

Activation of Rac1 by shear stress in endothelial cells mediates both cytoskeletal reorganization and effects on gene expression

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Hemodynamic shear stress is a fundamental determinant of vascular remodeling and atherogenesis. Changes in focal adhesions, cytoskeletal organization and gene expression are major responses of endothelial cells to shear stress. Here, we show that activation of the small GTPase Rac is essential for gene expression and for providing spatial information for shear stress-induced cell alignment. Fluorescence resonance energy transfer (FRET) localizes activated Rac1 in the direction of flow. This directional Rac1 activation is downstream of shear-induced new integrin binding to extracellular matrix. Additionally, Rac1 mediates flow-induced stimulation of nuclear factor κ B (NF- κ B) and the subsequent expression of intercellular cell adhesion molecule 1 (ICAM-1), an adhesion receptor involved in the recruitment of leukocytes to atherosclerotic plaque. These studies provide a unifying model linking three of the main responses to shear stress that mediate both normal adaptation to hemodynamic forces and inflammatory dysfunction of endothelial cells in atherosclerosis.

Keywords: cytoskeleton/fluid shear stress/mechanotransduction/NF- κ B/Rac GTPase

Introduction

Endothelial cells (ECs) that line the vasculature are constantly subjected to hemodynamic forces imposed by blood flow, including fluid shear stress. Shear stress plays an active role in leukocyte/ECs interaction and subsequent leukocyte extravasation into inflamed tissue (Harlan, 1985; Osborn, 1990). These effects are mediated in part by modulating surface expression of adhesive proteins on the endothelium (Lawrence *et al.*, 1987; Walpole *et al.*, 1995). In this way, hemodynamic forces play a critical role in determining the distribution of atherosclerotic plaques that appear almost exclusively at regions of the vasculature

where flow is disturbed due to curvature or at the proximal region of branching vessels.

Shear stress regulates expression of a variety of genes (Resnick and Gimbrone, 1995; Shyy *et al.*, 1995a; Gimbrone *et al.*, 1997; Khachigian and Collins, 1997; Resnick *et al.*, 1997; Traub and Berk, 1998). Several *trans*-acting factors are activated by shear stress and subsequently induce the expression of target genes. Nuclear factor κ B (NF- κ B) was the first such factor to be described (Lan *et al.*, 1994; Khachigian *et al.*, 1995; Bhullar *et al.*, 1998; Nagel *et al.*, 1999). NF- κ B is composed of protein dimers of the Rel/NF- κ B family, with the p50/p65 dimer being the predominant form *in vivo*. In unstimulated cells, Rel/NF- κ B dimers are sequestered in the cytoplasm by binding to members of the I κ B family of inhibitor proteins. Upon activation, I κ B is degraded and NF- κ B translocates to the nucleus, where it regulates the transcription of multiple target genes by binding to promoter elements in many genes (Resnick and Gimbrone, 1995). An NF- κ B consensus promoter was identified as the shear stress response element (SSRE) within the PDGF gene that mediated induction of PDGF by flow (Resnick and Gimbrone, 1995; Verma *et al.*, 1995). Activated NF- κ B was identified in smooth muscle cells, macrophages and ECs of human atherosclerotic tissue specimens (Brand *et al.*, 1996) as well as in humans with unstable angina pectoris (Ritchie, 1998), suggesting a pathophysiological role for NF- κ B in atherosclerosis (Brand *et al.*, 1997).

Rac1 contributes to reactive oxygen species (ROS) production in response to shear stress (Yeh *et al.*, 1999), and ROS production in response to shear stress leads to increased expression of intercellular cell adhesion molecule 1 (ICAM-1) gene (Chiu *et al.*, 1997). It has been shown previously that Rac mediates a cytokine-stimulated, redox-dependent pathway necessary for NF- κ B activation (Sulciner *et al.*, 1996). Additionally, Rac, Rho and Cdc42 induce transcriptional activity of NF- κ B by phosphorylation of I κ B (Perona *et al.*, 1997), and activation of Rac induces NF- κ B binding and activity and enhances expression of cyclin D1 (Joyce *et al.*, 1999). Toll-like receptor 2-mediated NF- κ B activation also depends on Rac (Arbibe *et al.*, 2000). More recently, Reyes-Reyes *et al.* (2001) reported that integrin signaling to NF- κ B is mediated by Rac.

We showed previously that shear stress induces conformational activation of integrins followed by new binding of integrins to extracellular matrix (ECM) (Tzima, 2001). These newly occupied integrins subsequently initiate signals, including a transient inactivation of Rho. Here, we test the involvement of the related GTPase Rac1 in shear stress signaling. Our results show that Rac1 mediates both cytoskeletal alignment and the upregulation of ICAM-1, the latter through activation of

NF- κ B. These results show that many of the key shear stress responses are linked within a single, integrin-mediated pathway.

Results

Regulation of Rac1 activity by shear stress

Binding of integrins to ECM proteins stimulates transient activation of Rac1 (Del Pozo *et al.*, 2000). Since shear stress induces new integrin binding to ECM (Tzima, 2001), we examined whether shear stress could activate Rac1. Pull-down assays were performed in which Rac1 GTP loading was determined by specific binding of the active GTPase to the p21-binding domain of PAK1 (PBD) fused to glutathione *S*-transferase (GST) (Glaven *et al.*, 1999; Del Pozo *et al.*, 2000). Serum-starved bovine aortic ECs (BAECs) were subjected to shear stress for the indicated times, lysates were prepared and the amount of Rac1 precipitated with the GST-PBD determined by western blotting. The results showed that shear stress stimulated a transient increase in Rac1 activity that peaked at 30 min and then returned to basal levels (Figure 1).

To visualize local Rac1 activation during shear stress, we used FLAIR (fluorescence activation indicator for Rho proteins) (Kraynov *et al.*, 2000). We introduced GFP-Rac1 into cells together with the biosensor Alexa568-PBD. Rac1 activation results in binding of these two polypeptides leading to increased fluorescence resonance energy transfer (FRET) from GFP to Alexa568. FRET images showed high activity at the edges of cells subjected to shear stress (Figure 2A). Quantitation revealed a substantial increase in the fraction of cells that scored positive for FRET at 30 min of shear stress. While the number of positive cells decreased substantially at 120 min, the signal intensity in those cells was similar to the 30 min time point (Figure 2B). Importantly, the FRET signals were localized primarily at the downstream edges of the cells. To quantitate this effect, those cells where Rac1 activity was above the baseline were scored for spatial distribution. We found that the low level of Rac1 activity in static cells was randomly oriented relative to flow (Figure 2B). In contrast, in the majority of cells with elevated Rac1 activity after 30 min of shear stress, activity was strongly localized to the downstream edge. After 120 min, the number of cells showing elevated Rac1 activity was much lower, but in those cells where the intensity was above baseline the remaining signal was still highly oriented. Expression of the Rac1 nucleotide exchange factor Vav activated Rac1, but few cells showed polarization.

New ECM binding mediates shear stress-induced Rac1 activation

Integrins regulate Rac1 (Clark *et al.*, 1998; Price *et al.*, 1998; Del Pozo *et al.*, 2000) and shear stress causes new integrin binding to ECM (Jalali *et al.*, 2001; Tzima, 2001). Moreover, the time course of Rac1 activation (Figure 1) is consistent with it being downstream of integrin binding to ECM, which is detectable at 2 min (Tzima, 2001). To test whether Rac1 activation requires new integrin binding, ECs fully spread on fibronectin (FN) were treated with anti-FN antibodies. This treatment prevents formation of new integrin-ligand connections without disrupting existing adhesions (Jalali *et al.*, 2001; Tzima *et al.*, 2001).

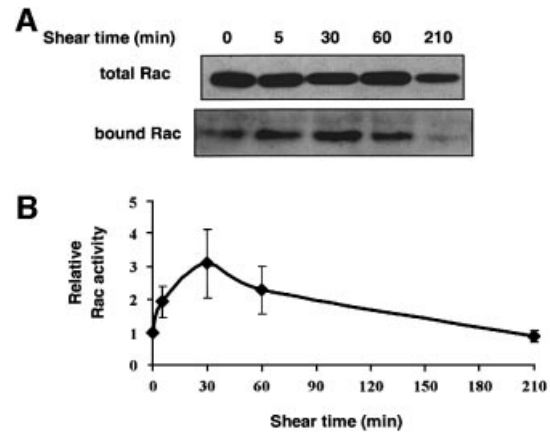


Fig. 1. Regulation of Rac activity by shear stress. (A) BAECs were subjected to shear stress for the indicated times. Rac activity is indicated by the amount of PBD-bound Rac, normalized to total Rac in whole cell lysates. (B) Quantitation of Rac activity relative to cells at time 0. Values are means \pm SEM from four independent experiments, each of which was performed in duplicate.

Shear stress was then applied and FRET assayed. The results showed that the blocking antibody 16G3 strongly inhibited the shear stress-induced increase in Rac1 activity, whereas the non-blocking antibody 11E5 had no effect (Figure 3). Interestingly, the residual FRET signal in the presence of blocking antibody showed no preferred direction. Taken together, these data demonstrate that new integrin binding to ECM determines the localized activation of Rac1 in response to shear stress.

Rac1 activation is required for shear stress-induced cell alignment

Cells exposed to shear stress for longer periods increase their stress fibers, elongate and align in the direction of flow (Galbraith *et al.*, 1998). To address the relevance of Rac1 to this process, the effect of Rac1 inhibition on stress fiber alignment in the direction of flow was examined. Cells were transiently transfected with expression vectors for GFP fused to dominant-negative (N17) or wild-type (WT) Rac1. N17Rac did not cause detectable apoptosis in these cells, as determined by caspase 3 assays (data not shown). ECs exposed to shear stress initially showed a decrease in stress fibers at 5 min, which is associated with a decrease in Rho activity (Tzima, 2001). Stress fibers returned within 1–2 h in a partially aligned state, although maximal alignment requires \sim 16 h. Transfected cells were subjected to shear stress for various times and F-actin visualized using rhodamine-phalloidin. Control cells initially showed a decrease in actin stress fibers followed by a recovery (Figure 4A). The restored stress fibers were noticeably aligned in the direction of flow at times as early as 1 h. N17Rac-expressing cells showed a similar decrease in actin staining followed by recovery; however, F-actin orientation was largely random. Even after 16 h, the orientation of actin stress fibers in cells expressing N17Rac was significantly inhibited (Figure 4B). Note that sheared cells expressing N17Rac showed increased linear stress fibers compared with unsheared cells; thus, the fraction that scores as aligned was higher than without shear, even though this fraction is only moderately above random (17%). To further test the idea that polarized Rac

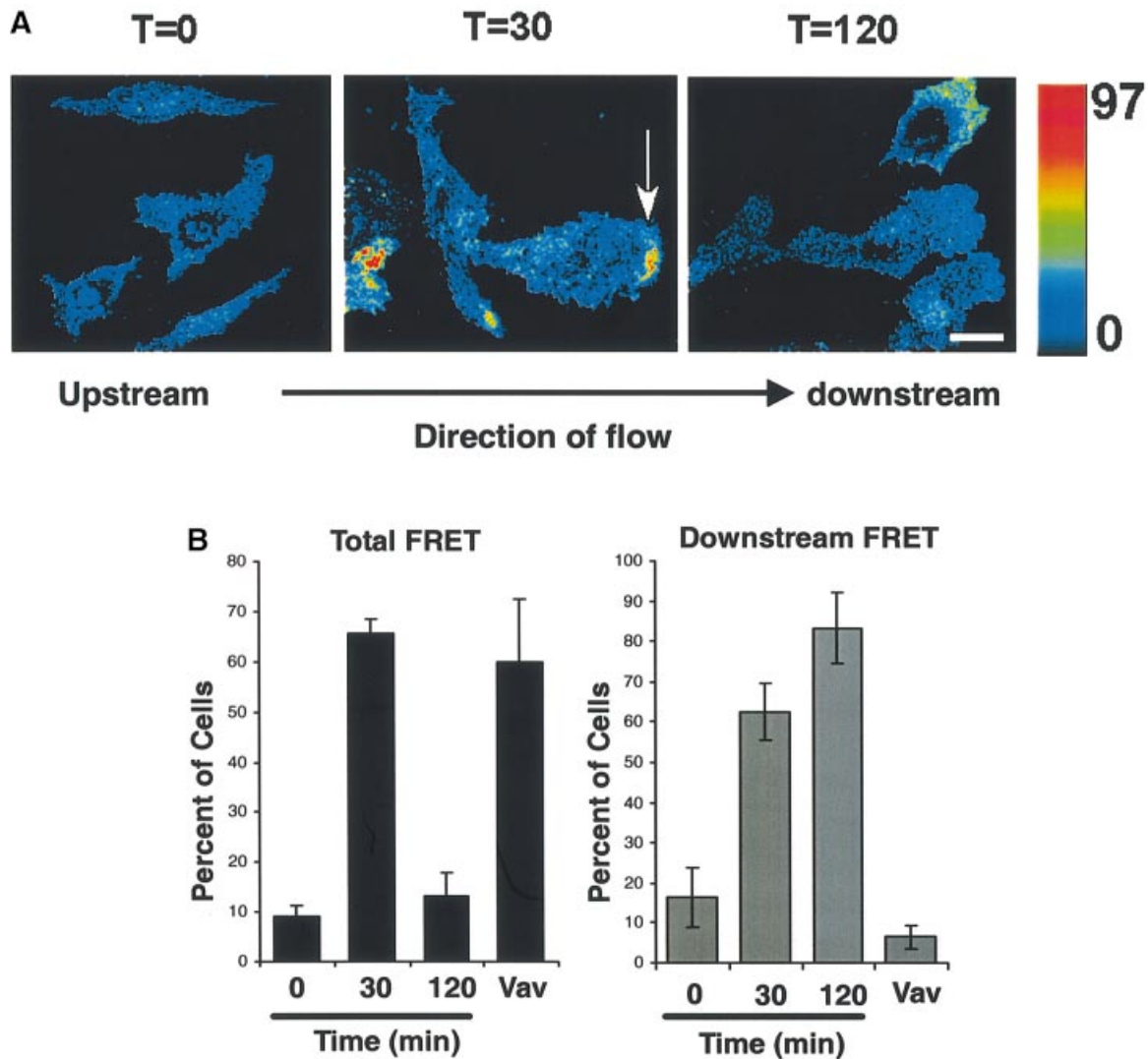


Fig. 2. Spatio-temporal localization of shear stress-induced Rac activity. (A) Cells transiently transfected with expression vectors for GFP fused to wild-type Rac1 (GFP-WTRac) were shear loaded with Alexa-PBD, plated on fibronectin for 2 h and subjected to shear stress or kept under static conditions for 30 min. Rac activation (FRET) is shown. In the color intensity scale, red represents high and blue low. Scale bar, 20 μ m. Arrow points to area of high FRET. (B) Cells were first scored for the presence of a FRET signal that was above background (left graph). Those cells showing a positive signal were then scored for whether the signal was predominantly localized to the downstream edge, i.e. flow was from left to right and the strongest FRET signals were present on the right (right graph). As a positive control, cells were transfected with the Rac1 nucleotide exchange factor Vav and examined without shear. Values are means \pm SEM, $n = 3$ and >100 cells were scored per condition.

activity may be important for cell alignment, we expressed V12Rac, which we reasoned should induce uniform high levels of Rac activity throughout the cells. FRET assays confirmed that, in cells expressing V12Rac, activity was high throughout the cell without detectable polarization toward the downstream edge (data not shown). When alignment was scored after 16 h of shear, cells transfected with V12Rac showed a significant decrease in alignment (Figure 4B). Taken together, these data show that Rac1 is not required for global changes in actin polymerization but is essential for providing the spatial information needed to align in the direction of flow.

Shear stress induces Rac1-dependent NF- κ B translocation

Shear stress leads to activation of NF- κ B (Bhullar *et al.*, 1998; Chien *et al.*, 1998; Nagel *et al.*, 1999), and there are numerous systems where NF- κ B activation is mediated

by Rac (Joyce *et al.*, 1999, 2001). To test whether shear stress-dependent NF- κ B activation requires Rac1, we investigated the localization of the p65 subunit after shear stress. Monolayers maintained under static conditions in low serum showed minimal NF- κ B activation, as documented by NF- κ B (p65) staining in the cytoplasm (Figure 5A). After exposure of cells to shear stress for 30 min, most cells exhibited nuclear localization of p65, consistent with NF- κ B activation noted in previous studies (Bhullar *et al.*, 1998). However, in cells transfected with N17Rac, flow induced minimal translocation of NF- κ B from the cytosol to the nucleus. In control experiments, expression of GFP-WTRac or GFP alone did not affect the nuclear translocation of NF- κ B. These results show that activation of NF- κ B in response to shear stress requires Rac1 activity.

To determine whether shear stress-induced NF- κ B transcriptional activity is regulated by Rac1, cells were

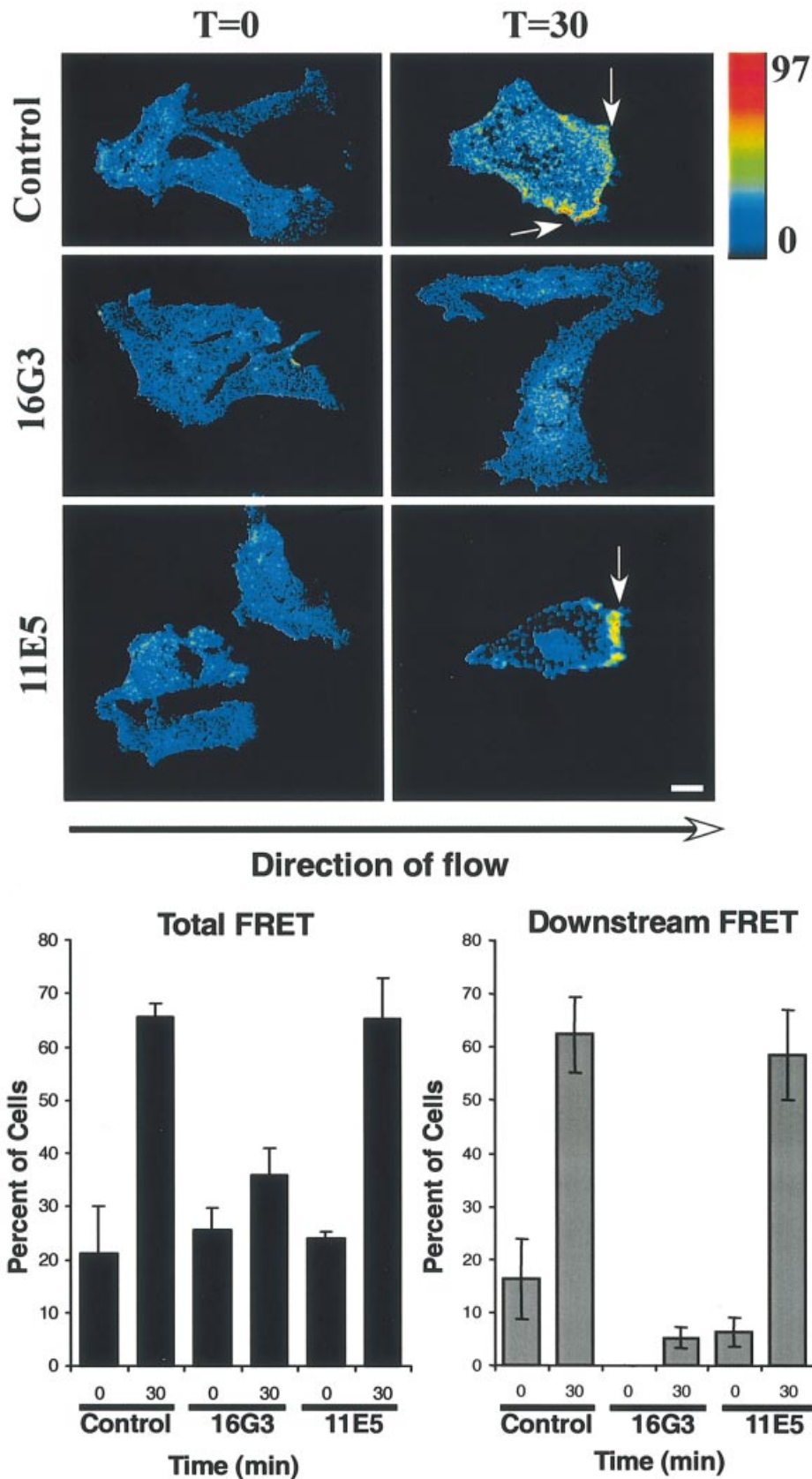


Fig. 3. Shear stress-induced Rac activation requires new ECM connections. Cells were transiently transfected with GFP-WTRac, shear loaded with Alexa-PBD and plated on FN for 2 h. Cells were incubated with anti-FN Fab fragments for 15 min and subjected to shear stress for the indicated times or kept under static conditions for an additional 30 min. Rac activation (FRET) is shown. Cells were scored for FRET-positive zones. Values are means \pm SEM, $n = 3$ and >100 cells were scored per condition. Scale bar, 20 μ m.

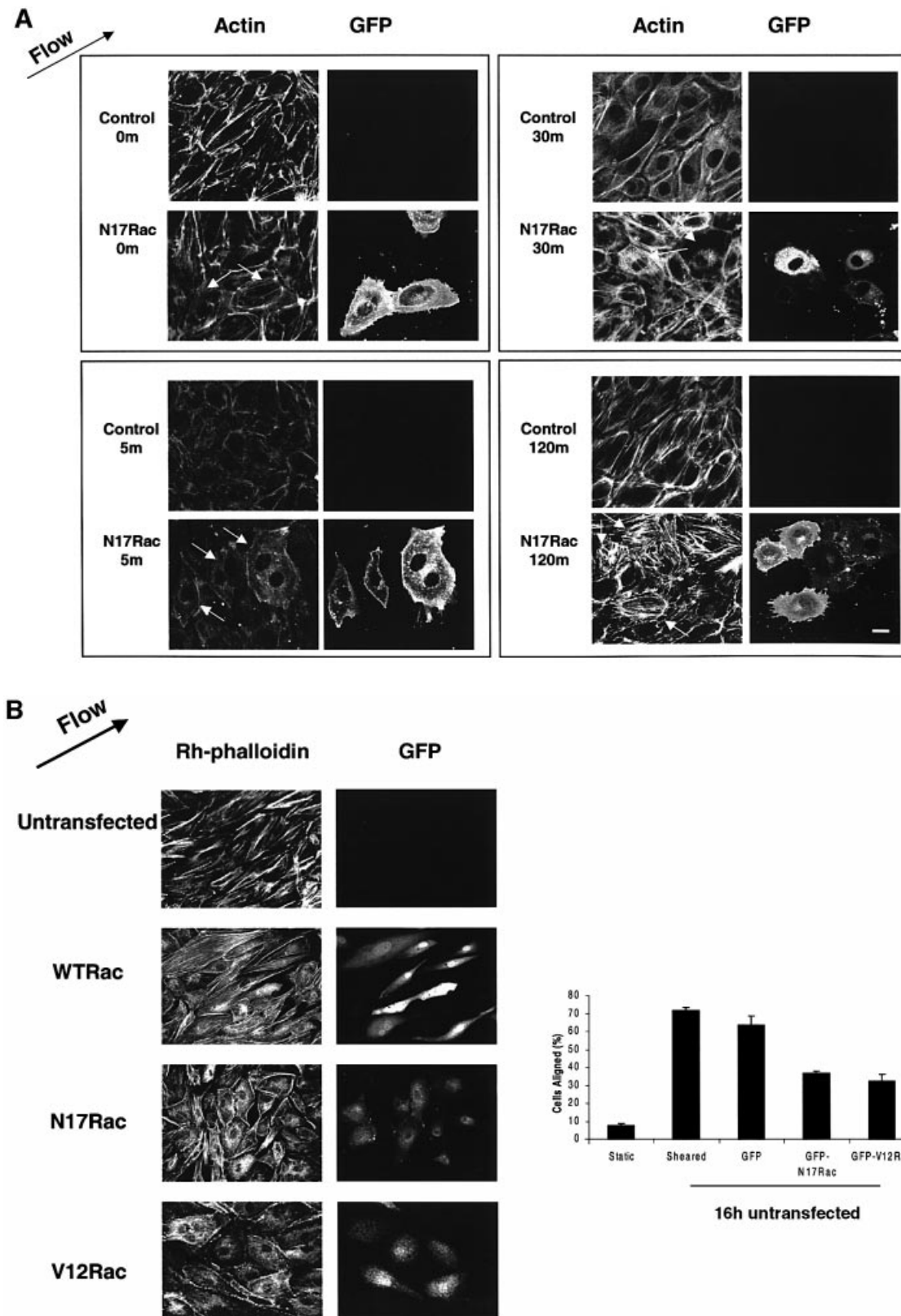


Fig. 4. Dominant-negative Rac blocks shear stress-induced cell alignment. **(A)** Untransfected cells or those transiently transfected with GFP-N17Rac were subjected to shear stress for the indicated times (min) or kept under static conditions. Cells were fixed and stained with rhodamine-phalloidin. Direction of flow is indicated by an arrow. Scale bar, 20 μ m. White arrows indicate transfected cells. **(B)** Untransfected or cells transiently transfected with GFP-WTRac, GFP-N17Rac or GFP-V12Rac were subjected to shear stress for 16 h. The cells were fixed and stained with rhodamine-phalloidin. Direction of flow is indicated by an arrow. The graph shows percentage of cells aligned in the direction of flow ($\pm 30^\circ$). Values are means \pm SEM, $n = 3$ and >100 cells were scored per condition.

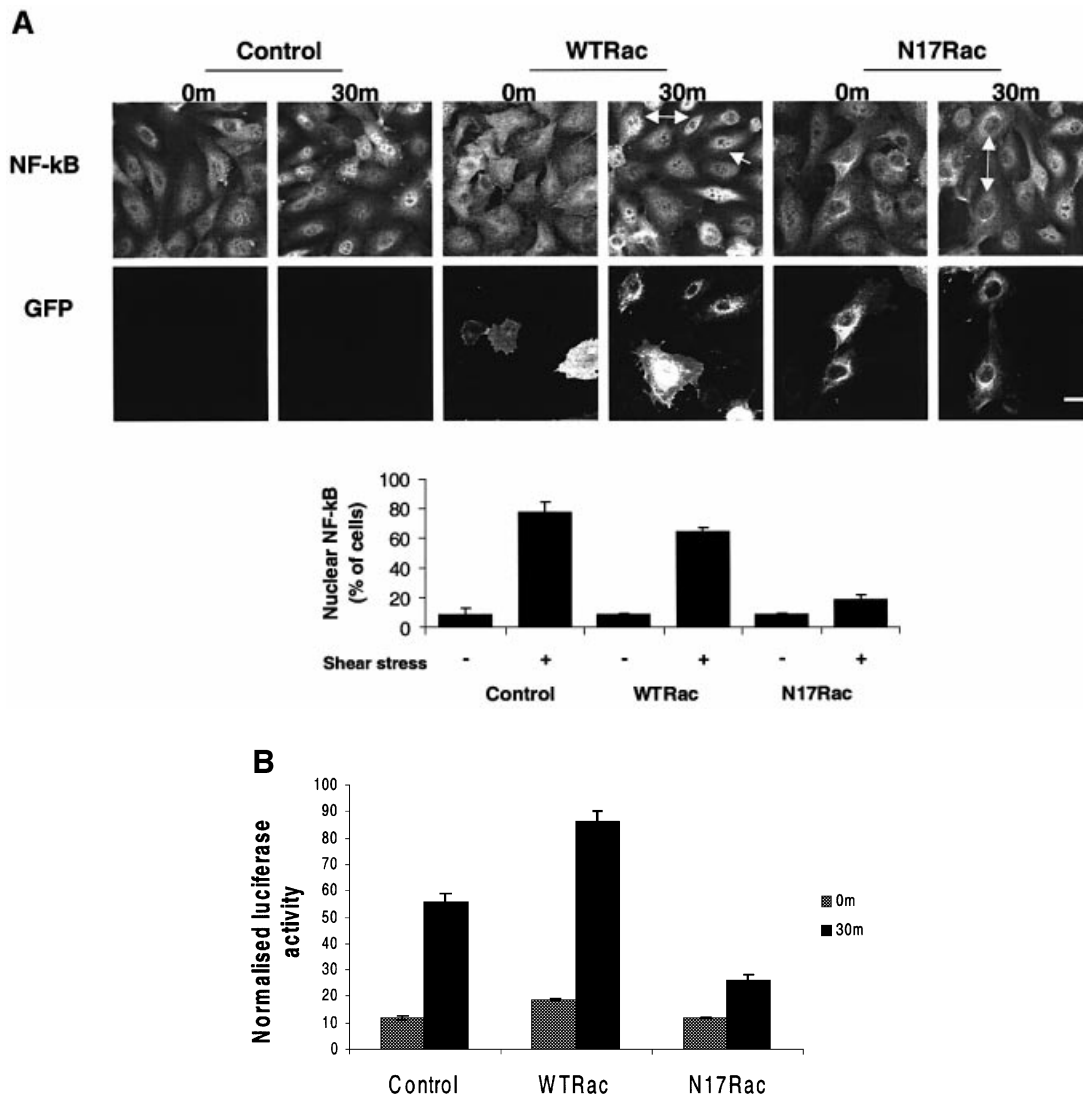


Fig. 5. Rac mediates shear stress-dependent NF- κ B activation. (A) BAECs transfected with GFP-WTRac or GFP-N17Rac were kept under static conditions or subjected to shear stress for 30 min, then fixed and stained for p65. Arrows point to transfected cells lacking nuclear p65. Nuclear translocation was scored ($n = 4$, >100 cells per condition). Values are means \pm SEM. Scale bar, 20 μ m. (B) BAECs transfected with HIV(LTR)-Luc and pSVRenilla plus WTRac or N17Rac were kept as static controls or subjected to shear stress for 30 min. Normalized luciferase activities represent means \pm SEM ($n = 3$).

co-transfected with a luciferase reporter for NF- κ B, HIV(LTR)-Luc, together with WTRac or N17Rac. In the presence of WTRac, shear stress increased luciferase activity 5-fold, which was somewhat higher than in vector-transfected cells. In contrast, much less luciferase was induced in cells co-transfected with N17Rac (Figure 5B). The increased luciferase activity due to WTRac overexpression in this assay contrasts with NF- κ B translocation where WTRac had no effect; this difference may be due to the quantitative nature of the luciferase assay, whereas scoring cells for translocation is done on an all-or-none basis.

Rac1 activation by shear stress mediates NF- κ B-dependent expression of ICAM-1

NF- κ B is responsible for the expression of several shear-responsive genes, including ICAM-1, VCAM-1 and the

macrophage chemoattractant protein 1 (MCP-1) (Landry *et al.*, 1997). To relate Rac1-dependent NF- κ B activation to these events, we addressed whether the flow-induced Rac1 activation is instrumental to the upregulation of ICAM-1 on vascular endothelium. Cells were transfected with GFP-WTRac or GFP-N17Rac and exposed to shear stress or kept as static controls. ICAM-1 was detected by immunofluorescence microscopy. Untransfected ECs or cells transfected with GFP-WTRac showed increased surface expression of ICAM-1 after 6 h of shear stress. In contrast, transfection with GFP-N17Rac clearly inhibited the shear-induced expression of ICAM-1 on the luminal surface of BAECs (Figure 6). Interleukin-1-induced upregulation of ICAM-1 was not inhibited by N17Rac (data not shown). These results demonstrate that Rac1 is required for cell surface expression of ICAM-1 in response to flow.

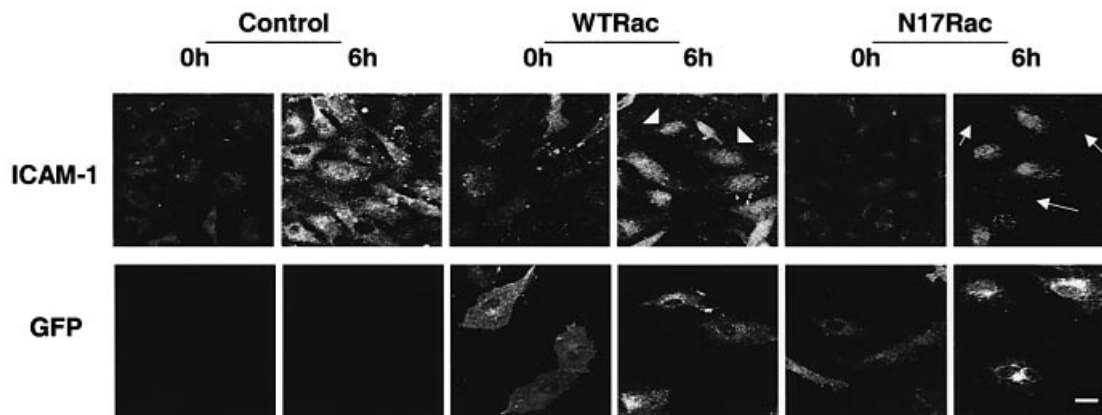


Fig. 6. Shear stress-induced Rac activation mediates NF- κ B-dependent expression of ICAM-1. Untransfected and BAECs transfected with GFP-WTRac or GFP-N17Rac were kept under static conditions or subjected to shear stress for 6 h. Cells were stained with polyclonal anti-ICAM-1 antibody followed by CY5-conjugated goat anti-rabbit IgG. Transfected cells were identified by GFP. Arrows point to transfected cells. Scale bar, 20 μ m.

Discussion

In this study, we investigated the role of Rac1 in shear stress-induced changes in cytoskeletal organization and gene expression. We report a transient upregulation of Rac1 activity by shear stress that is concentrated at the downstream edge of the cells. Similar to Rho (Tzima, 2001), regulation of Rac1 activity by shear stress was found to be due to integrin binding to ECM ligands. The prevalence of Rac1 activation in the direction of flow is of particular interest in light of the role of Rac1 in cell motility (Theriot and Mitchison, 1991; Michiels *et al.*, 1995; Nobes and Hall, 1995) and directed cell movement (Chung *et al.*, 2000). The localized rise in Rac1 activity and its requirement for new integrin–ligand connections suggest the attractive hypothesis that shear stress-induced new ECM connections determine the spatial localization of activated Rac1, which subsequently controls cell motility/morphology in a localized and directed fashion. Interestingly, Hahn and co-workers used FRET to show a gradient of Rac activity that is highest near the leading edge of migrating fibroblasts (Kraynov *et al.*, 2000). The EC membrane resists imposed shear stress by increasing tension (Fung and Liu, 1993), which has been shown to be higher at the upstream edge (Liu *et al.*, 1994). Spatial differences in shear stress (upstream versus downstream) have been detected within minutes after the application of shear stress by observing displacement of intermediate filaments (Helmke *et al.*, 2000). Microtubule remodeling due to shear stress occurs preferentially in the upstream side of the cell (Galbraith *et al.*, 1998). Likewise, shear stress increases membrane fluidity in the upstream side of cells (Butler *et al.*, 2001). Importantly, shear stress results in the remodeling of focal adhesions, with new adhesions forming preferentially toward the downstream edge of the cell (Davies *et al.*, 1994). Our data therefore suggest that the remodeling of focal adhesions originally observed by Davies and co-workers is responsible for the directional activation of Rac1.

In addition to Rac1 activity that is localized in the direction of flow, we observed that dominant-negative Rac1 inhibits cell alignment, in agreement with a recent report (Birukov *et al.*, 2002). It should be noted that Rho is also required for cell alignment in this system (Li *et al.*, 1999; Tzima, 2001), though Rho appears to be involved in the global dissolution and reformation of actin stress fibers. In contrast, Cdc42 was not found to influence actin stress fiber alignment (Li *et al.*, 1999). It is well established that Rac mediates the formation of focal complexes and lamellipodia, whereas actin stress fiber formation is dependent on Rho (Ridley and Hall, 1992; Nobes and Hall, 1994). Cell motility in fibroblasts is likely to be controlled by Rho GTPases in a sequential and coordinate manner: Cdc42 can activate Rac which then activates Rho (Nobes and Hall, 1995). Small focal complexes initiated by Rac can mature into Rho-dependent focal adhesions; thus, activation of Rho by Rac may occur in a spatially specific manner (Rottner *et al.*, 1999). Importantly, expression of N17Rac affected neither the initial dissolution nor the subsequent reformation of stress fibers during shear stress. Together with the ability of V12Rac to block alignment, these data suggest that correct spatial activation of Rac is required for the orientation of stress fibers with the direction of flow. Interestingly, although average Rac activity was much lower at later times, when evident it remained highly polarized; thus, Rac may continue to provide information about directionality at later times after initiation of flow.

Previous studies have indicated that shear stress can regulate the activities of NF- κ B, Egr-1, c-Jun and c-Fos in cultured ECs (Hsieh *et al.*, 1993; Lan *et al.*, 1994; Nagel *et al.*, 1994; Khachigian *et al.*, 1995, 1997; Resnick and Gimbrone, 1995; Shyy *et al.*, 1995b). NF- κ B can in turn regulate genes encoding vasoactive or inflammatory substances and adhesive molecules (Davies and Tripathi, 1993; Morigi *et al.*, 1995; Noris *et al.*, 1995; De Martin *et al.*, 2000). An SSRE has been identified within the promoter of ICAM-1, to which NF- κ B binds (Resnick

and Gimbrone, 1995) and which is responsible for the upregulation of ICAM-1 message by shear stress. Upregulation by shear stress of ICAM-1 is dependent on Rac1. ICAM-1 mediates leukocyte adhesion to ECs (Collins, 1993; Carlos and Harlan, 1994).

Our data define Rac1 as a major determinant of EC adaptation to shear stress and establish a pathway linking focal adhesion dynamics, cytoskeletal reorganization and gene expression in response to flow. We conclude that local modulation of integrin dynamics induces localized activation of Rac1. Rac1 subsequently provides directional information for cell alignment, a crucial adaptive process by which ECs reduce the local mechanical load and subsequent injury. In addition, Rac1, through its regulation of NF- κ B, directs the expression of genes involved in inflammation. Thus, changes in focal adhesion dynamics triggered by flow appear to mediate several important downstream responses.

The results we obtained in response to the sudden onset of flow are likely to be relevant to locations of disturbed or turbulent shear *in vivo*, where ECs initiate inflammatory reactions that lead to atherosclerotic lesions. Regions of disturbed flow have elevated levels of NF- κ B activity, increased uptake of lipoproteins, ICAM-1 expression and leukocyte recruitment as well as increased endothelial cell motility (Traub and Berk, 1998; Nagel *et al.*, 1999). We therefore hypothesize that chronic Rac1 activation is a critical factor in atherogenesis.

Materials and methods

Cell culture and shear stress

BAECs were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine (Gibco-BRL, Gaithersburg, MD) in a humidified 5% CO₂/95% air incubator at 37°C. BAECs on 38 × 76 mm slides at confluence were subjected to shear stress at 12 dynes/cm² in a parallel plate flow chamber (Frangos *et al.*, 1985; Tzima, 2001).

Plasmids and transfections

HIV(LTR)-Luc is a luciferase reporter driven by the human immunodeficiency virus long terminal repeat that contains two binding sites for NF- κ B (Nabel and Baltimore, 1987). For cytoskeletal alignment assays, BAECs at ~50% confluency in 60 mm tissue culture dishes were transfected with 2.5 μ g of GFP, GFP-N17Rac, GFP-V12Rac or GFP-WTRac (Del Pozo *et al.*, 1999) using Effectene according to the manufacturer's instructions (Qiagen, San Diego, CA). Transfections for FRET assays used 0.5 μ g of GFP-WTRac. After 10 h in growth medium, cells were starved overnight in 0.5% serum prior to shear stress.

GTPase assays

BAECs were seeded on slides coated with FN (5 μ g/cm²), grown to confluence and starved overnight in 0.5% serum. Pull-down assays for activated Rac1 were performed as described previously (Del Pozo *et al.*, 2000). FLAIR assays were performed as described previously (Del Pozo *et al.*, 2002), with slight modifications. Briefly, BAECs transiently transfected with GFP-Rac were serum starved overnight in 0.5% serum-containing media, loaded with Alexa-PBD as described previously (Clarke and McNeil, 1992), plated on FN-coated glass slides, allowed to adhere for 2 h and then subjected to shear. Images of fixed cells were acquired using a Bio-Rad 1024 Confocal Microscope. Calculations to account for bleedthrough and background were performed as described previously (Kraynov *et al.*, 2000). The resultant corrected 8-bit FRET images typically had a fluorescence intensity range of 0–78 and were displayed using pseudocolor, where blue was closest to 0 and red closest to 78. For blocking of unoccupied FN sites, the following antibodies were used as described previously (Tzima, 2001): 16G3, which blocks both the α v β 3 and α 5 β 1 binding sites for FN; and 11E5, which is a non-function blocking antibody (a generous gift from Dr K.M.Yamada).

Fluorescence microscopy

Cells were fixed for 30 min in 2% formaldehyde in PBS, permeabilized in 0.2% Triton X-100/PBS and blocked with 10% goat serum. Cells were stained with an antibody against the p65 subunit of NF- κ B (Santa Cruz Biotechnology) followed by CY5-conjugated goat anti-rabbit IgG (Sigma). Where indicated, cells were stained with TRITC-phalloidin (Sigma) to visualize F-actin or an antibody against ICAM-1, used without permeabilization. Confocal serial sectioned images were acquired using a Bio-Rad 1024 MRC Confocal Microscope.

Luciferase activity assays

HIV(LTR)-Luc, GFP-WTRac or GFP-N17Rac (2.0 μ g) were co-transfected into BAECs for the luciferase induction assay. The pSVRenilla plasmid was also co-transfected to monitor the transfection efficiency. Cells were lysed in buffer containing 1% Triton X-100. ATP and luciferin were then added to the lysate in a luminometer for measuring the total light output. The normalized luciferase activities represent the firefly luciferase activity corrected for renilla luciferase as a measure of transfection efficiency.

Acknowledgements

A special thanks to N.B.Alderson for preparing Alexa-PBD. This work was supported by PHS grants P01 HL48728 (to M.A.S.) and P01 HL64382 (to M.A.S. and S.C.). M.A.D.P. is a Leukemia & Lymphoma Society Special Fellow (3347-02).

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*Received May 28, 2002; revised October 29, 2002;
accepted October 30, 2002*