Shc coordinates signals from intercellular junctions and integrins to regulate flow-induced inflammation

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Atherosclerotic plaques develop in regions of the vasculature associated with chronic inflammation due to disturbed flow patterns. Endothelial phenotype modulation by flow requires the integration of numerous mechanotransduction pathways, but how this is achieved is not well understood. We show here that, in response to flow, the adaptor protein Shc is activated and associates with cell–cell and cell–matrix adhesions. Shc activation requires the tyrosine kinases vascular endothelial growth factor receptor 2 and Src. Shc activation and its vascular endothelial cadherin (VE-cadherin) association are matrix independent. In contrast, Shc binding to integrins requires VE-cadherin but occurs only on specific matrices. Silencing Shc results in reduction in both matrix-independent and matrix-dependent signals. Furthermore, Shc regulates flow-induced inflammatory signaling by activating nuclear factor κB–dependent signals that lead to atherogenesis. In vivo, Shc is activated in atherosclerosis-prone regions of arteries, and its activation correlates with areas of atherosclerosis. Our results support a model in which Shc orchestrates signals from cell–cell and cell–matrix adhesions to elicit flow-induced inflammatory signaling.

Introduction

Fluid shear stress, the frictional force from blood flow, acts directly on the endothelium to modulate vessel structure and function (Davies, 1997). In arterial regions with laminar flow, the endothelial cells (ECs) express various atheroprotective genes and suppress several proatherogenic ones, leading eventually to stability and quiescence (Malek et al., 1999). In contrast, in regions with slow or disturbed flow where low shear stress occurs, the atheroprotective genes are suppressed, whereas the proatherogenic genes are up-regulated, thereby promoting the atherosclerotic process (Malek et al., 1999). Importantly, acute onset of laminar flow stimulates many of the same responses as disturbed shear. However, over longer periods, the cells adapt to the unidirectional shear forces and down-regulate the stress signaling, whereas in the disturbed shear, continual changes in flow magnitude and direction lead to sustained signaling (Orr et al., 2006). Thus, the in vitro protocol in which cells under static conditions are exposed to an abrupt increase in flow has been widely used as a model for disturbed flow and is particularly useful in analyzing temporal responses to flow.

EC surfaces are equipped with numerous mechanoreceptors that are capable of detecting and responding to shear stress (Traub and Berk, 1998; Lehoux et al., 2006). After activation of mechanoreceptors, a complex network of several intracellular pathways is triggered, a process known as mechanotransduction. Forces from the apical surface must be transmitted through the cytoskeleton to points of attachment that resist shear stress and anchor the cell in place (Davies, 1995). In that regard, both cell–cell and cell–ECM adhesions have been implicated in shear stress signal transduction. The junction-localized, endothelial-specific cadherin, vascular endothelial cadherin (VE-cadherin), is required for transducing shear stress–dependent signals into the endothelium (Shay-Salit et al., 2002; Tzima et al., 2005). We recently reported that VE-cadherin forms a mechanosensory complex with the EC adhesion molecule PECAM-1 and tyrosine kinase VEGF receptor 2 (VEGFR2), and this minimal complex is necessary for a subset of endothelial shear stress responses, such as the activation of nuclear factor κB (NFκB) and proinflammatory target genes (Tzima et al., 2005). In addition to cell–cell junctions, cell–matrix adhesions have also been implicated in shear stress signaling. Acute onset of laminar flow stimulates the conversion of integrins to a high-affinity state

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Abbreviations used in this paper: AP, atheroprone; AR, atheroresistant; BAEC, bovine aortic endothelial cell; CL, collagen; EC, endothelial cell; ERK, extracellular signal–regulated kinase; FN, fibronectin; LN, laminin; NFκB, nuclear factor κB; VE-cadherin, vascular endothelial cadherin; VEGFR2, vascular endothelial growth factor receptor 2; VTI, 4-[(4′-chloro-2′-fluoro)phenylamino]-6,7-dimethoxyquinazoline.

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(Tzima et al., 2001) followed by their binding to the subendothelial ECM (Jalali et al., 2001; Tzima et al., 2001). The newly occupied integrins subsequently activate multiple signaling pathways that lead to cell and cytoskeletal alignment in the direction of flow as well as the activation of NFκB, which is important for the expression of inflammatory genes in the endothelium (Jalali et al., 2001; Tzima et al., 2001, 2002, 2003). Importantly, the activation of NFκB by flow is dependent on ECM composition (activated on fibronectin [FN] but not collagen [CL]; Orr et al., 2005), and certain types of matrix proteins, such as FN, are deposited at the atherosclerosis-prone sites in vivo (Sechler et al., 1998). Although the biochemical and mechanical consequences of integrin- and cadherin-mediated adhesions each have been described, how these adhesions cross-talk and cooperate, especially in response to flow, is less well understood.

Members of the Shc family of adaptor proteins are key components of the pathways that activate Ras and MAPKs downstream of growth factors, cytokines, integrins, and mechanical forces (Pelicci et al., 1992; Chen et al., 1999; Ravichandran, 2001). Shc is phosphorylated at tyrosine residues 239/240 and 317 and recruits the adaptor protein Grb2 and the nucleotide exchange factor SOS (Ravichandran, 2001). The assembly of Shc–Grb2–SOS complex provides a mechanism for the activation of Ras and the MAP kinases (Ravichandran, 2001). In addition, tyrosine-phosphorylated Shc associates with integrins $\alpha_5\beta_1$, $\alpha_1\beta_1$, and $\alpha_v\beta_3$ when they are conjugated to the appropriate ligands (Bhattacharya et al., 1995; Wary et al., 1996). Notably, ShcA is expressed primarily in the cardiovascular system of mouse embryos and is required for normal development of the heart and the vascular system (Lai and Pawson, 2000).

Here, we show that Shc associates with constellations of both cell–cell and cell–matrix adhesions in response to flow. Furthermore, activation of Shc occurs in areas of disturbed flow and correlates with atherosclerosis in vivo. Finally, we reveal a surprising role for Shc in flow-induced inflammatory signaling. Thus, Shc orchestrates signals from junctional and matrix adhesion complexes to mediate inflammatory signaling in response to fluid flow.

Results

Activation of Shc in atheroprone (AP) areas of the vasculature

Areas of disturbed flow in vivo are prone to inflammation and atherogenesis. To determine whether Shc is activated in a flow-dependent manner, we performed immunohistochemical analyses in different locations of C57BL/6 mouse aortas using a Shc phospho-Tyr239/240 antibody as a marker. The arch of the aorta, which corresponds to proinflammatory and AP regions (Suo et al., 2007), showed remarkably pronounced phospho-Shc staining. In contrast, the ascending aorta, which is atheroresistant (AR), displayed barely detectable levels of phospho-Shc (Fig. 1 A). The focal activation of Shc is not caused by differences in Shc expression levels as shown in Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200709176/DC1). Thus, activated Shc is localized in regions where blood vessels exhibit sharp curvatures and are therefore more likely to be susceptible to blood turbulence and to develop atherosclerotic lesions. To further test whether the activation of Shc correlates with atherosclerotic lesions in vivo, the ascending aortas from $ApoE^{-/-}$ and C57BL/6 mice were isolated and processed for immunohistochemistry. $ApoE^{-/-}$ mice develop atherosclerotic lesions throughout the aortic tree, with localization similar to the lesions seen in human atherosclerosis (Daugherty, 2002). Although Shc phosphorylation was robust in $ApoE^{-/-}$ mice, particularly in ECs overlying the atherosclerotic lesions, it was almost undetectable in C57BL/6 aorta (Fig. 1 B). Thus, Shc phosphorylation correlates with atherogenesis and atherosclerosis-prone regions near bifurcations.

The distinct activation pattern of Shc in areas of atherosclerosis in vivo prompted us to ask whether the activation of Shc is regulated by different flow patterns in vitro. Cells stimulated with prolonged oscillatory flow (which is proinflammatory and atherogenic) showed elevated Shc phosphorylation compared with cells stimulated with extended laminar flow (which is considered antiinflammatory and atheroprotective; Fig. 1 C). Interestingly, acute onset of laminar shear stimulates many of the same responses as disturbed/oscillatory flow (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200709176/DC1). However, in a prolonged laminar shear, these events are downregulated as cells adapt, whereas in a disturbed shear, they are sustained (Orr et al., 2005). Thus, a large number of in vitro studies have exploited the acute onset of laminar shear to model AP signaling, and a temporal map of signaling cascades can already be assembled (Chatzizisis et al., 2007). We therefore used the laminar flow protocol to assay the early responses downstream of Shc. To this end, we first examined whether the rapid initiation of flow also regulates Shc activation in vitro. In bovine aortic ECs (BAECs), Shc phosphorylation was readily detected upon the onset of flow, as assessed by immunoblotting the cell lysates with a phospho-Shc Tyr239/240 antibody (Fig. 1 E). Notably, all three isoforms of Shc are phosphorylated in response to flow. Immunofluorescence staining showed that a fraction of activated Shc localized to cell–cell junctions (Fig. 1 D) and colocalized with β-catenin (Fig. S3 A). Flow-induced phosphorylation of Shc Tyr317 was not observed in parallel experiments (unpublished data), which suggests that the downstream signaling elicited by Shc in response to flow is primarily mediated through its phosphorylation at Tyr 239/240.

Shc associates with components of EC junctions in response to shear stress

The distinct spatial activation of Shc in response to the onset of flow suggested that Shc might associate with components of interendothelial junctions. Recently, we identified a minimal complex necessary for a subset of EC shear stress responses, which requires PECAM-1, VE-cadherin, and VEGFR2 (Tzima et al., 2005). To further investigate the role of Shc in shear stress signaling, the association of Shc with crucial components of the VE-cadherin–VEGFR2 signaling pathway was examined. Rapid onset of flow induced an acute association of Shc with VE-cadherin as assessed by coimmunoprecipitation assays (Fig. 2 A). VEGFR2 was also present in these immune complexes (Fig. 2 A), which suggests its possible role in Shc activation and the existence

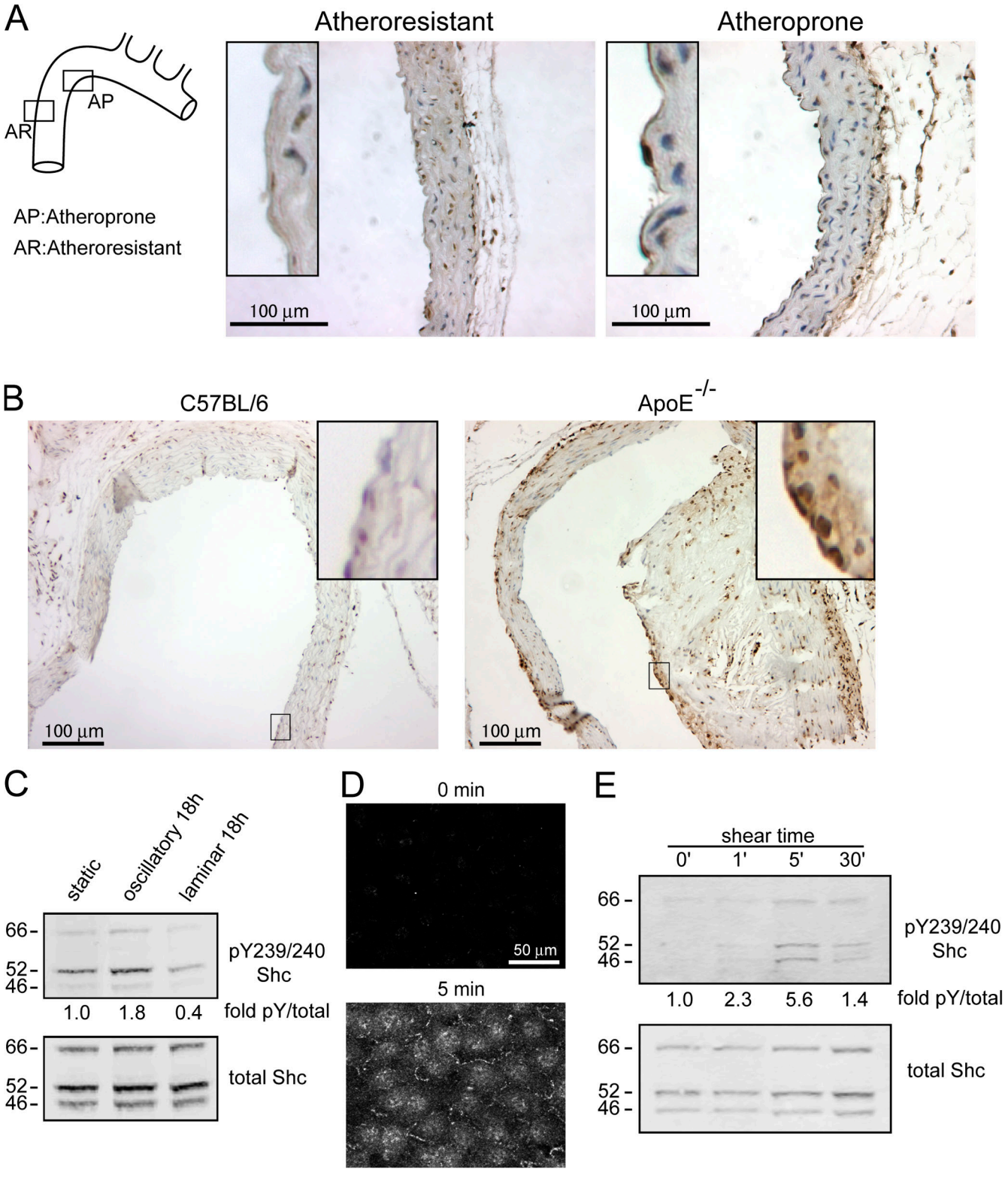

Figure 1. **Shc phosphorylation in vivo and in vitro.** (A) Aortas were isolated from C57BL/6 mice. Serial sections were obtained from AR and AP regions and stained for phospho-Tyr239/240 of Shc. Inserts show ECs lining up the aorta lumen. (B) Aortas were removed from ApoE$^{-/-}$ or C57BL/6 mice of the same age and embedded in paraffin. Serial sections were stained for phospho-Tyr239/240 of Shc. Boxes indicate the enlarged views shown in the insets. (C) BAECs were plated on FN-coated slides and subjected to oscillatory or laminar flow for 18 h or kept as static controls. Cell lysates were analyzed by immunoblotting with anti-Shc phospho-Tyr239/240 or anti-Shc antibodies. (D) BAECs were left untreated or subjected to laminar flow at 12 dyne/cm^2 for 5 min as described in the Materials and methods. Cells were subsequently fixed, permeabilized, and immunostained for phospho-Tyr239/240 of Shc. (E) BAECs were plated on FN-coated slides and sheared for 1, 5, or 30 min or kept as static controls. Cell lysates were subjected to SDS-PAGE and immunoblotting with anti-Shc phospho-Tyr239/240 or anti-Shc antibodies. Numbers to the left of the gel blots indicate molecular mass standards in kD.

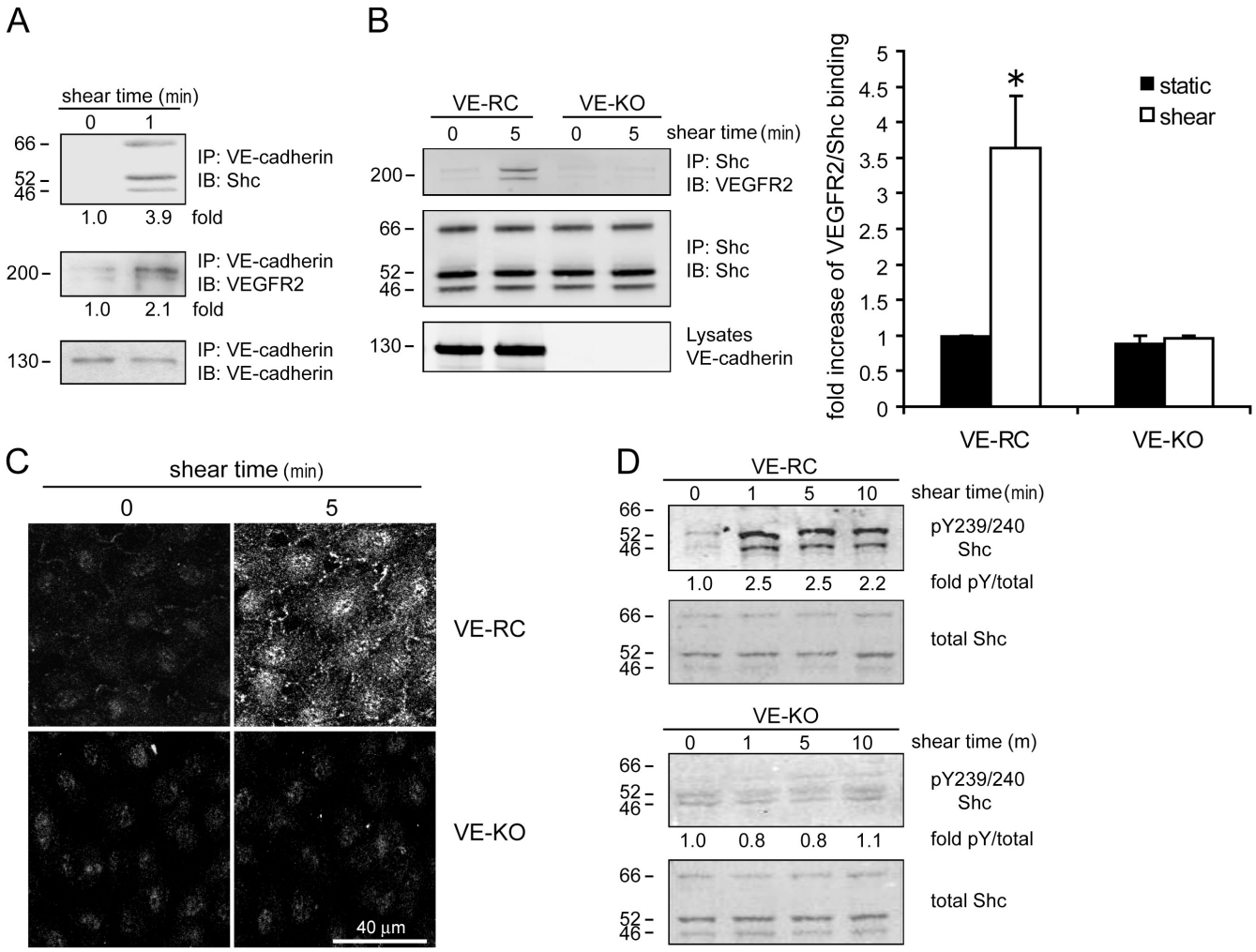

Figure 2. **Shear-induced interaction of Shc with VE-cadherin and VEGFR-2.** (A) BAECs were subjected to laminar flow for 1 min or left untreated. Cell extracts were immunoprecipitated with a VE-cadherin antibody and immunoblotted with antibodies specific for Shc, VEGFR-2, or VE-cadherin. Similar results were observed in three experiments. (B) VE-cadherin–null (VE-KO) and reconstituted (VE-RC) cells were left untreated or sheared for 5 min. Cell extracts were immunoprecipitated with anti-Shc antibody and analyzed by Western blotting with anti-VEGFR2 or anti-Shc antibodies. The quantification represents mean ± SD ($n = 3$; *, $P < 0.05$). (C) VE-RC and VE-KO cells were left untreated or exposed to shear stress for 5 min. Cells were fixed, permeabilized, and immunostained with an antibody specific for Shc phospho-Tyr239/240. (D) VE-RC and VE-KO cells were subjected to shear stress as in C. Cell extracts were analyzed by Western blotting with anti-Shc phospho-Tyr239/240 or total Shc antibodies. Numbers to the left of the gel blots indicate molecular mass standards in kD.

of a multiprotein complex induced by shear. Stimulation of ECs with oscillatory flow also induced the formation of a Shc–VEGFR2–VE-cadherin complex and the localization of activated Shc to junctions (Fig. S3). Importantly, the association of Shc with VE-cadherin was sustained under oscillatory flow (Fig. S3 C), which is similar to the sustained Shc phosphorylation (Fig. 1 C).

Because VE-cadherin is required for VEGFR2 activation by flow and downstream shear-dependent signaling (Shay-Salit et al., 2002; Tzima et al., 2005), we tested whether VE-cadherin is essential for the interaction of Shc with VEGFR2. VE-cadherin null (VE-KO) and cells reconstituted with human VE-cadherin (VE-RC) were used for these studies (Lampugnani et al., 2002). As shown in Fig. 2 B, application of shear stress to VE-RC cells stimulated Shc association with VEGFR2, which was not observed in VE-KO cells, suggesting that VE-cadherin is required for the formation of Shc-VEGFR2 complex in response to flow.

To investigate whether VE-cadherin is also required for flow-induced Shc activation, we examined Shc phosphorylation in VE-KO and VE-RC cells upon shear. As shown in Fig. 2 C, flow-induced Shc phosphorylation at Tyr 239/240 was not observed in VE-cadherin–null cells (Fig. 2, C and D), which indicates that this event is dependent on VE-cadherin.

We then sought to characterize the tyrosine kinases responsible for the flow-induced Shc activation. Flow rapidly activates several tyrosine kinases, including Src family kinases (Takahashi and Berk, 1996; Jalali et al., 1998; Okuda et al., 1999; Yan et al., 1999) and VEGFR2 (Chen et al., 1999; Jin et al., 2003). Because Src and VEGFR2 both localize to cell–cell junctions in response to flow, we tested their requirement for the flow-induced Shc activation. Pretreatment of ECs with Src inhibitor SU6656 abrogated flow-induced Shc tyrosine phosphorylation and translocation (Fig. 3). Similarly, treatment with the VEGFR2 kinase inhibitor 4-([4'-chloro-2'-fluoro]phenylamino)-6,7-dimethoxyquinazoline (VTI) abolished flow-induced Shc activation and localization to junctions (Fig. 3). These results indicated that tyrosine phosphorylation of Shc and its translocation

to EC junctions in response to shear stress require the kinase activities of Src and VEGFR2.

VE-cadherin is required for flow-induced Shc–integrin association

In addition to cell–cell junctions, signals from integrin–matrix adhesions are also important for shear stress–dependent responses. Acute onset of shear triggers conversion of integrins to a high-affinity state followed by their binding to the subendothelial ECM (Tzima et al., 2001). Resultant integrin signaling mediates cytoskeletal rearrangements (Tzima et al., 2001, 2002), gene expression (Chen et al., 1999), NFκB activation (Bhullar et al., 1998; Orr et al., 2005), and permeability (Orr et al., 2007). Shc is recruited to integrin–matrix adhesions upon cell attachment (Wary et al., 1996) and onset of flow (Chen et al., 1999). Interestingly, the association of Shc with $\alpha_v\beta_3$ integrin occurred at later times (30 min) after the onset of flow (Fig. 4). This association is absent in VE-KO cells (Fig. 4), which suggests that the ECM-dependent events in shear stress also require VE-cadherin. At this later time, no association of VEGFR2 with the Shc–$\alpha_v\beta_3$ integrin complex was detected (unpublished data), which is consistent with the association of Shc with VEGFR2 being transient (Chen et al., 1999). Collectively, these data show that both the early association of Shc with VEGFR2 and the later one with $\alpha_v\beta_3$ integrin require VE-cadherin, and that Shc may participate in both cell–cell and cell–matrix signaling in response to flow.

Shear stress–induced Shc-integrin association depends on specific integrin–ECM interactions (Fig. 5 A). Consistent with previous results, flow-induced Shc–integrin association was observed in cells plated on FN and vitronectin (both engage integrin $\alpha_v\beta_3$) but was absent in cells plated on CL or laminin (LN; both engage integrin $\alpha_2\beta_1$; Fig. 5 A). Importantly, the composition of the subendothelial ECM modulates inflammatory signaling and permeability in response to fluid flow (Orr et al., 2005, 2007). To determine whether Shc activation is also matrix specific, ECs were plated on either FN or CL, and Shc phosphorylation was assayed. Onset of flow triggered an increase in Shc phosphorylation irrespective of the matrix that the cells were plated on (Fig. 5 B). To test whether the flow-induced Shc association with cell–cell junctions is ECM dependent, immunoprecipitation assays were performed with lysates from cells plated on FN or CL. As shown in Fig. 5 C, Shc interaction with VE-cadherin was rapidly enhanced after the onset of flow regardless of the ECM composition. Cells plated on LN exhibited similar responses (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200709176/DC1). Thus, Shc activation and association with cell–cell junctions correlate closely and are independent of the matrix composition, whereas the later Shc–integrin association is ECM dependent.

Shc function is required for the activation of MAPKs by shear stress

The role of Shc in the activation of the Ras–MAPK pathway and mitogenic signaling has been well described (Ravichandran, 2001). The MAP kinases are activated by shear stress and mediate some of the effects of shear stress on ECs (Traub and Berk, 1998). To test whether Shc is involved in flow-induced extracellular signal–regulated kinase (ERK) activation, we suppressed cellular levels of Shc using siRNAs. As depicted in Fig. 6 (A and B), transfection of BAECs with Shc-specific siRNA resulted in a 90–95% decrease in the levels of all three isoforms of Shc. Decreasing Shc expression by siRNA dramatically inhibited the flow-induced ERK activation, whereas the control siRNA had no effect (Fig. 6, C and D). Interestingly, ERK activation was impaired to a similar extent in VE-KO cells when compared with VE-RC cells (unpublished data). In parallel experiments, the activation of another MAPK, p38, was also inhibited by attenuating Shc expression levels with siRNA, although p38 was inhibited to a lesser extent compared with ERK (unpublished data). Similar to the activation pattern for ERK, shear stress–induced p38 activation does not occur in ECs lacking VE-cadherin (Shay-Salit et al., 2002). As demonstrated (see Fig. 2 D), VE-cadherin is important for flow-induced Shc phosphorylation. These data are consistent with a model in which VE-cadherin–dependent Shc signaling contributes to the transient activation of ERK and p38 MAP kinases in response to fluid flow.

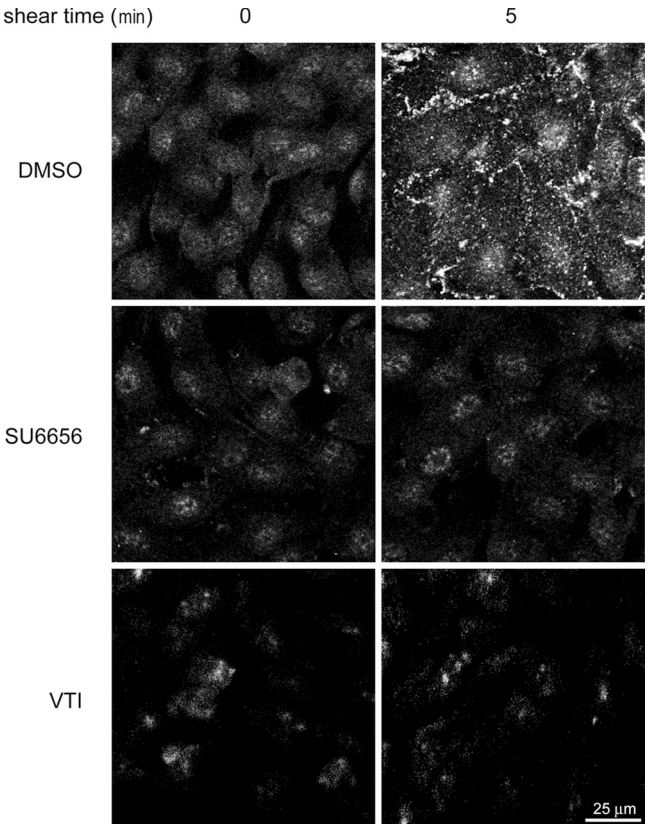

Figure 3. **Src and VEGFR2 kinase activities are required for flow-dependent Shc activation.** BAECs were pretreated with vehicle (DMSO), the Src inhibitor SU6656, or the VEGFR-2 inhibitor VTI for 30 min before their exposure to flow for the indicated times. Cells were fixed, permeabilized, and stained for Shc phospho-Tyr239/240 as described in the Materials and methods.

Shc mediates the flow-induced inflammatory responses

Shear stress regulates the chronic inflammation associated with atherogenesis (Caro et al., 1969; Ku et al., 1985; Glagov et al., 1988).

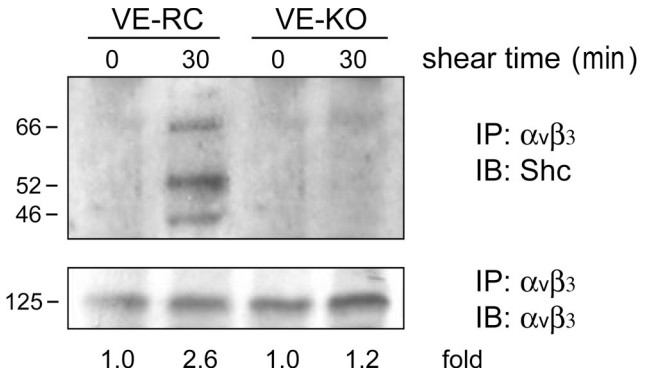

Figure 4. **VE-cadherin is required for shear-induced Shc–$\alpha_v\beta_3$ complex formation.** VE-cadherin–null (VE-KO) and reconstituted (VE-RC) cells were left untreated or sheared for 30 min. Cell extracts were immunoprecipitated with anti-$\alpha_v\beta_3$ antibody LM609 and analyzed by Western blotting with anti-Shc or anti-$\alpha_v\beta_3$ LM609 antibodies. Numbers to the left of the gel blot indicate molecular mass standards in kD.

The increased Shc phosphorylation in ApoE$^{-/-}$ aorta raises the possibility that Shc may participate in the regulation of the inflammatory response elicited by shear stress. NFκB is a key regulator of shear stress–induced inflammatory gene expression and contributes to the initiation of atherosclerosis by shear stress. We therefore tested whether Shc is upstream of NFκB activation in response to the onset of flow. NFκB is normally held inactive in the cytoplasm through its interaction with IκB. Degradation of IκB results in NFκB nuclear targeting and initiation of transcription. As shown in Fig. 7 A, attenuation of Shc expression abrogated nuclear translocation of NFκB, which suggests that Shc is upstream of NFκB activation in response to flow.

In addition to its nuclear targeting, phosphorylation of the p65 subunit of NFκB at Ser536 in its transactivation domain alters NFκB-dependent transcription. As shown in Fig. 7 B, Ser536 phosphorylation was stimulated by flow in cells transfected with control siRNA but showed deregulation in cells in which Shc levels were attenuated (Fig. 7 B). These data suggested that Shc function is important for flow-induced NFκB activation.

The NFκB dimer, particularly the p65/p50 heterodimer, binds to a shear stress–responsive element found in the promoter of several atherogenic genes, including ICAM-1 and VCAM-1, that regulate monocyte recruitment (Resnick et al., 1993; Khachigian et al., 1995). To test whether the Shc-dependent NFκB activation translates to altered gene expression, we assayed expression of both ICAM-1 and VCAM-1. Because cellular responses to laminar flow are transient, we examined the expression of these cell adhesion molecules in ECs exposed to oscillatory flow for longer times. As shown in Fig. 8 (A and B), under the extended oscillatory flow condition, although the expressions of ICAM-1 and VCAM-1 increased significantly in cells transfected with control siRNA, the up-regulation was strongly inhibited when Shc function was abrogated. Consistent with these observations, monocyte adhesion to EC monolayers was also inhibited as a result of reduced Shc expression levels (Fig. 8, C and D). Thus, flow-dependent NFκB nuclear translocation, phosphorylation, target gene expression, and monocyte adhesion correlate closely. We conclude that Shc function is important for the initial events in inflammation and atherogenesis induced by shear stress.

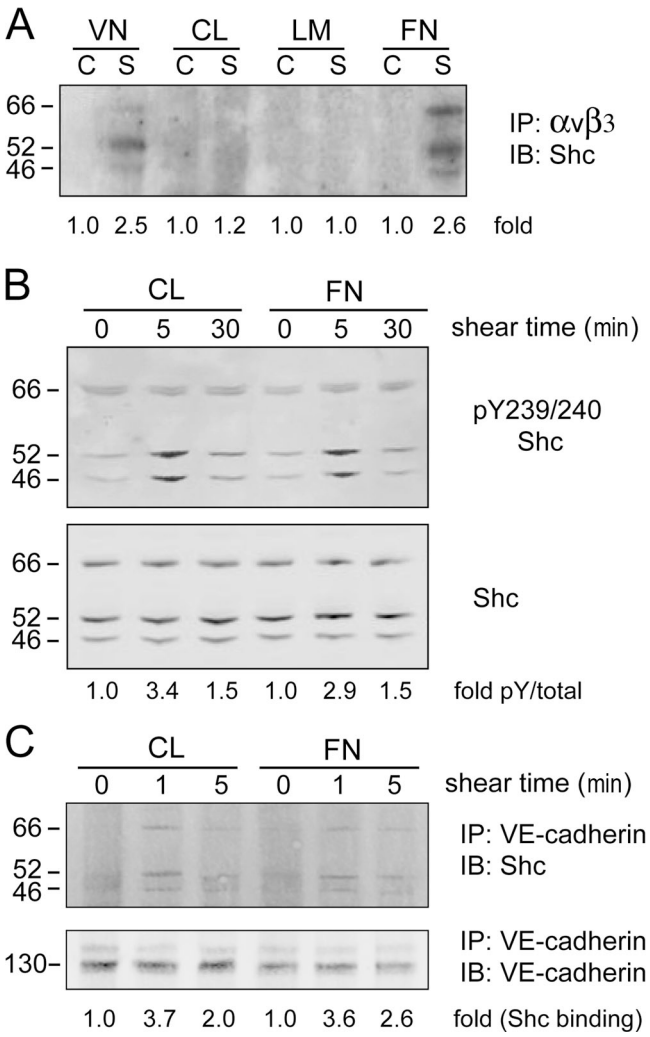

Figure 5. **Shear-induced Shc–integrin association, but not Shc phosphorylation or Shc–VE-cadherin association, is ECM dependent.** (A) Slides were coated with vitronectin, CL, LN, or FN. BAECs were sheared for 30 min or kept as static controls. Cell lysates were immunoprecipitated with LM609 anti-$\alpha_v\beta_3$ followed by immunoblotting with anti-Shc antibody. Each pair of lanes represents static control (C) and sheared (S) samples from cells plated on the indicated ECM. (B) BAECs were plated on CL- or FN-coated slides and sheared for 5 or 30 min, or kept as static controls. Cell lysates were subjected to SDS-PAGE and immunoblotting with anti-Shc phospho-Tyr239/240 or anti-Shc antibodies. (C) BAECs were plated on CL- or FN-coated slides and sheared for 1 or 5 min, or kept as static controls. Cell lysates were immunoprecipitated with a VE-cadherin–specific antibody and immunoblotted with anti-Shc or anti-VE-cadherin. Numbers to the left of the gel blots indicate molecular mass standards in kD.

Discussion

In the present study, we present evidence that Shc integrates signals from both cell–cell and cell–matrix adhesions to regulate flow-induced inflammatory signaling. Shc activation occurs in vivo and correlates with areas of disturbed flow and atherogenesis. Shc associates with components of the junctional complexes VE-cadherin and VEGFR2 at early times after the onset of flow and with integrin–ECM complexes at later times.

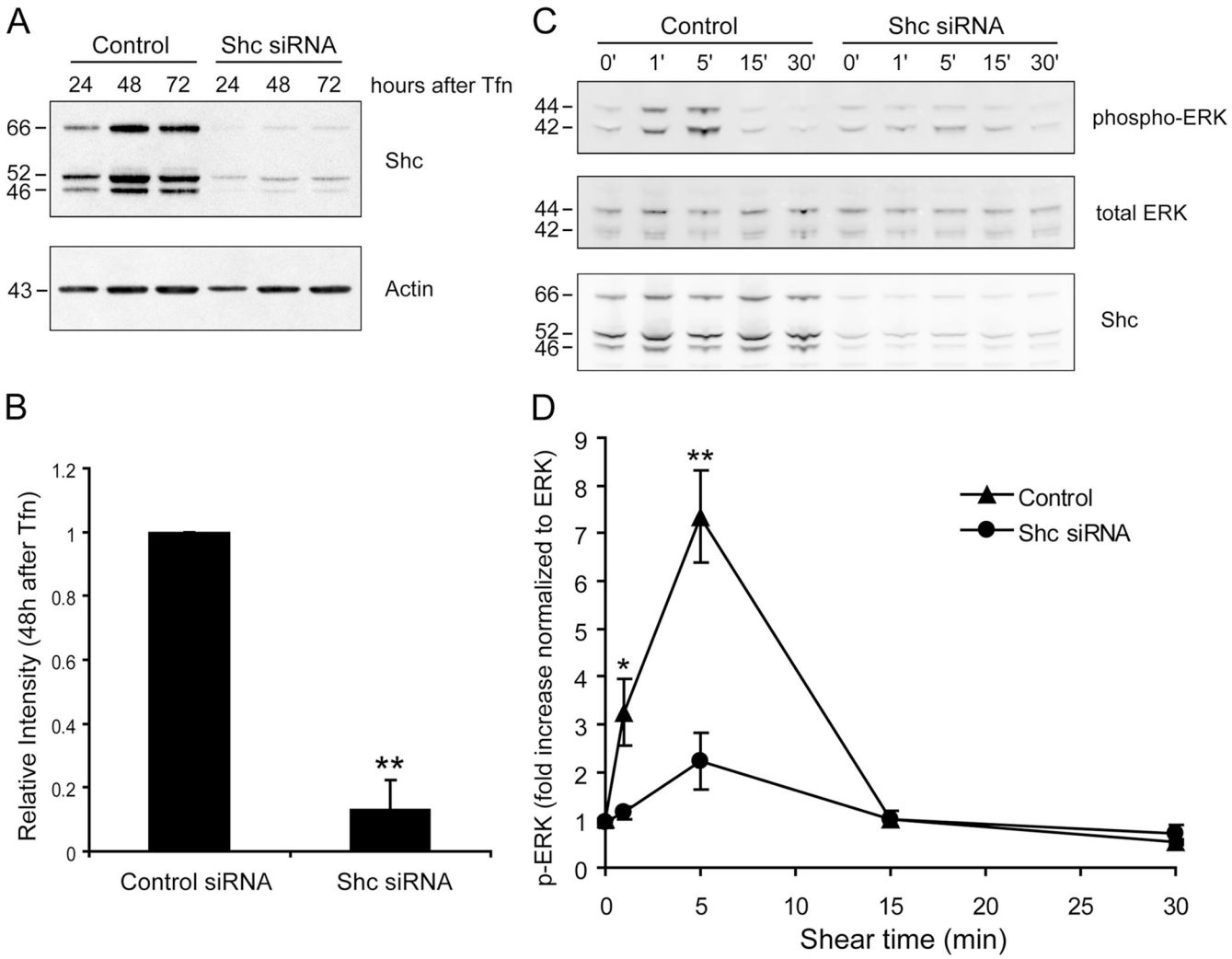

Figure 6. **Shc mediates flow-induced Erk activation.** (A) BAECs were transfected with control siRNA or ShcA siRNA as described in the Materials and methods. Cells were lysed at indicated times after transfection, and cell lysates were analyzed by immunoblotting with a Shc-specific antibody to confirm the knockdown effect of the Shc siRNA. Blots were stripped and reprobed with an antibody against actin as a loading control. (B) Quantitation of the RNAi efficiency 48 h after transfection. The data represent mean ± SD ($n = 5$; **, $P < 0.01$). (C) BAECs were transfected with control siRNA or Shc-specific siRNA as in A. 48 h after transfection, cells were subjected to laminar flow for indicated times. Cell lysates were analyzed by immunoblotting with a phospho-Erk–specific antibody or anti-Erk antibody. Numbers to the left of the gel blots indicate molecular mass standards in kD. (D) Quantitation of the fold increase for p-ERK levels normalized to total ERK levels. The data represent mean ± SD ($n = 4$; *, $P < 0.05$; **, $P < 0.01$).

The association with integrins requires VE-cadherin and the activation of Shc requires the activities of VEGFR2 and Src, two tyrosine kinases that localize to EC junctions in response to flow. Although Shc phosphorylation and its association with VE-cadherin are ECM independent, Shc binding to integrins occurs only on specific matrices. Depletion of Shc with siRNA inhibits the activation of flow-responsive signaling proteins including ERK and NFκB. The up-regulation of the EC adhesion molecules ICAM-1 and VCAM-1, as well as leukocyte adhesion to endothelial monolayers, are also significantly inhibited as a result of reduced Shc expression levels. Interestingly, the activation of downstream ERK signaling is ECM independent whereas the activation of NFκB signaling is ECM specific and correlates with the ECM specificity for the Shc–integrin association. Thus, we propose that Shc functions as a molecular switch to orchestrate signals from cell–cell and cell–matrix adhesions to elicit an inflammatory response in ECs under flow (Fig. 9).

Coordinated changes between cell adhesions to the ECM and those to neighboring cells are crucial for numerous physical transformations that the cells must undergo during development, tissue homeostasis, and wound healing. Although cell–cell junctions and cell–matrix adhesions mediate unique downstream signals, integrin and junctional signaling pathways are highly interwoven into complex signaling networks. In the context of shear stress signaling, there are many levels of cross talk. For instance, both cell–cell and cell–matrix adhesions activate common effectors such as NFκB (Shay-Salit et al., 2002; Tzima et al., 2002; Orr et al., 2005; Tzima et al., 2005), ERK and p38 MAPKs (Takahashi and Berk, 1996; Li et al., 1997; Osawa et al., 2002; Shay-Salit et al., 2002; Tai et al., 2005; Fleming et al., 2005), Akt and Src kinases (Okuda et al., 1999; Fleming et al., 2005; Tai et al., 2005; Tzima et al., 2005), and endothelial nitric oxide synthase (eNOS; Jin et al., 2003; Dusserre et al., 2004; Fleming et al., 2005; Bagi et al., 2005). Another level

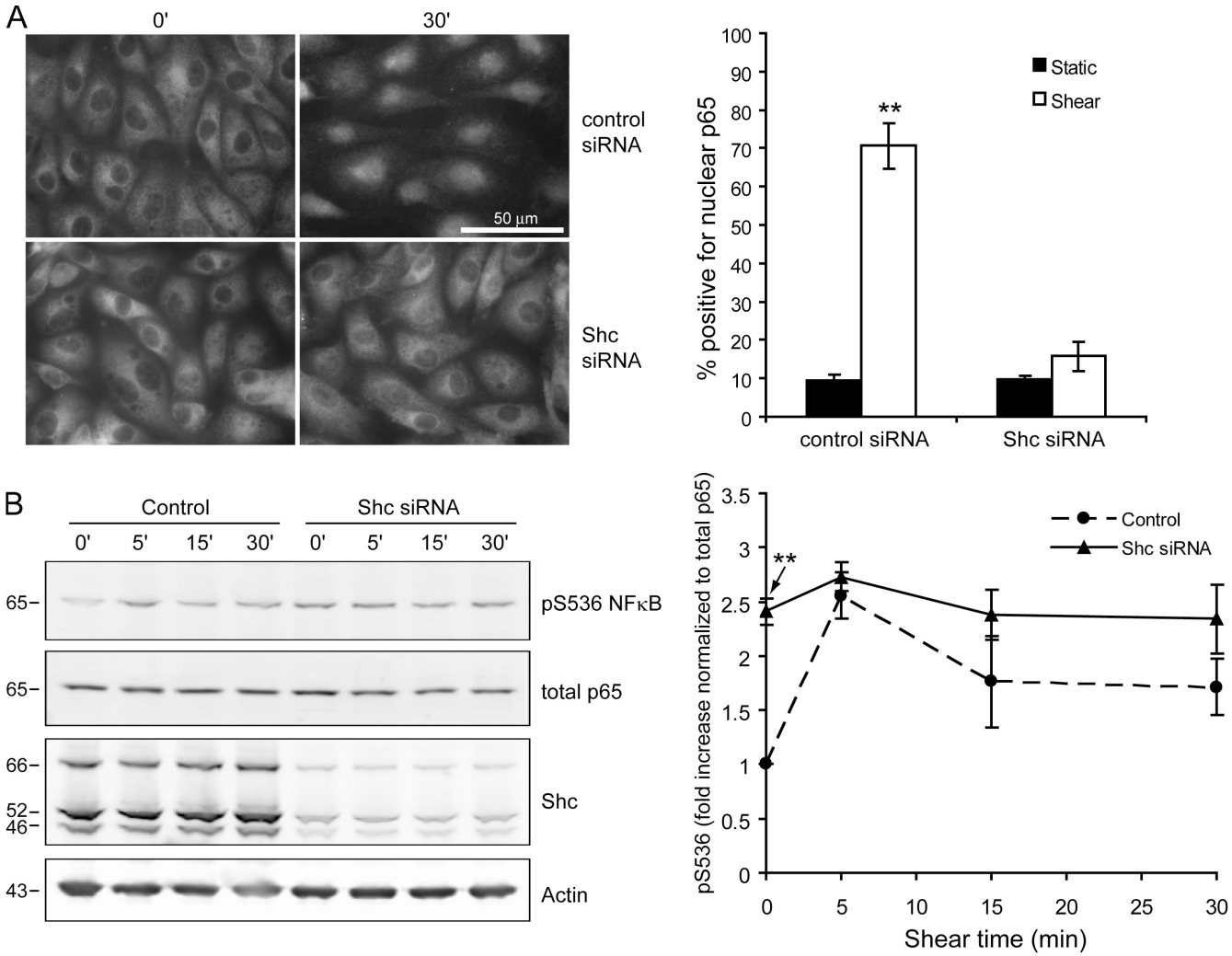

Figure 7. **Shc is required for flow-induced NFκB activation.** (A) BAECs were transfected with control siRNA or Shc-specific siRNA. 48 h after transfection, cells were exposed to laminar flow for 30 min or left as a static control. Cells were fixed, permeabilized, and stained for the p65 subunit of NFκB as described in the Materials and methods. Three independent experiments were performed and 100 cells were counted for each experiment (**, $P < 0.01$). (B) BAECs were transfected with control siRNA or Shc-specific siRNA. 48 h after transfection, cells were exposed to laminar flow for indicated times or left as a static control. Cells were lysed and analyzed by immunoblotting with anti-p65 phospho-Serine536, anti-p65, anti-Shc, or anti-actin antibodies ($n = 3$; **, $P < 0.01$). Numbers to the left of the gel blot indicate molecular mass standards in kD. The data represent mean ± SD.

of cooperation lies in the commonality of binding partners. VEGFR2 binds to both adherens junctions (through VE-cadherin) and integrins, and thus, at any given time, VEGFR2 may regulate two distinct signaling modules by interacting with either VE-cadherin or $\alpha_v\beta_3$ integrin (Bussolino et al., 2001). More recently, integrins were implicated as intermediates that are activated downstream of junctional signaling that leads to phosphoinositide 3-kinase–induced integrin activation and increased ECM binding (Tzima et al., 2001, 2005). We now provide evidence that the adaptor protein Shc plays a critical role in the cross talk between cell–cell junctions and integrins during flow.

The function of Shc in flow may be tightly regulated by tyrosine phosphorylation/dephosphorylation events. Shear stress stimulates the activation of Src kinases, which transactivate VEGFR2 (Jin et al., 2003). VEGFR2 activation may result in the recruitment and tyrosine phosphorylation of Shc, which is dependent on VE-cadherin. As demonstrated (see Fig. 4), VE-cadherin is required for the association of Shc with integrins, which mediates Ras–ERK activation and the flow-dependent transcriptional responses. It is worth noting here that upon VEGF treatment, VE-cadherin becomes phosphorylated and binds to Shc, which is dephosphorylated (Zanetti et al., 2002). The functional importance of this association may be that it facilitates Shc dephosphorylation through a VE-cadherin–associated phosphatase. In contrast to the stimulation by VEGF, the temporal response of Shc tyrosine phosphorylation induced by shear stress is sustained (Chen et al., 1999).

The contribution of Shc to both integrin- and growth factor–induced activation of ERK is well documented (Wary et al., 1996; Barberis et al., 2000; Lai and Pawson, 2000), but this is the first study to reveal a role for Shc in the inflammatory signaling through NFκB. It has recently been shown that flow-induced NFκB activation is ECM dependent and is only observed in cells plated on FN but not on CL. ECM composition is a crucial factor in atherogenesis and may regulate the early changes

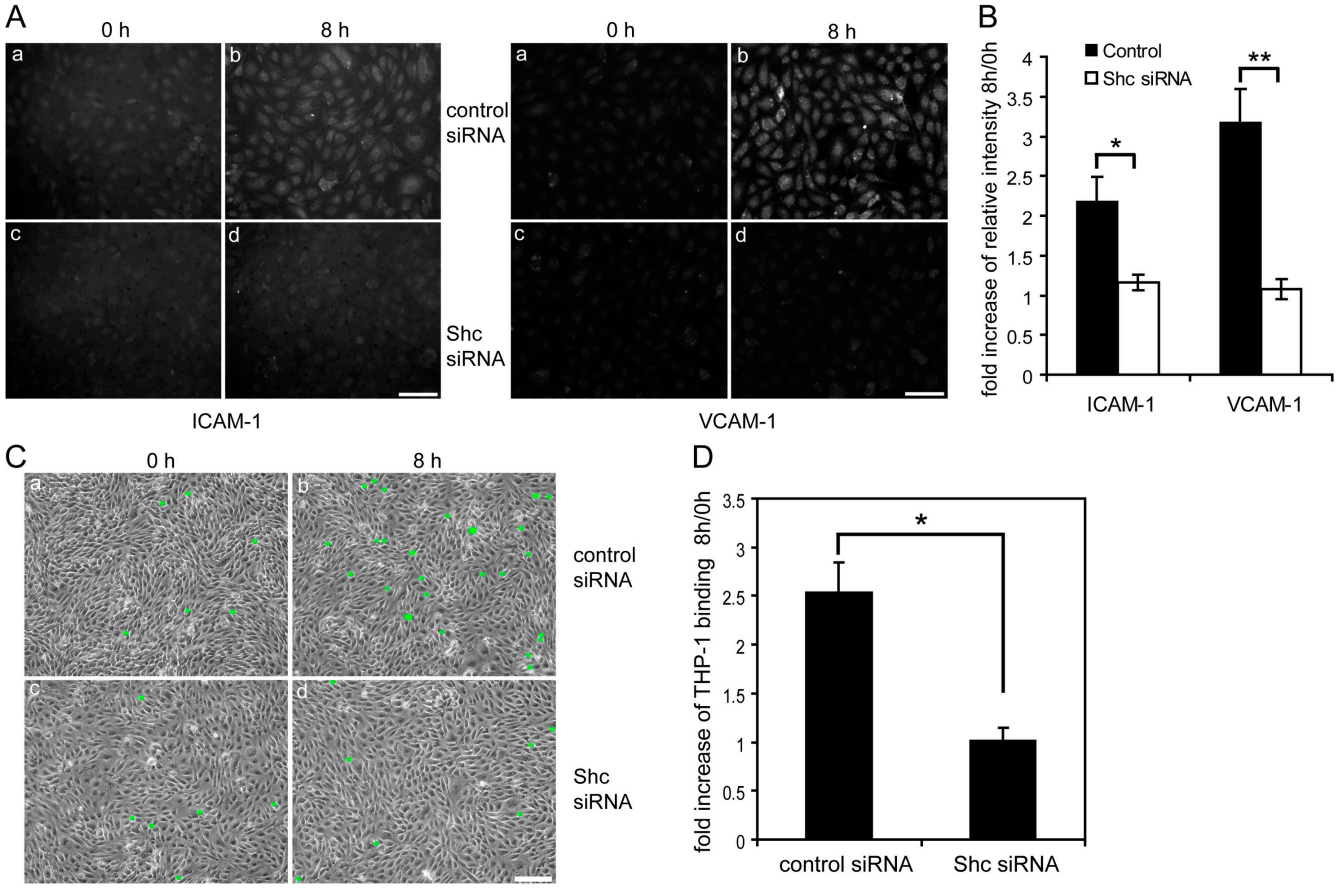

Figure 8. **Shc is required for the flow-induced up-regulation of endothelial adhesion molecules ICAM-1 and VCAM-1 as well as flow-induced monocyte adhesion.** (A) BAECs were transfected with control siRNA (a and b) or Shc-specific siRNA (c and d). 48 h after transfection, cells were exposed to oscillatory flow for 8 h or left as a static control. Cells were fixed and stained for adhesion molecules ICAM-1 or VACM-1 as described in the Materials and methods. Bars, 50 µm. (B) Quantitation of the change in staining intensity after shear ($n = 3$; *, $P < 0.05$; **, $P < 0.01$). (C) BAECs were transfected and sheared as in A. THP-1 monocytes prelabeled with CellTracker green were added to BAEC monolayers, and a monocyte binding assay was performed as described in the Materials and methods. Bar, 100 µm. (D) Quantitation of monocyte binding assays in C. The data represent mean ± SD for three independent experiments (*, $P < 0.05$).

in inflammation associated with atherogenesis (Orr et al., 2005). Interestingly, NFκB activation in Shc-attenuated cells closely resembles cells plated on CL, whereas cells transfected with control siRNA emulate the FN phenotype observed by Orr et al. (2005). In addition, the association of Shc with integrins in response to flow is ECM specific (Fig. 5). Taken collectively, these data raise the possibility that Shc may function as a molecular switch to translate ECM specificity into ECs through its regulated interaction with integrin receptors engaged with the appropriate ECM.

Mutant mice lacking all three Shc isoforms die at embryonic day 11.5 due to cardiovascular defects (Lai and Pawson, 2000), whereas mice selectively missing the p66 ShcA isoform are long-lived (Migliaccio et al., 1999) due to the role of p66 Shc in oxidative stress signaling (Pinton et al., 2007). The exact contribution of each Shc isoform to development and signaling is still unclear. Most recently, pioneering work has shown that combinatorial differences in ShcA docking interactions may yield multiple signaling mechanisms to support diversity in tissue morphogenesis (Hardy et al., 2007).

In conclusion, our data provide a molecular description of the coordination of mechanochemical signals between cell–cell and cell–ECM adhesions that drive the complex inflammatory signaling elicited by disturbed shear stress. As ECM deposition and leukocyte adhesion to the AP sites are instrumental to early events in atherogenesis, our observations together with previously published results point to Shc as a potential therapeutic target in the treatment of atherosclerosis and coronary artery diseases.

Materials and methods

Cell culture, transfections, and shear stress

BAECs were maintained in DME (Invitrogen) with 10% FBS (Invitrogen), 10 µg/ml penicillin, and 0.25 µg/ml streptomycin (Invitrogen). VE-cadherin null (VE-KO) and reconstituted (VE-RC) cells were prepared as described previously (Carmeliet et al., 1999) and grown in DME containing 10% FBS, 5 µg/ml EC growth serum, and 100 µg/ml heparin and penicillin/streptomycin. THP-1 leukocytes were maintained in RMPI 1640 medium (Invitrogen) with 10% FBS, 10 µg/ml penicillin, 0.25 µg/ml streptomycin, and 2 mM glutamate (Invitrogen). Control siRNA or Shc siRNA (Thermo Fisher Scientific) were transfected into BAECs as described previously (Liu et al., 2005). For shear stress experiments, BAECs were plated on appropriate matrix proteins (10 µg/ml FN or 20 µg/ml Coll I) and allowed to grow for 10 h in medium containing 10% FBS or 4 h in 0.5% FBS. Cells were then starved overnight in medium containing 0.5% FBS. Slides were loaded onto a parallel plate flow chamber in 0.5% FBS, and 12 dynes/cm^2 of shear stress was applied for indicated times. To examine upstream kinases

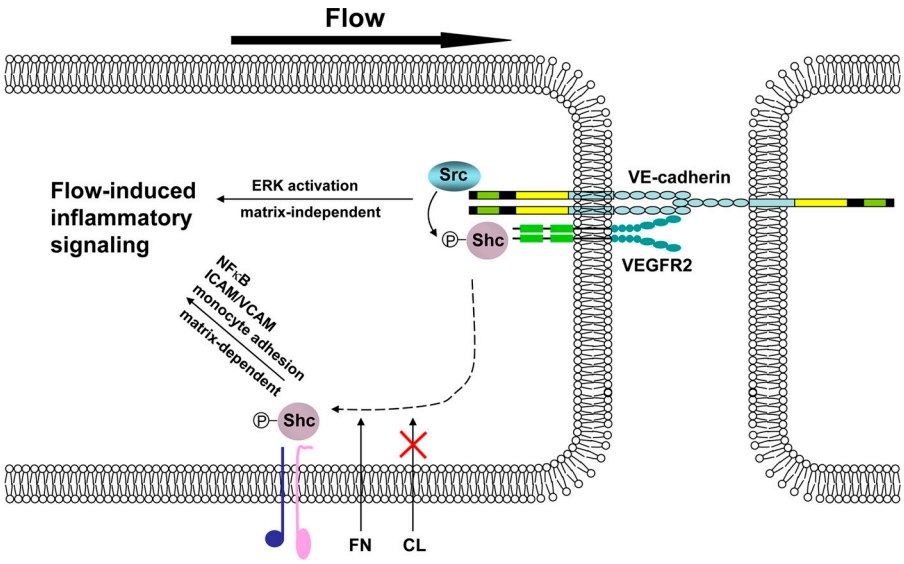

Figure 9. **Coordination of cell–cell and cell–matrix flow-induced inflammatory signals through Shc.** Shc functions as a molecular switch to coordinate signals from cell–cell junctions and cell–matrix adhesions in the regulation of flow-induced inflammatory responses.

required for Shc functions, cells were incubated with 10 µM of the VEGF receptor tyrosine kinase inhibitor VTI or 5 µM SU6656 (EMD) for 30 min at 37°C.

Oscillatory flow

To perform oscillatory flow, cells were cultured on 2 × 3 inch slides. After cells reached 100% confluence, the slides were attached to parallel chambers. The chambers were subsequently connected to an NE-1050 bidirectional pump (New Era Pump Systems, Inc.). Cells were sheared at ±6.5 dyne/cm^2, 1 Hz.

Immunoprecipitations, Western blotting, and antibodies

Cells were harvested in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS) supplemented with 1 mM aprotinin, 1 µg/ml leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, 1 mM sodium pyrophosphate, and 1 mM β-glycerophosphate. Lysates were precleared with 50 µl protein A/G–Sepharose beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C. Supernatants were then incubated with 30 µl of protein A/G–Sepharose previously coupled to the primary antibodies for 2 h at 4°C with continuous agitation. The beads were washed three times with lysis buffer supplemented with protease and phosphatase inhibitors, and the immune complexes were eluted in 2× SDS sample buffer. Associated proteins were subjected to SDS-PAGE and Western blotting using the appropriate primary antibodies and HRP-conjugated anti–mouse or anti–rabbit antibodies (Jackson ImmunoResearch Laboratories). Immunoreactive proteins were visualized by enhanced chemiluminescence (GE Healthcare). The phospho-Shc (Tyr239/240 or Tyr317), phospho-ERK (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-p65 (Ser536), and ERK antibodies were obtained from Cell Signaling Technology. An anti-Shc phosphoTyr239/240 antibody from BioSource (Invitrogen) was tested and generated similar results to the Cell Signaling Technology phospho-Shc antibody. VEGFR-2 and p38 antibodies were obtained from Santa Cruz Biotechnology. Anti–VE-cadherin was purchased from Qbiogene. Anti-Shc and anti-NFκB (p65) were obtained from BD Biosciences. The ICAM-1 and VCAM-1 antibodies were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. FITC-conjugated goat anti–mouse IgG and rhodamine-conjugated goat anti–rabbit IgG were obtained from Jackson ImmunoResearch Laboratories and used at 1:200 dilution.

Immunofluorescence microscopy

To examine the tyrosine phosphorylation of Shc and the nuclear translocation of NFκB, cells were fixed for 20 min in PBS containing 2% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with PBS containing 10% goat serum and 1% BSA for 1 h at room temperature. Antibody incubations were performed as described previously (Tzima et al., 2001), and slides were mounted in Vectashield mounting medium (Vector laboratories). Images were obtained using the 60× 1.40 NA oil objective on a microscope (Eclipse E800; Nikon) equipped with a digital camera (ORCA-ER; Hamamatsu) and MetaMorph software (MDS Analytical Technologies). To examine the expression levels of adhesion molecules ICAM-1 or VCAM-1, cells were stained without Triton X-100 permeabilization, and the 20× 0.75 NA objective on the same microscope was used to acquire the images.

Leukocyte adhesion assay

For each adhesion assay, 1× 10^6 THP-1 cells were collected by centrifugation. Cells were resuspended in serum-free RMPI 1640 medium containing 1 µM CellTracker green 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) and incubated at 37°C for 20 min. Cells were then spun down and resuspended in RMPI 1640 medium containing 10% FBS. After the ECs were sheared for the required times, the prelabeled THP-1 cells were added onto the monolayers of ECs and incubated at 37°C for 15 min. The unbound cells were rinsed off with PBS and the bound cells were fixed with 2% formaldehyde. To quantify the assays, five random fields under the 10× 0.30 NA objective on an inverted microscope (DMIRB; Leica) were counted for each assay, and representative images were acquired using a RETIGA 1300 camera (QImaging).

Immunohistochemistry

5-µm serial sections were obtained from paraffin-embedded mouse aortas. After antigen retrieval with antigen unmasking solution, anti–phospho-Shc (1:30, Cell Signaling Technology) was applied to the sections. Detection of antibody was performed using a Vectastain Elite ABC kit (Vector Laboratories), and the epitopes were visualized by DAB reaction. Images were acquired using the 10× 0.30 NA or 20× 0.40 NA objective on a DMIRB inverted microscope equipped with a RETIGA 1300 camera and QCapture software (QImaging).

Quantification and statistical analysis

Band intensity of immunoblots was quantified using the ImageJ program. Each experimental group was analyzed using single factor analysis of variance. P-values were obtained by performing two-tailed Student's *t* test using Excel (Microsoft). Statistical significance was defined as $P < 0.05$.

Online supplemental material

Fig. S1 shows that en face staining of phospho-Shc is enhanced in the AP region of the C57BL/6 aorta compared with the AR region. No significant differences in total Shc levels were observed as assessed by en face staining, immunoblotting of tissue homogenates, and immunohistochemistry staining. Fig. S2 shows that acute onset of oscillatory flow induces similar responses to those observed with laminar flow. Fig. S3 shows that similar to laminar flow, onset of oscillatory flow stimulates Shc phosphorylation, Shc translocation to EC junctions, and the formation of the Shc–VE-cadherin–VEGFR2 complex; however, over longer periods, the responses are more sustained under oscillatory flow compared with laminar flow. Fig. S4 shows that onset of laminar flow induces acute Shc association with VE-cadherin in ECs plated on LN, which is also observed in cells on FN or CL. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200709176/DC1.

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