co-immunoprecipitated with Shc in ECs subjected to shear stress for 1 min. This increased association of Grb2 with Shc was sustained. In a separate experiment, cell lysates immunoprecipitated with a polyclonal anti-Shc and immunoblotted with the polyclonal anti-Sos revealed that Shc was also associated with Sos in sheared cells (data not shown). The results in Fig. 6 demonstrate that shear stress induces a sustained interaction of integrins with Shc, which not only results in the tyrosine phosphorylation of Shc, but also the association of Shc with the Grb2·Sos complex.

Shc Regulates ERK, JNK, and AP-1/TRE in Response to Shear Stress—Shear stress activates mitogen activated protein kinases, including ERK and JNK (5–7), which in turn cause the transcriptional activation of AP-1 acting on the TRE in the 5′ promoter of some of the shear-inducible genes, *e.g.* the MCP-1 gene (6, 26). Through its association with Grb2·Sos, Shc can be upstream of these events. We constructed Shc-SH2 that functions as a negative mutant of Shc (28) to investigate its inhibitory effects on the shear stress activation of ERK, JNK, and on the TRE-driven luciferase reporter. Shc-SH2 was co-transfected with either Myc-ERK2 or HA-JNK1 into BAECs, and pcDNA3 parental plasmid was used as parallel controls. The transfected cells were either kept under static condition or subjected to a shear stress of 12 dynes/cm^2 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) fusion protein as the respective substrate. As shown in Fig. 7, shear stress activated Myc-ERK2 and HA-JNK1 in BAECs transfected with pcDNA3 by 2- and 3-fold, respectively. Co-transfection of Shc-SH2 drastically attenuated the shear stress activation of Myc-ERK2 and HA-JNK1. These results indicate that Shc is involved in the upstream signaling for the shear stress induction of ERK and JNK.

BAECs were co-transfected with Shc-SH2 and the chimeric construct 4XTRE-Pl-Luc consisting of luciferase reporter driven by four copies of TRE linked to the rat prolactin mini-

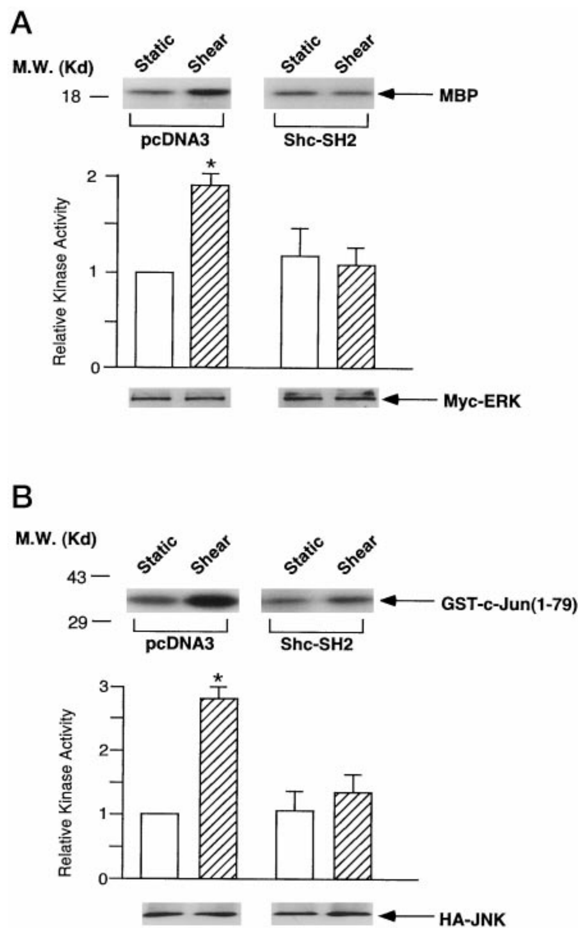

FIG. 7. **Negative mutant of Shc attenuates shear stress activation of Myc-ERK2 and HA-JNK1 in BAECs.** In *A*, 3 μg of epitope-tagged Myc-ERK2 were co-transfected with 9 μg of Shc-SH2 or pcDNA3 empty vector into BAECs in 75-cm^2 tissue culture flasks. The transfected cells were passed onto slides until confluence before subjected to a shear stress of 12 dynes/cm^2 for 10 min. The cell lysates were immunoprecipitated with anti-Myc mAb for IP kinase assays using MBP and [γ-^{32}P]ATP as substrates. The bands indicated by the *arrow* represent the phosphorylated MBP after SDS-PAGE and autoradiography. Shown in the *bottom panel* is IB with anti-Myc mAb, indicating that comparable amounts of Myc-ERK2 were expressed in the various samples. *B* is the result of a parallel set of experiments in which 3 μg of HA-JNK1 were co-transfected with 9 μg of pcDNA3 or Shc-SH2 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-^{32}P]ATP as substrates. Shown in the *bottom panel* is IB with anti-HA mAb. *Bar graphs*, representing mean ± S.E. from three separate experiments, show the kinase activities of the various samples relative to those in the pcDNA3-transfected, static controls.

mum promoter. In parallel experiments, cells were co-transfected with pcDNA3 together with 4XTRE-Pl-Luc. A shear stress of 12 dynes/cm^2 caused 33-fold induction of luciferase activities (relative to those in the static controls kept for 8 h); this shear stress induction of luciferase activity was drastically reduced in cells co-transfected with Shc-SH2 (Fig. 8*A*). We also tested whether Shc-SH2 can attenuate shear stress induction of MCP1-Luc-540 by using a chimeric construct which contains luciferase under the control of the 540-base pair 5′ promoter of the MCP-1 gene (26). As shown in Fig. 8*B*, Shc-SH2 caused a significant reduction of shear stress induction of MCP1-Luc-540, indicating that Shc plays a significant role in shear stress induction of the MCP-1 gene. Together, the data from Figs. 7 and 8 confirm that Shc mediates the shear stress activation of ERK and JNK pathways and the ensuing AP-1/TRE-mediated transcriptional activation.

DISCUSSION

This study demonstrates that receptor tyrosine kinases such as Flk-1 and integrins, including $\alpha_v\beta_3$ and those containing β_1 or β_5 subunit, can serve as mechanosensors in ECs in response to shear stress. Shear stress activates the Shc-dependent pathways through its association with Flk-1 and these mechanosensitive integrins. In contrast to the transient association of Flk-1 with Shc, the association of the various integrins with Shc is sustained. The docking of Shc induced by shear stress is functionally linked to the activation of ERK and JNK pathways, and the AP-1/TRE-mediated transcriptional activation at downstream. These findings, summarized in Fig. 9, provide new insights into the molecular basis of mechanically induced signal transduction and gene expression.

Binding of cognate growth factors results in dimerization of various RTKs, activation of their tyrosine kinase activity, and autophosphorylation of their cytoplasmic domain (see Ref. 29 for review). VEGF binds to Flk-1 and fms-like tyrosine kinase-1 (Flt-1) and induces the autophosphorylation of these RTKs (27, 30). It is not likely that the shear stress induced-tyrosine phosphorylation of Flk-1 (peaked at 5 min) results from a VEGF autocrine stimulation, because it is too rapid to be regulated by the *de novo* synthesis of VEGF, which requires hours. Furthermore, Flk-1 tyrosine phosphorylation was not found in BAECs incubated in the shearing media nor in cells treated with anti-VEGF. A ligand-independent activation of RTKs is also observed when cells are exposed to environmental stresses (see Ref. 31 for review). UV irradiation and osmotic shock cause the clustering and internalization of receptors for EGF, tumor necrosis factor, and interleukin-1 (32). In addition, the activity of cellular phosphotyrosine phosphatase has been shown to be inhibited by UV irradiation, which may contribute in part to the activation of receptors for EGF and platelet-derived growth factor (33). The molecular mechanism by which shear stress increases the tyrosine phosphorylation of Flk-1 remains to be determined. But the data presented in Fig. 3 suggest that the clustering of Flk-1 on the luminal side of ECs is one of the earliest events of mechanotransduction. In addition to Flk-1, shear stress also induced the clustering of Flt-1 in BAECs.[2] It seems that shear stress imposes a perturbation on the membrane to cause the aggregation of Flk-1 and Flt-1. Since this form of activation by mechanical stimulation does not require the binding of their specific ligands, it is reasonable to postulate that shear stress may also activate other RTKs. Thus, shear stress activation of FlK-1 shown in this study may be a specific example of a more general phenomenon for other membrane receptors.

Treating ECs with VEGF promotes the tyrosine phosphorylation of several SH2-containing molecules, including phospholipase C-γ (PLC-γ), GTPase-activating protein of Ras, phosphatidylinositol 3-kinase (PI 3-kinase), and Nck (34). Among these, PLC-γ has been shown to be recruited to Flk-1 (35). The SH2-containing molecules can be classified into two main groups. The first group consists of proteins with enzymatic functions, *e.g.* GTPase-activating protein of Ras, PI 3-kinase, PLC-γ, c-Src, and protein-tyrosine phosphatases (*e.g.* SH-PTP1 and SH-PTP2) (36). The second group comprises adaptor proteins that are composed of almost exclusively SH2 and SH3 domains (*e.g.* Grb2, p130^{cas}, and Shc). The results in Fig. 2 indicate that shear stress increases the association of Shc with Flk-1. Presumably, this is through the binding of the SH2 domain of Shc to the phosphorylated tyrosines of Flk-1. Many signaling events in ECs would be activated if multiple RTKs are activated by shear stress through the ligand-independent

[2] S. Li and J. Y.-J. Shyy, unpublished result.

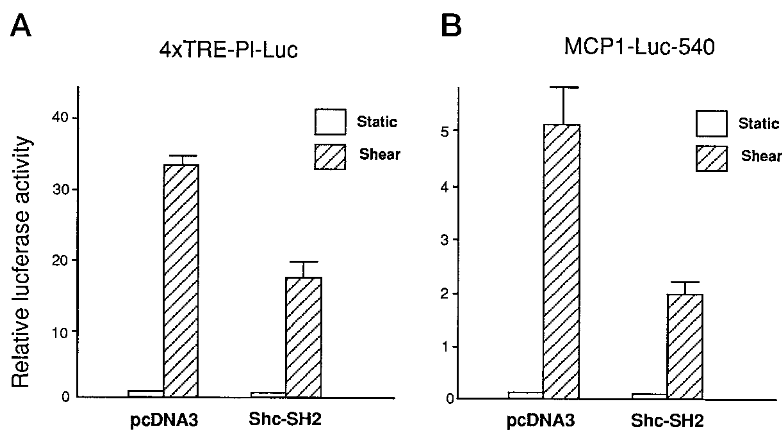

FIG. 8. **Negative mutant of Shc attenuates the shear stress induction of AP-1/TRE in BAECs.** Nine micrograms of pcDNA3 empty vector or Shc-SH2 were co-transfected with 3 μg of 4xTRE-Pl-Luc (*A*) or MCP1-Luc-540 (*B*), together with 3 μg of pSVβ-gal, into BAECs in 75-cm^2 tissue culture flasks. The DNA-transfected cells were reseeded on slides until confluence and either subjected to a shear stress of 12 dynes/cm^2 for 8 h or kept as static controls, followed by luciferase activities assays. The normalized luciferase activities are the luminometer readings of luciferase activity normalized for transfection efficiency based on β-galactosidase activity. The results represent the mean ± S.E. from at least three experiments.

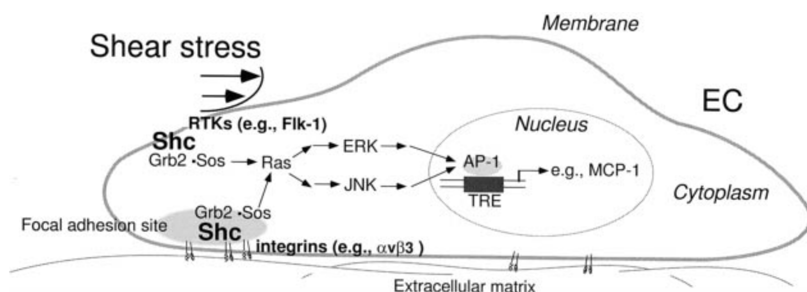

FIG. 9. **The proposed mechanism of mechanotransduction in ECs in response to shear stress.** RTKs (*e.g.* Flk-1) and integrins (*e.g.* $\alpha_v\beta_3$) in ECs convert the mechanical stimulation into chemical signals by associating with Shc. Subsequently, Ras is activated by the complex of Shc·Grb2·Sos. As a result, ERK and JNK pathways are activated, ultimately leading to the transcriptional activation of AP-1/TRE-mediated gene expression (as exemplified by the MCP-1 gene).

mechanism, and then associate with many SH2-containing enzymes and adaptor proteins. As a result, many signal transduction pathways would be activated by shear stress. For example, the PI 3-kinase pathway can lead to the generation of intracellular diacylglycerol and inositol 1,4,5-trisphosphate, and PLC-γ can activate the protein kinase C pathway. Indeed, inositol 1,4,5-trisphosphate and protein kinase C have been shown to be activated by shear stress (37–40). Although other stimuli such as growth factors and environmental stresses also activate RTKs (*i.e.* aggregation and autophosphorylation), but their effects on cells are different from those by shear stress. VEGF is an EC mitogen (41, 42), UV can lead to apoptosis (43), whereas laminar shear stress is vital for endothelial homeostasis in blood vessels and has been shown to protect ECs from undergoing apoptosis *in vitro* (44).

Shear stress not only increases the association of Shc with Flk-1, but also its interaction with mechano-sensitive integrins ($\alpha_v\beta_3$ and those containing β_1 or β_5 subunit). The co-immunoprecipitation of Shc with integrins in the sheared cells suggests an increased association rate or a decreased dissociation rate. We did not find an increased association of Shc with integrins in cells treated with VEGF.[3] This result is consistent with the previous finding that treatment of A431 cells with EGF did not result in the association of $\alpha_6\beta_4$ integrin with Shc or Grb2 (45). Thus, the binding of growth factors to their receptors promote the recruitment of Shc to the RTKs, but not to integrins. In contrast, shear stress increases the association of Shc to both RTKs and integrins.

It has been shown by immunostaining that integrin aggregation caused by beads coated with ligands (*e.g.* fibronectin, RGD peptide, and anti-integrin antibody) triggers the accumulation of protein-tyrosine kinases (*e.g.* FAK and c-Src) and signaling molecules (*e.g.* Grb2, Sos, PLC-γ) in focal adhesion sites (46–48). In addition, biochemical analysis has revealed that Shc is tyrosine-phosphorylated and is associated with $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ when these integrins are conjugated to their ligands (23, 49). The similarities in cellular responses to shear stress and to integrin-mediated cell adhesion have led us to propose that integrins serve as mechanosensors (4). Although the Shc-integrin association and Shc tyrosine phosphorylation are responses common to cell adhesion and shear stress, they are transient during cell adhesion (Fig. 4*B*; Refs. 23 and 49), but sustained with shear stress. The molecular basis underlying the temporal dynamics in the association of Shc with integrins is unknown. Presumably, both shear stress and the integrin-mediated cell adhesion induce conformational changes of integrins to facilitate their association with Shc. However, the events resulting from mechanical stimuli are unique in the sustained nature of the response, which does not occur following stimulation by growth factors or cell adhesion. The dynamic remodeling of the adhesion plaques in ECs exposed to shear stress (16) requires constant association and dissociation of integrins with ECM. It is likely that the enhanced "on-off" rates of integrin/ECM interaction result in a sustained conformational change of integrins, which in turn increases the association of Shc. Under static condition, the anchorage-dependent adhesion of ECs relies on the interaction of integrins with ECM. When cells are exposed to shear stress, such interaction needs to be reinforced to withstand the shearing forces. This reinforcement is probably achieved by moving dynamically the adhesion plaques to the strategic positions while sending signals to the cytoplasm through Shc recruitment.

The activation of Flk-1 leads to the recruitment of Grb2 through SH2 binding (35). Overexpression of Flk-1 or the association of integrins with various ligands causes ERK activation (23, 35, 50). The integrin-mediated ERK activation involves FAK autophosphorylation on Tyr-397, which leads to c-Src recruitment. The association of FAK with Src family protein-tyrosine kinases at focal adhesions further increases the phosphorylation of FAK at Tyr-925, creating a Grb2 binding site (51–54). The data shown in Fig. 7 suggest that Shc regulates not only ERK but also JNK. We have previously shown that shear stress causes Ras activation which regulates both ERK and JNK (6, 10). By interacting with Grb2·Sos, Shc plays a pivotal role in activating Ras, which in turn regulates

[3] K.-D. Chen and J. Y.-J Shyy, unpublished results.

mitogen activated protein kinases and the AP-1/TRE-mediated transcriptional activation. It is to be noted that the shear stress activation of Ras is transient (6), but the association of Shc with Grb2 is sustained (Fig. 6*B*). Shear stress not only regulates immediate early responses but also other late events such as the formation of stress fibers and the alignment of ECs and their cytoskeletal elements with the direction of flow (see Ref. 1 for review). The binding of integrins to both ECM and actin-associated cytoskeletal proteins (*e.g.* talin, vinculin, α-actinin, and paxillin) has been suggested to provide a path for mechanical signaling (55, 56) and thus may be important in morphological remodeling.

In addition to RTKs and integrins, other molecules on the membrane and at cell junctions may also be involved in the mechano-chemical transduction processes. Angiotensin II receptor plays an important role in mechanical stress-induced cardiac hypertrophy (57), whereas platelet endothelial cell adhesion molecule-1 is tyrosine-phosphorylated in response to shear stress (58). Some of these molecules may also regulate Shc. For example, the G_q-coupled angiotensin II receptor activates Ras via the Shc·Grb2·Sos pathway in cardiac myocytes (59) and $G\beta\gamma$ subunits of G proteins mediate the tyrosine phosphorylation of Shc and the formation of Shc·Grb2 complex (60). Thus, while Shc is important in the RTK- and integrin-mediated responses of endothelial cells to mechanical stimuli, it may also be involved in other mechanotransduction pathways, with some of them yet to be identified.

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