Polarized downregulation of the paxillin-p130^{CAS}-Rac1 pathway induced by shear flow

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Summary

Exposure of sparsely plated endothelial cells or a wounded monolayer to shear flow induces an instantaneous inhibition of 'upstream' lamellipodial protrusion and suppresses cell migration against the flow. This phenomenon is caused by the inhibition of Rac1 activity in the upstream lamellae, as demonstrated by fluorescence resonance energy transfer experiments, and by the capacity of constitutively active Rac1 to abolish flow-induced cell polarization. The local inactivation of Rac1 coincides with rapid dephosphorylation of paxillin and the adapter protein p130^{CAS}, which, in their phosphorylated state, participate in the activation of the Rac1 exchange factor complex DOCK180/ELMO. Indeed, overexpression of DOCK180 and ELMO rescue upstream protrusion in cells exposed to flow. Searching for the mechanosensors responsible for the polarized p130^{CAS} dephosphorylation, we discovered that shear stress stimulates the turnover and overall growth of upstream focal adhesions, whereas downstream adhesions tend to shrink. We propose that polarized, shear stress-induced signaling from focal adhesions at the upstream lamellae, leads to the local inactivation of Rac1 by inhibiting paxillin and p130^{CAS} phosphorylation, and consequently blocking the DOCK180/ELMO pathway.

Supplementary material available online at
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Key words: Shear flow, Polarity, Mechanotransduction, Focal adhesions, Rho GTPases

Introduction

Endothelial cells develop and function in a highly stressed environment. At their unique position along the inner surface of blood vessels, they are constantly exposed to blood flow, as well as to changes in hydrostatic pressure and in basement membrane dimensions. These mechanical perturbations have a profound effect on endothelial cell physiology, morphology and gene expression (Davies, 1995; Fisher et al., 2001; Resnick et al., 2003). Shear stress was shown to induce cell elongation and polarization of the cytoskeleton (Dewey et al., 1981; Eskin et al., 1984; Langille and Adamson, 1981; Nerem et al., 1981); specifically, the actin network assumes a uniform pattern of parallel stress fibers, running along the direction of flow (Franke et al., 1984; Galbraith et al., 1998; Kim et al., 1989; Noria et al., 2004). This alignment depends on intact microfilament and microtubule systems, tyrosine kinase activity and intracellular calcium ions, while being apparently independent of intermediate filaments and stretch-activated ion channels (Malek and Izumo, 1996). Several models have been proposed for endothelial cell alignment under flow (Civelekoglu-Scholey et al., 2005; Dieterich et al., 2000; Galbraith et al., 1998; Suciu et al., 1997), yet the mechanism responsible for sensing flow direction remains elusive.

Shear flow also affects endothelial cell migration. Flow running parallel to a wound introduced into an intact or cultured endothelial monolayer facilitates motility (Albuquerque et al., 2000; Vyalov et al., 1996), whereas flow applied at right angles to a wound enhances cell migration in the direction of flow and blocks migration in the opposite direction (Ando et al., 1987; Dewey et al., 1981). In sparse endothelial cell cultures, migration in the direction of flow was also observed (Li et al., 2002; Masuda and Fujiwara, 1993b; Wojciak-Stothard and Ridley, 2003). Although increased traction forces associated with elevated RhoA activity may account in part for the increased motility rates (Shiu et al., 2004), the mechanism underlying the directionality of flow-induced 'mechanotaxis' is still not clear. Specifically, are cells passively drifting, or does the flow trigger polar signaling events?

Prime regulators of cell adhesion and migration are the different Rho family GTPases (Raftopoulou and Hall, 2004; Ridley, 2001). Activated Rac1 induces actin polymerization and formation of peripheral focal complexes (Rottner et al., 1999), whereas RhoA triggers acto-myosin contractility and induces focal adhesion formation (Chrzanowska-Wodnicka and Burridge, 1996). Focal adhesions and focal complexes in turn have been shown to regulate Rho GTPases (reviewed by DeMali et al., 2003; Schwartz and Shattil, 2000). One conserved pathway linking adhesion-based signaling to Rac1 activation is the phosphorylation-dependent formation of a paxillin-crk-p130^{CAS} complex that activates a Rac1 guanine exchange factor (GEF) complex consisting of DOCK180 and ELMO (Grimsley et al., 2004; Kiyokawa et al., 1998a; Kiyokawa et al., 1998b). It was shown that Rac1 activity increases rapidly and remains elevated for at least 30 minutes

after application of flow (Hu et al., 2002; Tzima et al., 2002; Wojciak-Stothard and Ridley, 2003). This Rac1 activity was shown, by fluorescence resonance energy transfer (FRET), to be concentrated in the 'downstream' region of cells after 30 minutes of flow (Tzima et al., 2002).

The present study addresses the molecular mechanism responsible for this polarized Rac1 activity. We show here that shear flow differentially activates focal adhesion turnover and overall assembly at the upstream aspect of the cells. Concomitantly, paxillin and $p130^{CAS}$ undergo rapid dephosphorylation and, consequently, Rac1 becomes inactivated in this region of the cell.

Materials and Methods

cDNAs, antibodies and chemical reagents

YFP-actin and YFP-paxillin were constructed by re-cloning the published GFP constructs of these proteins (Choidas et al., 1998; Zamir et al., 2000) into the pEYFP vector (Clonetech, Palo-Alto, CA). Vinculin-GFP was re-cloned into pECFP (Clonetech).

Constructs encoding GFP-Rac1(L61), GFP-Rac1(N17) and GFP-RhoA(N19) were a generous gift of Michael Way (Cancer Research UK, London, UK). The FRET constructs, Raichu-Rac1 and Raichu-Rac1(N19), as well as plasmids containing DOCK180 and DOCK180-PS were generously provided by Michiyuki Matsuda (Osaka University, Osaka, Japan). GFP-Elmo-1 was kindly provided by Kodi S. Ravichandran (University of Virginia, Charlottesville, VA), the plasmid for Tiam1 was kindly provided by John G. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands), and the plasmid encoding C3-toxin was obtained from Alan Hall (University College, London, UK).

Primary antibodies used were mouse anti-PY 4G10 (Upstate Biotechnology, Charlottesville, VA); mouse anti-human vinculin (Sigma, St Louis, MO); rabbit anti-GFP (Abcam, Cambridge, UK); mouse anti-paxillin (BD Transduction Laboratories, Lexington, KY); rabbit anti-phosphorylated (pY165) p130Cas (Cell Signaling Technology, Beverly, MA); rabbit anti-phosphorylated (pY118/pY31) paxillin and rabbit anti-phosphorylated (pY397) FAK (Biosource, Camarillo, CA). Phosphospecific antibodies were used for western blotting against whole cell lysates to verify their specificity. Secondary antibodies were goat anti-mouse or anti-rabbit, conjugated to either Cy3 or Alexa 488 (Jackson ImmunoResearch Laboratories, West Grove, PA). Actin was labeled with phalloidin, conjugated to FITC or TRITC (Sigma).

H-7 [1-(5-iso-quinolinylsulfonyl)-2-methylpiperazine] was from Sigma and Y27632 was from Calbiochem (San Diego, CA).

Flow chamber apparatus

A parallel plate flow chamber (GlycoTech, Rockville, Maryland, USA) was connected to two reservoirs, each containing 25 ml warm medium. A height difference of ~20 cm between the reservoirs created a gravity-driven flow from the upper to lower reservoir. A peristaltic pump (Minipuls3; Gilson, Middleton, WI) returned medium from the lower to the upper reservoir to maintain a constant level. Cells for flow experiments were plated on 35 mm number 1 coverslips (Marienfeld Laboratory Glassware, Germany), which were held in place under the flow chamber by a vacuum pump (ABM, Germany). The shear stress τ was calculated (in dyn/cm^2) from the volumetric flow rate, using the equation $\tau=6\eta Q/a2b$, where η is the apparent viscosity of the media (taken to be 0.76 cP); a, channel height (0.025 cm); b, channel width (0.5 cm); and Q, volumetric flow rate (0.14 ml/second for 20 dyn/cm^2). A Perspex holder was custom made to hold the flow chamber steady on the microscope stage.

Cell culture, transfection and staining

Primary porcine aortic endothelial cells (kindly provided by Avrum Gotlieb, Toronto General Hospital, Toronto, Canada) were cultured in M199 medium with Earle's salts, L-glutamine and $NaHCO_3$ (Sigma) supplemented with 10% FCS and antibiotics (Biological Industries, Beit Haemek, Israel), at 37°C in a 5% CO_2 humidified atmosphere. Only early passages (4-12) were used. For wound closure experiments 4×10^5 cells were plated on 35 mm round coverslips, pre-coated with fibronectin (5 μg/ml; Sigma) for 1 hour. Wounds were introduced into the confluent monolayer using a plastic micro tip 36-48 hours after plating. For sparse culture experiments 4×10^4 cells were plated and experiments were conducted 24 hours later. Transfections (40-60 μg plasmid DNA per 10 cm plate) were conducted by electroporation using a Gene Pulser II (Bio-Rad, Hercules, CA), resulting in at least 60% plasmid-expressing cells. Transfected cells were plated directly onto flow coverslips and experiments were carried out 24-36 hours later. For staining, cells were fixed for 2 minutes in warm 3% paraformaldehyde with 0.5% Triton X-100 and then in paraformaldehyde alone for an additional 40 minutes. After fixation, cells were immunolabeled as described (Zaidel-Bar et al., 2003).

Image acquisition and live cell imaging

Phase-contrast, FRET and double color movies, as well as images of fixed cells, were taken with a DeltaVision system (Applied Precision, Issaquah, WA), consisting of an Axiovert 100TV inverted microscope (Zeiss, Oberkochen, Germany) equipped with a temperature-controlled box (Life Imaging Services, Switzerland), using Resolve3D software (Applied Precision). DMEM containing 25 mM HEPES, without Phenol Red and riboflavin (Biological Industries), supplemented with 10% FCS (Biological Industries) was used to maintain a pH of 7.04 in live cell experiments.

Image analysis and statistics

For quantification of polarity, the number and direction of protrusions was recorded. Protruding regions with a convex shape and a band of actin at their rim were taken to be active protrusions. For each cell, the number of protrusions pointing in each of four sectors (upstream, downstream, right or left) was recorded. At least 60 cells were included in the analysis of each treatment.

Kymographs were produced from time-lapse movies by spreading all time points of a two-pixel-wide line on the x-axis. This was done with a program we added to Priism software package (www.msg.ucsf.edu/IVE).

FRET of Raichu-Rac was calculated as described (Yoshizaki et al., 2003) and was quantified by measuring the mean normalized FRET value in four sample regions in the vicinity of the upstream or downstream cell edges. Quantification of phosphorylation levels of specific focal adhesion proteins was assisted by automatic segmentation of the double stained images using the 'water' algorithm (Zamir et al., 1999). Focal adhesions in the upper and lower thirds of each cell were separated by manually drawing polygons around each region. Total intensity, divided by area was used to calculate the average intensity for each individual focal adhesion and the average for a treatment was calculated from ~1000 focal adhesions from 20 different cells.

Analysis of upstream and downstream focal adhesion dynamics was carried out by recording cells expressing CFP-vinculin and YFP-actin by time-lapse photography at 2-minute intervals. Out of these movies 80 prominent vinculin-containing focal adhesions from eight different cells were chosen and categorized as 'upstream' or 'downstream' focal adhesions based on their orientation relative to the actin stress fibers. Working on a Silicon Graphics workstation model O2 (Mountain View, CA) and using the Priism software package (www.msg.ucsf.edu/IVE) we produced pixel-by-pixel ratio images of frame at $t+x$ divided by frame at t, where time point t was the first

frame in control movies and the last frame before application of flow. Manually marking polygons around focal adhesions, we calculated the area and total intensity of each focal adhesion at both time points, as well as the difference between them, i.e. the fraction of area and intensity that was present in frame *t* but not in frame *t+x* ('blue' pixels in ratio image), and the fraction of area and intensity present in frame *t+x* but not in frame *t* ('red' pixels in ratio image). Average intensity was calculated by dividing total intensity by area.

Data were regularly registered using Microsoft Excel 98 and statistical analysis and graphs were drawn in JMP IN software version 4.0.3 (SAS Institute, Cary, NC). Images were prepared for publication using Adobe Photoshop v5.0.2ME (Adobe Systems, San Jose, CA).

Results

Flow inhibits the upstream migration of endothelial cells

Introduction of an artificial 'wound' into an endothelial cell monolayer triggers rapid polarization of cells and migration into the wound. When flow is applied, perpendicular to the wound axis, cells at the 'upstream edge' of the wound, with their leading edge oriented in the direction of flow, continue to migrate into the wound at a slightly increased rate, whereas cells at the other edge, stop migrating (Fig. 1A). Careful examination of phase-contrast time-lapse movies and their kymographs, indicated that the response to flow was essentially instantaneous, shown by a complete arrest of the extension of upstream membrane protrusions (Fig. 1B). This effect was evident for as long as flow was applied (up to several hours) and new protrusions were reformed starting 1 minute after cessation of flow. The arrest of migration into the wound was shear stress-dependent in the range of 3-20 dyn/cm^2, reaching an essentially complete arrest at 20 dyn/cm^2. Cells migrating in the direction of flow increased their migration velocity by up to 25% (Fig. 1C). Visualization of the actin cytoskeleton and the associated matrix adhesions in cells at the two sides of the wound, revealed morphologies consistent with the migratory activity: at the upstream side, many large actin protrusions were observed, outlined by numerous focal complexes, whereas the edges of all cells facing the flow were dominated by large focal adhesions (Fig. 1D).

Polarization of endothelial cells under flow is attributable to inhibition of protrusion in the upstream direction

To explore the differential effect of shear stress on the upstream and downstream edges of single cells we exposed sparse cultures of porcine aortic endothelial cells (PAECs) to flow. Time-lapse movies revealed that single endothelial cells rapidly retract their upstream protrusions, and within less than 5 minutes of flow, lamellipodial protrusions are restricted to the downstream aspect of cells (Fig. 2A and supplementary material Movie 1). In some, but not all, cases, downstream migration was also observed. Fluorescent labeling for actin and vinculin showed that focal complexes are restricted to the downstream side of cells, whereas the upstream region contained large focal adhesions (see supplementary material Fig. S1). To quantify this effect we exposed sparse PAECs to flow for 5 minutes, and then fixed the cells and stained for actin (Fig. 2B). Over 80 control and flow-treated cells, from three separate experiments, were scored for the number and direction of their lamellipodial protrusions. Analysis of the data indicated that flow inhibits upstream protrusion (>90% reduction), with only marginal effect (~10% increase) on downstream protrusion (Fig. 2C). Cell polarization under flow is not unique to endothelial cells, and a similar polarization was observed in all cell lines tested, including B16 melanoma and primary human and mouse fibroblasts (Fig. 2C).

Cell polarization under flow is not due to increased RhoA activity or acto-myosin contractility

The absence of focal complexes and prominence

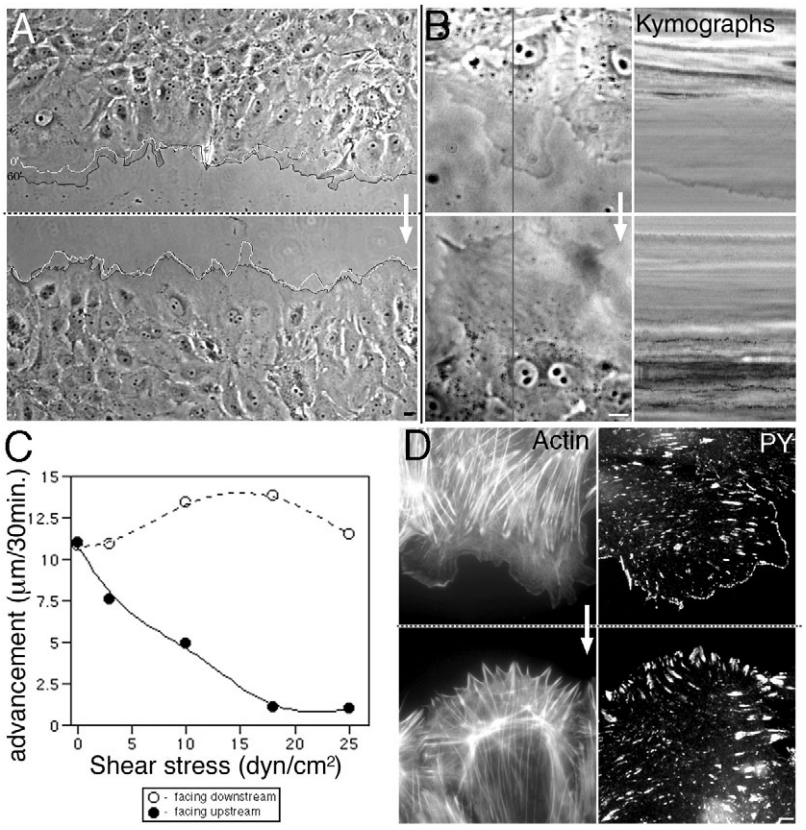
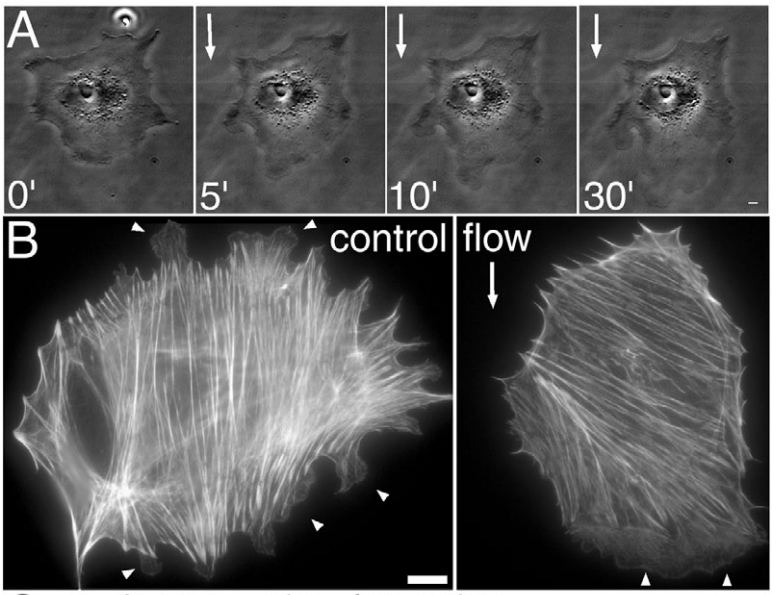


Fig. 1. Flow inhibits the upstream migration of endothelial cells in an in-vitro wound. A confluent PAEC monolayer was wounded, and 4 hours later placed under perpendicular flow (from top to bottom in all figures). Migration of cells into the wound was monitored by time-lapse phase-contrast microscopy. (A) Image of a wound, 1 hour after the application of flow (25 dyn/cm^2), with the white lines marking the contour of the front of the cell at time 0 and the black lines marking the front after 60 minutes. Cells migrating downstream advanced on average more than 15 μm, whereas cells facing upstream did not advance but retracted. (B) A kymograph of two cells, located at either side of a wound, depicting the dynamics of the leading edge at 16-second resolution over a period of 70 minutes under flow (20 dyn/cm^2). (C) The relationship between migration speed and flow rate (average values, based on 40 cells). Empty and solid circles represent cells migrating downstream and upstream, respectively. (D) Immunolabeling of cells after 30 minutes of flow (20 dyn/cm^2) for actin and phosphotyrosine (PY). Bar, 10 μm (A,B); 5 μm (D).



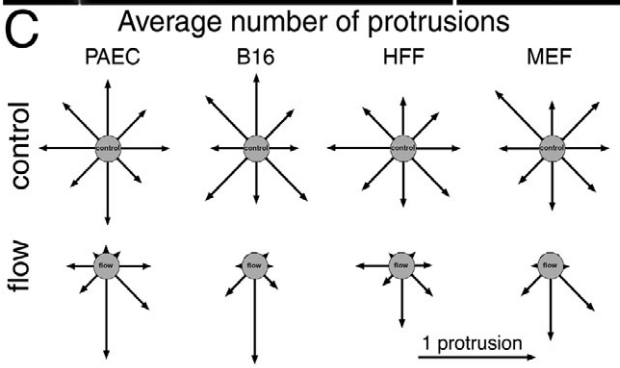


Fig. 2. Inhibition of upstream protrusion leads to polarization of single endothelial cells under flow. (A) Frames from a phase-contrast time-lapse movie of a single PAE cell exposed to flow (arrow, 20 dyn/cm^2). (B) PAE cells from cultures under stationary condition (control) or after 5 minutes of flow (arrow, 20 dyn/cm^2), stained for actin with phalloidin. Arrowheads mark protrusions. (C) Quantitative analysis of the number and direction of protrusions in control cells and cells exposed to flow. The average number of protrusions in each of eight directions is displayed. Notice that polarization under flow is primarily due to inhibition of upstream protrusions, rather than to increase in downstream protrusion. Bar, 5 μm.

of large focal adhesions at the upstream side of cells raised the possibility that local upregulation of RhoA activity and a consequent increase in cell contractility might play a role in the inhibition of protrusion. To examine this hypothesis we treated sparse cells with inhibitors of myosin light chain kinase (H7) or Rho kinase (Y27632). Both drugs inhibit intracellular tension, as evident from the destruction of stress fibers, and induce conspicuous cell spreading (Fig. 3A,B). Exposure of these cells to flow results in rapid polarization, evidenced by arrest of protrusion of the upstream lamellipodium. In the wound context, addition of either drug caused rapid advancement of the lamellae of cells at the upstream edge of the wound, whereas at the other edge cells remained stationary on the whole, only occasionally drifting downstream (Fig. 3B and supplementary material Movie 2). These experiments suggest that increased acto-myosin contractility is not responsible for the arrest of upstream protrusion. To explore the possibility that RhoA might be involved through other effectors, we overexpressed in PAECs either a dominant-negative form of RhoA (N19) or C3-toxin, an inhibitor of RhoA/B/C (Fig. 3C,D). Single cells expressing either construct showed a drastic reduction in the

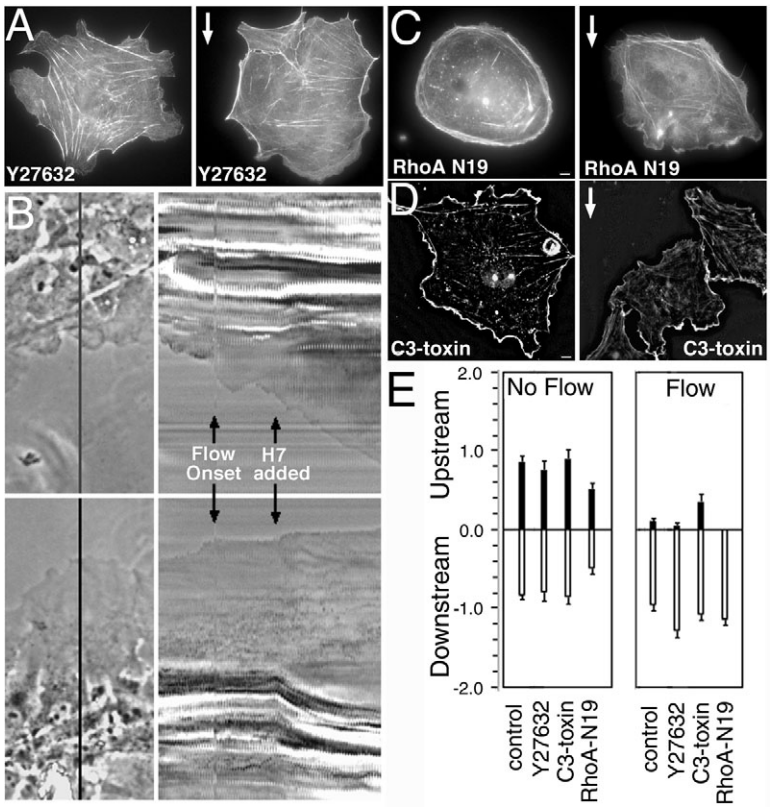


Fig. 3. Inhibition of upstream protrusion is not due to increased RhoA activity and/or acto-myosin contractility. (A) Sparse PAE cells were treated for 30 minutes with 10 μM Y27632, a ROCK inhibitor, then exposed to flow for 5 minutes before being fixed and stained for actin. Notice the reduction in stress fibers, yet maintenance of polarized protrusion under flow (panel with arrow). (B) Kymograph demonstrating the effect of the ROCK/myosin light chain kinase inhibitor H7 (300 μM) on PAE cells in a wound, exposed to flow (arrow, 18 dyn/cm^2). Notice that although the addition of H7 had a significant effect on downstream migration, it did not affect the flow-induced inhibition of upstream migration. (C,D) Sparse PAE cells expressing a dominant-negative form of RhoA (N19) (C) or C3-toxin (D) were exposed to flow for 5 minutes before being fixed and stained for actin. (E) At least 60 cells in each treatment were scored for the number and direction of protrusions. To visualize the degree of cell polarization the mean number of protrusions in the upstream or downstream directions (±45°) is presented. Error bars indicate s.e.m. Bar, 5 μm.

number of stress fibers and focal adhesions and normal peripheral membrane ruffling. However, when exposed to flow, these cells stopped ruffling at their upstream edges while continuing to produce protrusions at the downstream side. These cells failed to migrate. Quantification of the degree of polarity of cells treated with RhoA or Rho kinase inhibitors under control and flow conditions was performed by counting actin protrusions in any direction in over 60 cells and comparing the number of protrusions in the upstream and downstream (±45°) directions (Fig. 3E).

Rac1 activity is inhibited in the upstream region of endothelial cells under flow

Our observation of upstream inhibition of protrusion by flow (rather than enhancement of downstream membrane activity) raised the possibility that flow locally inhibits upstream Rac1 activity. To test this possibility we used an intramolecular FRET reporter of Rac1 activity (Yoshizaki et al., 2003). In the absence of flow, high and stable Rac1 activity was detected throughout the cell and in particular along the peripheral lamellae (Fig. 4A). Upon application of flow a rapid decrease

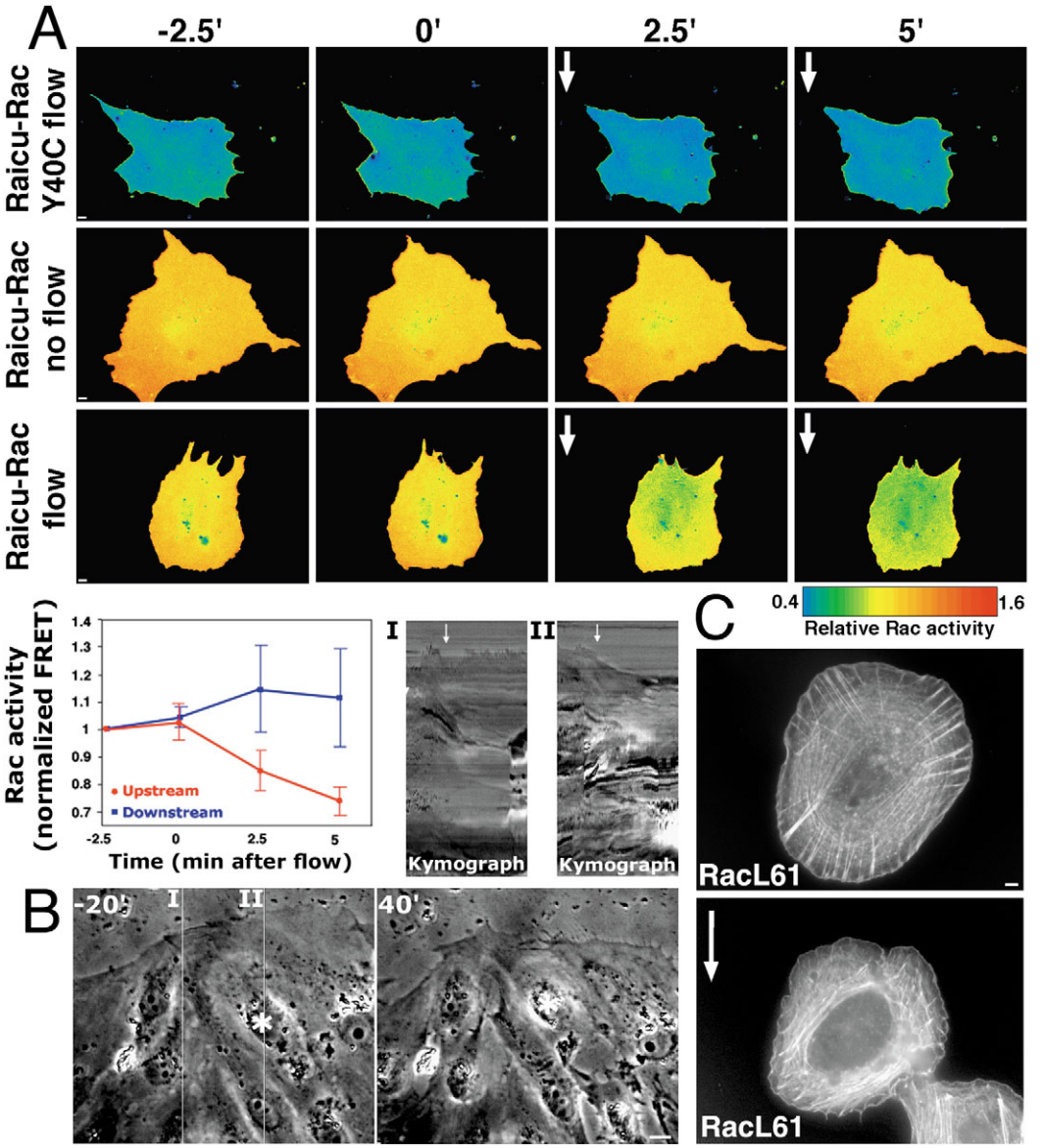


Fig. 4. Rac1 activity is inhibited in the upstream region of endothelial cells under flow. (A) Rac1 activity in live PAECs was monitored using the FRET reporter Raichu-Rac. The negative control (Raichu-RacY40C) shows stable and low FRET levels. With no flow, Raichu-Rac shows a relatively high level of activity throughout the entire cell, and specifically in the leading lamella. With the application of flow (arrow, 20 dyn/cm^2) a dramatic decrease in Rac1 activity is observed in the upstream region of the cell. Rac FRET activity levels in upstream or downstream sides of three cells, normalized to their values 2.5 minutes before flow onset, are shown in the graph. Error bars denote s.d. (B) A cell expressing constitutively active Rac1-L61 (asterisk) along the edge of a wound. Kymographs I and II correspond to the lines in the movie frame, where line I crosses the cell expressing active Rac. Arrows in the kymographs denote initiation of flow. Note that the transfected cell retracts and then advances upstream, despite the flow (20 dyn/cm^2) whereas its neighbors only retract (see also supplementary material Movie 3). (C) Single PAE cell, expressing Rac1-L61 under no flow conditions, or after five minutes of flow (arrow, 20 dyn/cm^2). Under both conditions, lamellar protrusions are formed in all directions. Bar, 10 μm (B); 5 μm (C).

in Rac1 activity, as measured by the FRET signal, was recorded at the upstream region of the cell. This polarity persisted upon long exposure to flow, and did not occur in the negative control Raichu-Rac (Y40C).

The relationship between the inactivation of Rac1 and the arrest of protrusion at the cell edge facing the flow was substantiated by showing that cells expressing a constitutively active mutant of Rac1 (the GTPase activating protein-resistant mutant, L61) do not undergo flow-induced polarization (Fig. 4B,C and supplementary material Movie 3).

The paxillin-p130^{CAS}-DOCK180/ELMO pathway is downregulated in upstream focal adhesions in response to flow

Rac1 activity can be downregulated either by increased GTPase activating protein (GAP) activity or by decreased GEF activity, and multiple GEFs and GAPs could be involved in this process (reviewed by Etienne-Manneville and Hall, 2002). A prominent Rac1 activation pathway involves the tyrosine phosphorylation of the focal adhesion proteins paxillin and p130^{CAS} (Turner, 2000). Paxillin, phosphorylated at tyrosine residues 31 and 118, binds CrkII (Petit et al., 2000), which binds p130^{CAS}, which in its phosphorylated state forms a complex with the GEF complex DOCK180 and ELMO (Kiyokawa et al., 1998b), which, in turn, can activate Rac1 (Kiyokawa et al., 1998a; Grimsley et al., 2004; Lu et al., 2005; Valle et al., 2004).

To explore the relevance of this pathway to flow-induced cell polarization we compared the phosphorylation of three focal adhesion proteins, namely paxillin, p130^{CAS} and focal adhesion kinase (FAK) in stationary PAECs and in cultures subjected to flow. All three proteins were associated with focal adhesions, irrespective of the presence or orientation of flow (Fig. 5A,B and supplementary material Fig. S2). However, upon exposure to flow, the levels of phosphorylated paxillin (pY^{118}) and p130^{CAS} (pY^{165}) decreased (by 40-50%) at the upstream aspects of the cells and increased (by 15-30%) at the opposite side. The phosphorylation of FAK (pY^{397}), on the other hand, nearly doubled on both sides of the cells following exposure to flow (Fig. 5B). Dephosphorylated p130^{Cas} was reported to dissociate from focal adhesions (Fonseca et al., 2004), which

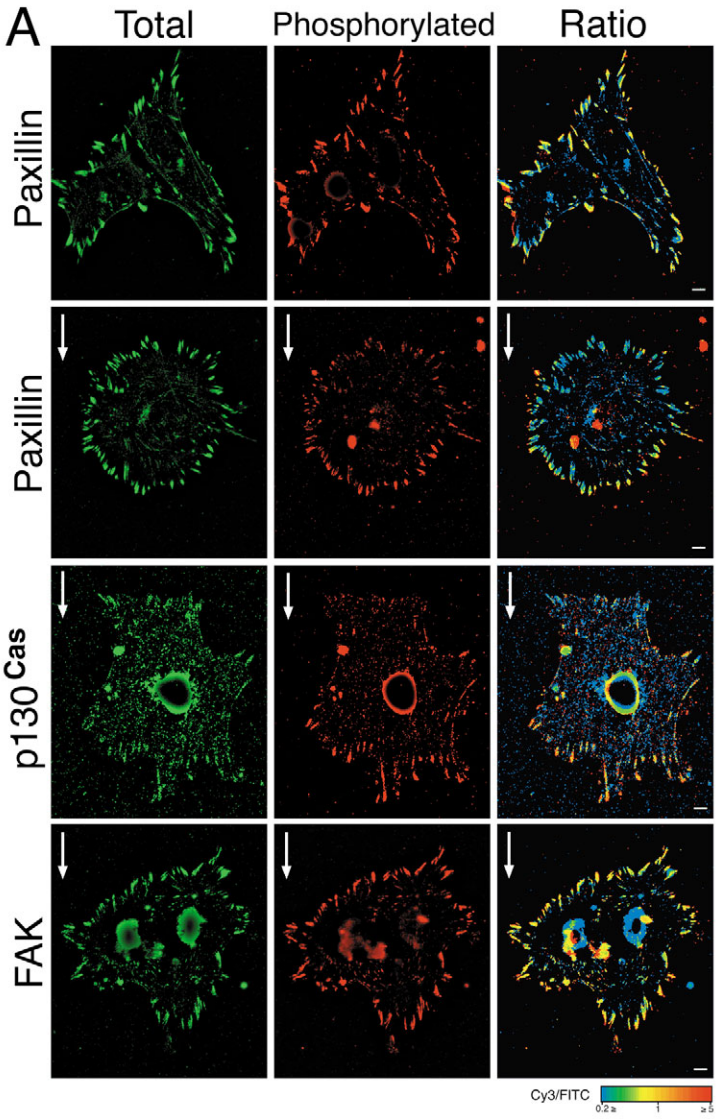


Fig. 5. p130^{CAS} and Paxillin are dephosphorylated in upstream focal adhesions in response to flow. (A) Sparse PAECs were exposed to shear flow for five minutes (arrow, 20 dyn/cm^2), and then immediately fixed and double stained for paxillin, p130^{CAS} and FAK (left column) and the corresponding phosphorylated forms, namely, pY118, pY165 and pY397, respectively (middle column). Ratio images (right column) highlight the relative levels of the phosphorylated proteins compared to total protein. Images of p130^{CAS} and FAK under no flow conditions can be seen in supplementary material Fig. S2. (B) Quantification of the average intensity of total and phosphorylated forms of paxillin, p130^{CAS} and FAK in upstream and downstream focal adhesions was calculated for more than a thousand focal adhesions from 20 different cells, under no flow conditions or after 15 minutes of flow. The average intensity after 15 minutes of flow, normalized by the intensity under no flow conditions, is presented. Bars indicate s.e.m. Bar, 5 μm.

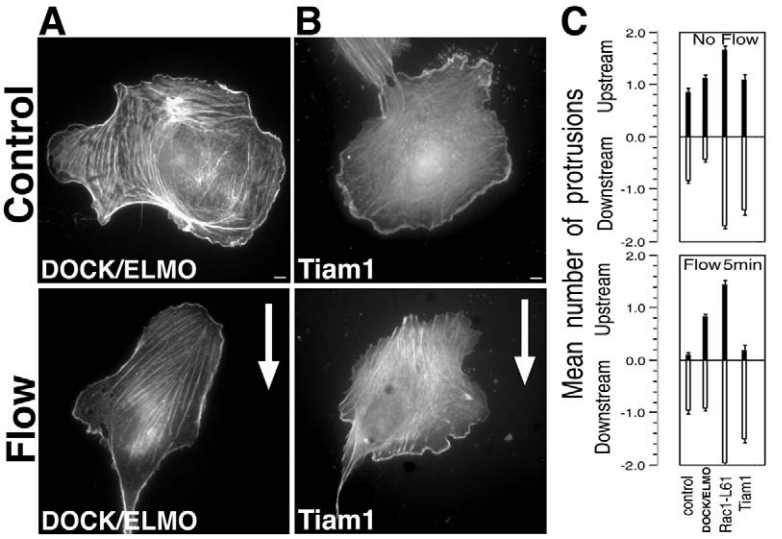


Fig. 6. DOCK180/ELMO overexpression can overcome flow-induced cell polarization, whereas Tiam1 overexpression does not. Single PAE cells overexpressing both subunits of the Rac1 GEF DOCK180/ELMO (A) or Tiam1 (B) were placed under control or flow conditions (arrow, 20 dyn/cm^2) for 10 minutes before being fixed and stained for actin. Notice the large upstream lamellipodium in the DOCK/ELMO expressing cell. (C) Quantification of degree of polarity for these cells, as well as cells expressing active Rac1 was performed as described in Fig. 3. Bar, 5 μm.

might account for the ~25% decrease in p130^{CAS} levels in upstream focal adhesions. The levels of FAK and paxillin in focal adhesions were not affected by flow.

DOCK180 expression in PAECs was verified by western blotting (data not shown). To demonstrate that signaling through DOCK180 is a viable pathway in these cells we expressed full-length DOCK180 and a dominant-negative deletion mutant of DOCK180 (DOCK-PS), which lacks the CrkII binding site (Kiyokawa et al., 1998b) and has a truncated 'docker' domain (Lu et al., 2005). DOCK-PS complexes with ELMO, but cannot bind or activate Rac1, and its expression was shown to inhibit NBT-II cell migration (Valle et al., 2004). Expression of DOCK-PS in PAECs significantly reduced the protrusive activity of sparse cells in comparison to expression of GFP alone, whereas expression of full-length DOCK180 dramatically enhanced lamellae activity (supplementary material Movie 4). To further establish the involvement of this particular pathway in flow-induced polarization of the cells, we overexpressed DOCK180 together with ELMO (in equal amounts) in cells before exposing them to flow. The protrusions induced by overexpression of DOCK180/ELMO persisted even under the presence of flow (Fig. 6A,C). In contrast, overexpressing the Rac1 GEF Tiam1 increased overall protrusions under control conditions, yet upstream protrusions stimulated by Tiam1 were suppressed under flow conditions (Fig. 6B,C).

Shear stress induces high turnover and growth of upstream focal adhesions and reduction of downstream focal adhesion area

The differential effect of shear stress on the phosphorylation of p130^{CAS} and paxillin in upstream and downstream focal adhesions raised the possibility that these adhesion sites respond to flow differently in these two regions of the cell, thus providing a link between mechanosensing and polar signaling. To explore this idea, we monitored focal adhesion dynamics in PAECs transfected with CFP-vinculin by time-lapse video microscopy before and after application of flow (Fig. 7A). For quantitative analysis we randomly selected over 80 'upstream' and 'downstream' focal adhesions from eight different cells, and calculated the gain or loss of area and intensity at every time point for each focal adhesion. Under control conditions focal adhesions appear to be quite dynamic (typically, 3-4% area change per minute), however they maintain their total area quite constant. The application of flow induced a dramatic increase in both assembly and disassembly rates in upstream focal adhesions, yet their growth rate exceeded the rate of

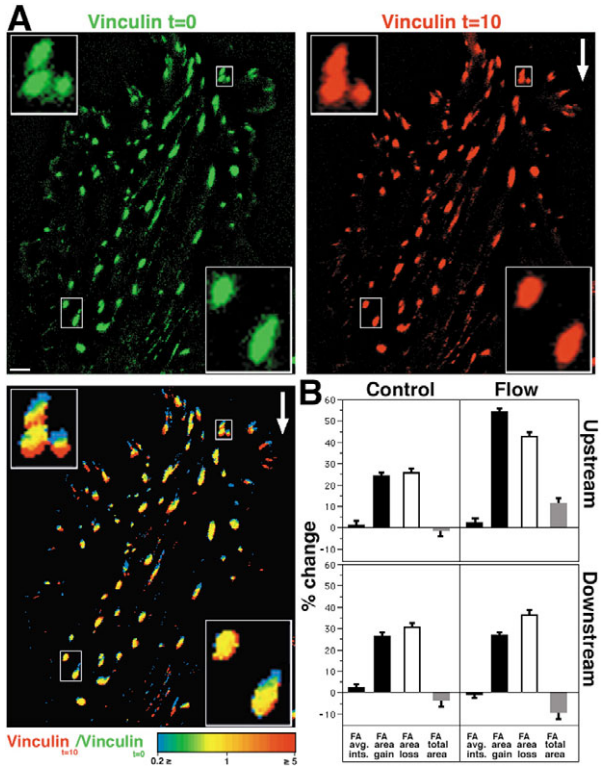


Fig. 7. Shear stress induces growth of upstream focal adhesions and reduction of downstream focal adhesion area. PAECs expressing CFP-vinculin were monitored at 2-minute intervals before and after application of flow (arrow, 20 dyn/cm^2). (A) Frames from such a movie, before and after 10 minutes of flow, and a ratio image of the two time points. In this ratio image, extension of focal adhesions appears red whereas loss of focal adhesion area appears blue. (B) Gain and loss of focal adhesion area are quantified by examination of a large number of focal adhesions (80 for flow, 60 for no flow controls). For each focal adhesion the area and intensity at two time points, 6-9 minutes apart were compared. Here, the average area 'lost' or 'gained' is presented, normalized to the area of the focal adhesion at time 0, in addition to the change in average intensity. Error bars indicate s.e.m. Bar, 5 μm.

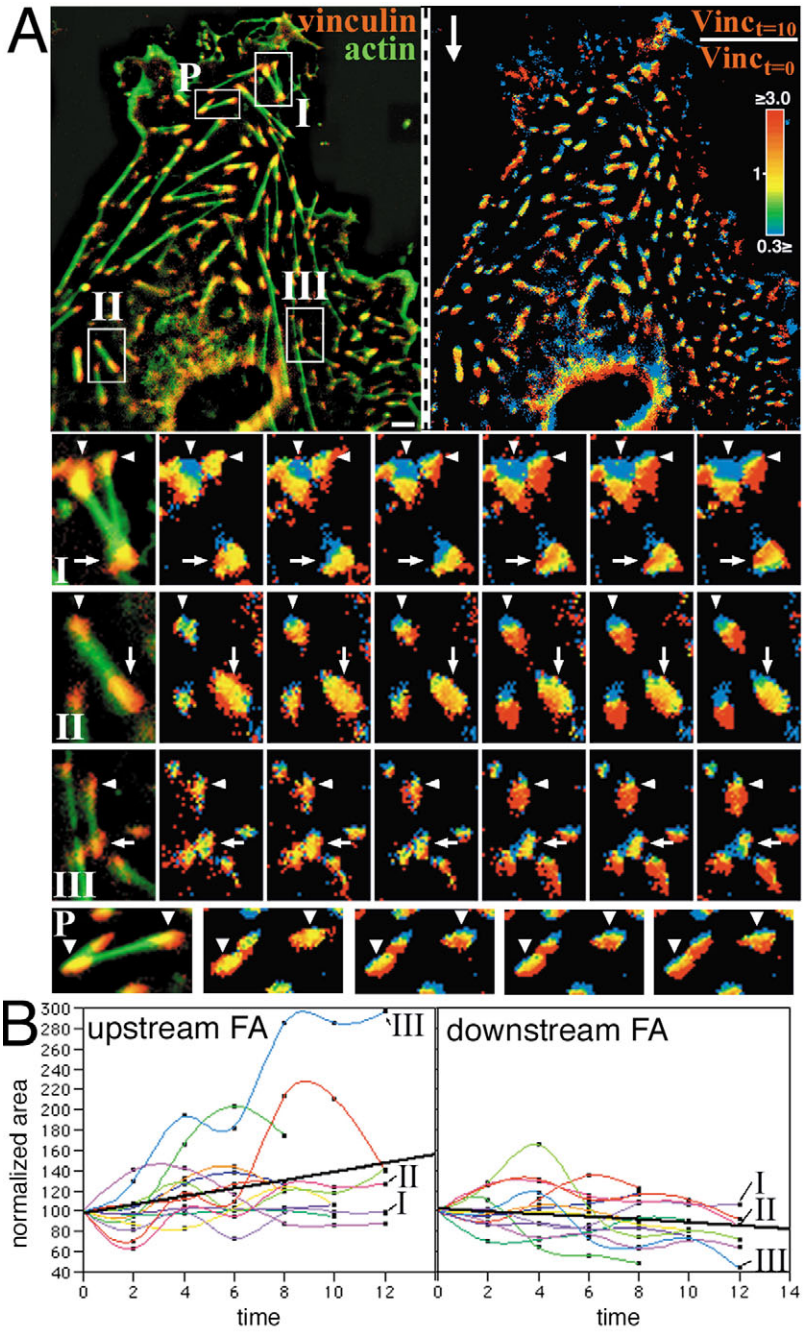


Fig. 8. Differential effect of flow on focal adhesion dynamics depends on focal adhesion orientation and not on their subcellular localization. PAECs expressing both CFP-vinculin and YFP-actin, were monitored, at two-minute intervals, before, and after application of flow (arrow, 20 dyn/cm^2). (A) The last frame before application of flow is shown at the top left. To visualize flow-induced changes in focal adhesion structure, ratio images were generated for vinculin by dividing frame *t*+X by frame *t* (top right). For three vertical focal adhesion pairs (roman numbers) plus one perpendicular pair (P), a series of ratio images (2 minutes apart) is shown. Arrows and arrowheads indicate the upstream and downstream focal adhesions, respectively. (B) The change in area of each focal adhesion, as a function of time under flow (normalized to pre-flow values) is shown for the three pairs in A along with another nine pairs from other cells. The same color is used in both graphs to identify paired focal adhesions. Notice that the overall trend (marked by a black line) is growth of upstream focal adhesions and reduction is size of downstream focal adhesions. Bar, 5 μm.

disassembly resulting in a net increase of ~10% in focal adhesion area within 10 minutes (Fig. 7B). Notably, the expansion of focal adhesions always occurred in the direction of the cell center whereas the loss of area was observed at the other end of focal adhesions (Fig. 7A). In downstream focal adhesions, there was only an increase in turnover, resulting in a ~10% net decrease in size under flow conditions (Fig. 7B).

Effect of shear stress on focal adhesions depends on their orientation rather than their subcellular localization

Forces applied to focal adhesions by the associated stress fibers and by shear stress are both highly directional. We thus reasoned that the effect of flow on the overall tension applied at a focal adhesion depends on the relative orientations of the two forces. Thus, flow oriented in the same direction as the stress fiber pulling will increase local tension, whereas flow in the opposite direction will reduce it, irrespective of where in the cell the particular focal adhesion is located. To test this hypothesis, we co-transfected PAECs with CFP-vinculin and YFP-actin and examined the flow-induced effects in pairs of focal adhesions, connected by a single stress fiber (Fig. 8A). We selected 13 such pairs, located in different regions of the cell, and analyzed them by temporal ratio imaging. In addition, we calculated the change in area of each focal adhesion as a function of time under flow, normalized to pre-flow area values (Fig. 8B). This analysis indicated that the effect of flow on focal adhesion dynamics is primarily determined by their orientation relative to the flow, rather than by their position within the cell. Thus, focal adhesions at the upstream end of a stress fiber grow in size by elongating in the direction of flow, whereas focal adhesions at the other end of the same stress fiber either remain unchanged or decrease in size by shrinking at their upstream side (Fig. 8A,B). In pairs of focal adhesions with stress fibers perpendicular to the direction of flow we observed increased assembly on the downstream side (‘widening’) of the adhesion sites, equally in both focal adhesions (Fig. 8A, insert P).

Discussion

It has long been known that endothelial cells can respond to shear stress by changing their shape and reorganizing their cytoskeleton, both in a confluent monolayer context (Dewey et al., 1981; Franke et al., 1984; Galbraith et al., 1998; Goode et al., 1977; Kim et al., 1989) and as individual cells (Li et al., 2002; Masuda and Fujiwara, 1993a; Masuda and Fujiwara, 1993b). However, despite the wide interest in this phenomenon, the

mechanism underlying this response and the nature of the 'shear stress mechanosensor' are still largely unknown. An emerging paradigm is that flow deforms the apical cell surface, producing force that is transmitted to different regions of the cell where mechanotransduction takes place (Davies, 1995; Davies et al., 2003). According to this 'decentralized' model, stress-induced signaling is triggered in regions connected to the cytoskeleton, such as cell-cell junctions, cell-matrix adhesion sites, the nuclear envelope and the apical membrane. Candidate molecules, suggested to be involved in such responses include PECAM-1 (Fujiwara et al., 2001), integrins (Shyy and Chien, 2002) and mechanosensitive ion channels (Barakat, 1999; Fisher et al., 2001).

Here we concentrated on early flow-induced polarization of actin-based protrusion of the lamellipodium, as manifest in wounded monolayers and in single cells. In an attempt to define the molecular mechanism underlying this response we first considered the possibility that flow directly blocks protrusion by applying pressure to the tip of the lamellipodium. However, the pressure generated by actin polymerization was calculated to be in the range of 5-15 kPa (Abraham et al., 1999), whereas the local pressure exerted by the flow applied in our experiments was three orders of magnitude weaker (~2 Pa). Thus, it seems highly unlikely that flow mechanically blocks protrusion at the leading edge, and hence it appears conceivable that Stokes forces and shear stress, exerted by flow, induce localized signaling events, despite their largely 'global' effects on the cells.

In this paper we show that polarized Rac1 signaling plays a critical role in the cellular response to flow. This is supported by the capacity of overexpression of constitutively active Rac1 to overcome the polarized suppression of protrusion, and by the reduction in active Rac1 levels at the cell margin facing the flow. Flow-induced Rac1 activation has been reported (Tzima et al., 2002), and it was proposed that Rac1 is activated at the downstream region of the cells, driving a downstream cell migration. Our experiments, however, indicate that this polarity is primarily attributable to inhibition of Rac1 activity at the upstream region. This notion is supported by the finding that after 5 minutes of flow, the mean number of downstream protrusions increases by only 10% whereas upstream protrusion is inhibited by ~90%, compared to levels in the control.

The central question addressed in this study concerns the molecular nature of the 'mechanosensor' and signaling systems activated by flow. Prime candidate sites for such 'force-sensing' are focal adhesions. This notion is based on several considerations: (1) focal adhesions are mechanosensitive organelles that grow upon application of force (Bershadsky et al., 2003; Geiger and Bershadsky, 2001; Katsumi et al., 2004); (2) focal adhesion organization is altered by shear flow (Davies et al., 1994; Girard and Nerem, 1995); (3) integrin-mediated signaling promotes flow-mediated effects (Jalali et al., 2001; Muller et al., 1997; Tzima et al., 2001); (4) focal adhesions are asymmetric structures subjected to unidirectional force applied by the attached stress fiber. It is this last feature of focal adhesions that affords them the capacity to respond differentially to flow from different directions.

Indeed, the application of shear stress to cells exerted a highly polarized effect on focal adhesions, manifest by an increase in turnover and net growth of focal adhesions pointing upstream, and decrease in the size of focal adhesions oriented in the opposite direction, relative to the flow. Accompanying the shear-induced increase in upstream focal adhesion dynamics was a major dephosphorylation of paxillin and $p130^{CAS}$. This might seem surprising in light of the literature of flow-induced phosphorylation of various signaling proteins, such as VEGFR, PECAM-1, Src, Pyk2, FAK and even $p130^{CAS}$ (Chen et al., 1999; Fujiwara et al., 2001; Li et al., 1997; Okuda et al., 1999). However, all these studies relied primarily on biochemical analysis of cell lysates, and therefore they were unable to identify differences in the subcellular distribution of the phosphorylated molecules. The increased phosphorylation of paxillin and $p130^{CAS}$ we observed in downstream adhesions might be responsible for the increase in migration rate in the downstream direction by upregulating DOCK/ELMO and hence Rac1 activity in the downstream region.

Integrin engagement, during spreading and at the leading edge, induces paxillin and $p130^{CAS}$ phosphorylation (Panetti, 2002), however force-dependent transition of focal complexes to focal adhesions involves a major decrease in overall phosphotyrosine levels (Zaidel-Bar et al., 2003). Our results differ from those of Tamada and colleagues who recently reported that stretching of triton cytoskeletons resulted in recruitment and phosphorylation of $p130^{CAS}$ and activation of Rap1 (Tamada et al., 2004). Although our cellular system is very different, it would be of interest to check the involvement of Rap1 in flow-induced cell polarity. We observed a striking correlation between paxillin phosphorylation levels and focal adhesion dynamics. Namely, a decrease in phosphorylation correlated with an increase in focal adhesion area. This is in line with a published report that focal adhesion turnover decreases in cells expressing a non-phosphorylatable mutant of paxillin (Webb et al., 2004). The levels of phosphorylation of paxillin and $p130^{CAS}$ could be regulated, in principle, by an increase in the activity of phosphatases such as PTP-PEST, which is known to dephosphorylate these two proteins (Garton et al., 1996; Shen et al., 1998) and was shown to inhibit Rac1 (Sastry et al., 2002), or SHP2, which was shown to affect focal adhesion dynamics (von Wichert et al., 2003). Alternatively, increased stress at focal adhesions might lead to a reduction of the activity of kinases, such as Src. Preliminary experiments indicated that flow-induced cell polarization occurred in RPTPα-null cells; cells expressing dominant-negative PTP-PEST and with Src- and FAK-null mouse embryonic fibroblasts, suggesting that these phosphatases and kinases do not play a critical role in the arrest of protrusion in the upstream lamellae.

The identity of the putative phosphatase or kinase remains to be discovered, however our work suggests that this dephosphorylation inhibits the formation of a complex consisting of paxillin-Crk-$p130^{CAS}$-DOCK180/ELMO, and the absence of this active complex accounts for the local inhibition of Rac1 activity in the upstream lamella. This is supported by the capacity of DOCK/ELMO overexpression to specifically reverse the effect of flow on membrane protrusion. Another GEF of Rac1 (namely, tiam1) did not exert such an effect. It is noteworthy that increased tension at focal adhesions was shown to inhibit Rac1 and suppress lamellar activity in a different experimental setting, following equibiaxial stretching of a flexible matrix (Katsumi et al., 2002). The data presented

in this study support the ability of such mechanical stimulation to induce highly orientation-specific reorganization of the adhesive machinery of the cells. We further show here that this process involves a mechanically induced downregulation of the paxillin-Crk-p130^{CAS}-DOCK180/ELMO-Rac1 pathway, leading to the local inhibition of Rac1.

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