Retrograde Fluxes of Focal Adhesion Proteins in Response to Cell Migration and Mechanical Signals[□] [▽]

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Recent studies suggest that mechanical signals mediated by the extracellular matrix play an essential role in various physiological and pathological processes; yet, how cells respond to mechanical stimuli remains elusive. Using live cell fluorescence imaging, we found that actin filaments, in association with a number of focal adhesion proteins, including zyxin and vasodilator-stimulated phosphoprotein, undergo retrograde fluxes at focal adhesions in the lamella region. This flux is inversely related to cell migration, such that it is amplified in fibroblasts immobilized on micropatterned islands. In addition, the flux is regulated by mechanical signals, including stretching forces applied to flexible substrates and substrate stiffness. Conditions favoring the flux share the common feature of causing large retrograde displacements of the interior actin cytoskeleton relative to the substrate anchorage site, which may function as a switch translating mechanical input into chemical signals, such as tyrosine phosphorylation. In turn, the stimulation of actin flux at focal adhesions may function as part of a feedback mechanism, regulating structural assembly and force production in relation to cell migration and mechanical load. The retrograde transport of associated focal adhesion proteins may play additional roles in delivering signals from focal adhesions to the interior of the cell.

INTRODUCTION

Recent studies suggest that mechanosensing is involved in several physiological processes, including embryogenesis (Newman and Comper, 1990; Beloussov *et al.*, 1994) and wound healing (Hinz *et al.*, 2001; Tomasek *et al.*, 2002), and in pathological processes such as fibrosis and carcinogenesis (Paszek *et al.*, 2005). Adherent cells seem capable of both responding to applied mechanical forces (Tzima *et al.*, 2005) and applying contractile forces to probe mechanical properties of the environment (Discher *et al.*, 2005). The downstream responses include changes in migration (Pelham and Wang, 1997; Sheetz *et al.*, 1998; Lo *et al.*, 2000), cell–cell interactions (Guo *et al.*, 2006), proliferation (Wang *et al.*, 2000; Nelson *et al.*, 2005), differentiation (Engler *et al.*, 2004, 2006), and apoptosis (Wang *et al.*, 2000). For cultured adherent cells, focal adhesions are thought to mediate mechanosensing through integrin-mediated anchorage to the extracellular matrix (Larsen *et al.*, 2006). The molecular repertoire of focal adhesions includes >100 adaptor and signaling proteins (Bershadsky *et al.*, 2006), in addition to associated actomyosin bundles that provide mechanical forces for probing the environment (Choquet *et al.*, 1997), inside-out signaling (Chrzanowska-Wodnicka and Burridge, 1996; Schwartz and Ginsberg, 2002), and cell migration (Ridley *et al.*, 2003). However, few details are available concerning

how these components work in concert to generate the responses to mechanical signals.

Several aspects have emerged recently as the key features of integrin-mediated mechanosensing. First is the increase in cortical organization and contractility in response to mechanical signals. Application of mechanical forces to integrin-associated beads causes “reinforcement” of cortical resistance (Wang *et al.*, 1993; Choquet *et al.*, 1997), whereas pushing the dorsal cortex or pulling the focal adhesion causes enlargement of focal adhesions (Riveline *et al.*, 2001). In addition, substrate rigidity induces the appearance of large focal adhesions, prominent stress fibers, and strong traction forces (Pelham and Wang, 1997; Discher *et al.*, 2005). Such responses are often accompanied by the induction or enhancement of centripetal cortical movements, which may manifest as flow of actin arcs in lamella or retrograde movements of integrin-associated beads (Felsenfeld *et al.*, 1996). Chemical signals involved in these processes include the activation of small GTPases, including Rho and Rac (Chrzanowska-Wodnicka and Burridge, 1996), and tyrosine kinases, including Src family kinases (Suter and Forscher, 2001) and focal adhesion kinase (FAK; Burridge *et al.*, 1992; Schmidt *et al.*, 1998), as well as the entry of Ca²⁺ through stretch-activated ion channels (Guharay and Sachs 1984; Lee *et al.*, 1999; Munevar *et al.*, 2004).

A more intriguing response is the redistribution of some focal adhesion proteins upon mechanical stimulations. Several proteins, including zyxin and vasodilator-stimulated phosphoprotein (VASP), are known to localize to a variable extent at focal adhesions (Crawford *et al.*, 1992; Rottner *et al.*, 2001), or to shuttle between focal adhesions and the nucleus (Nix and Beckerle, 1997; Aplin and Juliano, 2001). Recent studies further indicated that mechanical stimulation can induce the change in distribution of zyxin from focal adhesions to either actin stress fibers in cultured fibroblasts (Yoshigi *et al.*, 2005), or the nucleus of vascular smooth muscle cells (Cattaruzza *et al.*, 2004). The latter may be related to the

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Abbreviations used: FAK, focal adhesion kinase; FRAP, fluorescence recovery after photobleaching; VASP, vasodilator-stimulated phosphoprotein.

suspected ability of zyxin to regulate gene expression (Degenhardt and Silverstein, 2001). However, the mechanism of redistribution from focal adhesions is unclear, because these studies showed only the steady-state localization after mechanical stimulation.

To reach a better understanding of mechanosensing, we have examined the dynamics of actin and several focal adhesion proteins suggested to play a role in mechanosensing, by combining live cell imaging, fluorescence recovery after photobleaching (FRAP), substrate engineering, and mechanical stimulation. We show that actin filaments and some focal adhesion proteins, including zyxin and VASP undergo retrograde fluxes in response to the state of cell migration and mechanical signals. The common aspect of conditions that stimulate the flux lead us to propose a mechanism that controls structural assembly at focal adhesions based on migration- or force-induced structural shear within the focal adhesion. The flux may in turn provide feedback regulation of force production in relation to cell migration, and/or facilitate the delivery of long-range signals released by focal adhesions.

MATERIALS AND METHODS

Cell Culture, Plasmids, and Drug Application

NIH3T3 mouse embryonic fibroblasts NIH3T3 (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% donor calf serum (Hyclone Laboratories, Logan, UT), 50 μ g/ml streptomycin, 50 U/ml penicillin, and 2 mM L-glutamine. Normal rat kidney (NRK) epithelial cells (American Type Culture Collection) were cultured in F12K medium (Sigma-Aldrich) containing 10% fetal bovine serum (Hyclone Laboratories), 2 mM L-glutamine, 50 μ g/ml streptomycin, and 50 U/ml penicillin. All cells were maintained in an incubator with 5% CO₂ at 37°C.

Red Fluorescent protein (RFP)-zyxin was constructed by recloning the enhanced green fluorescent protein (EGFP) construct (Rottner *et al.*, 2001) into the pDsRed1-N1 vector (Clontech, Palo Alto, CA). RFP-VASP and EGFP-tagged paxillin (Rottner *et al.*, 2001), and yellow fluorescent protein (YFP)-vinculin were kindly provided by Dr. Juergen Wehland (Deutsche Forschungsgemeinschaft, Braunschweig, Germany), and YFP-dSH2 was kindly provided by Dr. Benny Geiger (Weizmann Institute; Israel; Kirchner *et al.*, 2003). EGFP-tagged N-fragment of tensin was kindly provided by Dr. Shin Lin (University of California, Irvine). EGFP-actin was purchased from Clontech. NIH3T3 and NRK were nucleofected using Amaxa Nucleofector I and kit R (Amaxa Biosystems, Gaithersburg, MD).

Y-27632 (Calbiochem, San Diego, CA) was prepared as 10 mM stock solution in phosphate-buffered saline (PBS). Stock solutions of 100 mM blebbistatin (Calbiochem), 100 mM genistein (Calbiochem), 10 mM PP2 (Calbiochem), and 10 mM PP3 (Calbiochem) were prepared in DMSO and stored at -20°C. All reagents were diluted from the stock solution into medium immediately before use. The final concentration was 50 μ M for Y-27632, 5 μ M for blebbistatin, 100 μ M for genistein, and 20 μ M for PP2 and PP3.

Preparation of Micropatterned Substrates and Local Mechanical Stimulation by Stretching

Patterned substrates with 40- $\times$ 40- μ m² islands were generated by spin coating of SU-8 2002 photoresist (MicroChem, Newton, MA) on a dried polyacrylamide film, which was prepared with 50 μ l of 10% acrylamide, 0.26% bis-acrylamide spread over a circular area 32 mm in diameter on a glutaraldehyde-activated 40- $\times$ 50-mm² coverslip (Pelham and Wang, 1997). The thickness of the film was 6.2 ± 1.0 μ m after air-drying. After further drying at 95°C for 2 min, the surface was spin coated with 200 μ l of SU-8. The photoresist was prebaked at 95°C for 2 min and exposed to 365-nm UV from a high-flux UV LED (OTLH-0480-UV) for 1 min through an opaque photomask with an array of 40- $\times$ 40- μ m² clear squares (CAD/Art Services, Bandon, OR). After postbaking at 95°C for 2 min, the photoresist was developed in SU-8 developer (MicroChem) for 1 min, rinsed with ethanol, and air-dried. A second layer of 8% polyacrylamide was polymerized on top of the first layer at a volume of 15 μ l, to further reduce cell adhesion to areas outside the SU-8 islands (Mader *et al.*, 2007). Omitting bis-acrylamide and N,N,N',N'-tetramethylethylenediamine (TEMED) generated linear polyacrylamide chains that grew off the dried layer of polyacrylamide without covering the SU-8 islands. We subsequently found that the first layer of polyacrylamide may be omitted, by directly spin coating SU-8 on a glutaraldehyde-activated coverslip. After photolithography to generate the island pattern of SU-8 as described above, the remaining area was blocked by

grafting 8% linear polyacrylamide (with TEMED) directly onto the exposed glass surface.

Micropatterned polyacrylamide gels were prepared by activating the gel surface with 50 mM sulfosuccinimidyl 6 (4'-azido-2'-nitrophenyl-amino) hexanoate (Pierce Chemical, Rockford, IL) in 200 mM HEPES, pH 8.5, as described previously (Wang and Pelham, 1998). Excess liquid on the surface was removed by blowing with nitrogen gas, and the surface was stamped for 1 min with a patterned polydimethylsiloxane stamp inked with collagen. The stamp was made as described by LeDuc *et al.* (2002), by using a molding made from the SPR 220-3.0 photoresist (Shipley, Marlboro, MA), patterned with the same photomask as for the SU-8 substrates. The stamp was soaked in 100 μ g/ml type I collagen (USB, Cleveland, OH) for 45 min and rinsed with PBS briefly. Excess liquid was blown off with nitrogen gas before the stamp was applied to the gel surface. The same procedure was applied to gels with 10% acrylamide/0.26% bis-acrylamide (Young's modulus >50 kPa; referred to as the stiff gel), and gels with 10% acrylamide gel/0.03% bis-acrylamide (Young modulus ~5 kPa; referred to as the soft gel).

Local stretching forces were applied to NIH3T3 cells plated on polyacrylamide gel of 5% acrylamide/0.08% bis-acrylamide as described previously (Lo *et al.*, 2000). Briefly, glass capillary tubing was pulled into needles with a vertical micropipette puller (David Kopf Instruments, Tujunga, CA). The tip of the needle was then melted and shaped with a microforge (Narishige, East Meadow, NY). The blunted needle tip was gently pressed into the gel near the cell and moved away from the cell with a micromanipulator (Leitz, Wetzlar, Germany), to generate pulling forces. The manipulation caused an ~10% overall increase in cell length, which was prominent at the end of the cell proximal to the needle but became undetectable at the opposite end (Lo *et al.*, 2000). Based on an estimated Young's modulus of 1–5 kPa for fibroblasts (Engler *et al.*, 2004), the induced cell strain reflected an applied stress of $1\text{--}5 \times 10^{-10}$ N/ μ m² cellular cross-sectional area.

Immunofluorescence Staining and Imaging, FRAP, and Image Analysis

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Samples were blocked with 1% bovine serum albumin/PBS for 15 min at room temperature, and then they were incubated for 1 h at 37°C with 1:200 dilution of primary antibodies, against fibronectin (F3648; Sigma-Aldrich), paxillin (H-114; Santa Cruz Biotechnology, Santa Cruz, CA), vinculin (clone VIN11-5; Sigma-Aldrich), FAK (C-20; Santa Cruz Biotechnology), FAK-pY397 (Santa Cruz Biotechnology), phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY), or zyxin (Synaptic Systems, Gottingen, Germany). Alexa dye-conjugated anti-rabbit or anti-mouse secondary antibodies were obtained from Invitrogen (Carlsbad, CA) and applied at a dilution of 1:400. Alexa 488-conjugated phalloidin (Invitrogen) was applied following manufacturer's procedure. Images were collected with a 100 $\times$ Plan-Nufluor numerical aperture (N.A.) 1.3 oil lens, and a low-light electron multiplication charge-coupled device (CDD) camera iXon DV887DCS-BV (Andor Technology, South Windsor, CT), or a conventional cooled CCD camera (NTE/CCD-512-EBFT; Princeton Instruments, Trenton, NJ), attached to an inverted microscope (Axiovert 200M; Carl Zeiss, Thornwood, NY), equipped with a 100 $\times$ Plan-Nufluor N.A. 1.3 oil lens and a stage incubator for live cell time-lapse imaging. Fluorescent images were recorded every 2 min.

Green fluorescent protein (GFP) fluorescence was bleached with an Argon ion laser (Lexel, Palo Alto, CA), by using 10-ms pulses of the 457-nm line at 50 mW. To bleach RFP-zyxin, we used the 514-nm line at a power of 200 mW and 10-ms duration. Fluorescence images before bleaching and during fluorescence recovery were captured every 5 s with spinning-disk confocal optics (QLC100; Solamere Technology, Salt Lake City, UT), using 488 nm for the excitation of GFP and 532 nm for the excitation of RFP and the corresponding filter set provided by the manufacturer. To allow simultaneous bleaching and imaging, the epifluorescence filter cube was mounted with a 525-nm dichroic mirror (525DRLP; Omega Optical, Brattleboro, VT) for RFP signals, or a 475-nm dichroic mirror (z457rdc; Chroma Technology, Brattleboro, VT) for GFP signals. These mirrors allowed the excitation and emission wavelengths for confocal imaging to pass through while directing the bleaching beam at the sample. Conventional fluorescence imaging was carried out with the Omega Optical 100-3 filter set for GFP and Chroma Technology 41002 filter set for RFP. Fluorescence intensity was measured by integrating signals within a user-defined, closed boundary of any shape, using custom software written in C++. Kymographs also were produced and analyzed using custom software, which allowed the user to define the location, orientation, length, and width of a rectangular area in an image series. Images within the defined area were then extracted from the series, reoriented vertically, and combined side by side into a kymograph. All experiments involving single cell time-lapse and immunofluorescence assays have been performed at least three times with consistent results. Statistical significance was determined using Student's *t* test.

Figure 1. Retrograde fluxes of zyxin and VASP observed in some fibroblasts and epithelial cells. RFP-VASP expressed in a NIH3T3 fibroblast (A) or RFP-zyxin expressed in an NRK epithelial cell (C) form long comet-like tails off some (arrows) but not other (arrowheads) focal adhesions. The focal adhesion at the tip of the flux remained largely stationary (B, arrows). Proteins in the tail show striking fluxes away from focal adhesions, as shown in the kymograph (B, for the boxed region in C). Bar, 20 μm .

RESULTS

Retrograde Flux of Zyxin at Focal Adhesions in Relation to Cell Migration

We started by examining the dynamics of zyxin and VASP in living NIH3T3 cells transiently expressing DsRed-labeled zyxin or VASP (referred to as RFP-zyxin and RFP-VASP). The hypothesis was that their variable association with focal adhesions and stress fibers, as shown in previous studies (Crawford *et al.*, 1992; Rottner *et al.*, 2001), may reflect different states of focal adhesions in responses to mechanical signals and/or cell migration. We observed two discrete patterns of the distribution of these proteins near focal adhesions in the lamella region of NIH3T3 cells. In most cells they were confined to the typical plaque-shaped focal adhesions; however, in a small percentage ($\sim 10\%$ of 100 cells examined) of cells they were present both at focal adhesions and along a “comet tail” that extended away from some focal adhesions toward the cell center. An example of cells in this subpopulation is shown in Figure 1A.

Time-lapse recording and kymograph analysis indicated that both zyxin and VASP underwent active retrograde flux along these tails (Figure 1B), whereas focal adhesion at the tip of the flux remained largely stationary (Figure 1B, arrows). In addition, the tails showed lateral swings while fluxing (Supplemental Movie 1). Similar results were obtained with NIH3T3 fibroblasts (Figure 1A) and NRK epithelial cells (Figure 1C and Supplemental Movie 1), although the latter showed zyxin tails in a higher percentage of cells ($>20\%$ of 100 cells examined), with an average flux rate of $0.71 \pm 0.04 \mu\text{m}/\text{min}$ ($n = 9$). Furthermore, immunofluorescence staining of zyxin showed similar tail structures, which colocalized with RFP-zyxin in transfected cells over a wide range of expression levels (Supplemental Figure 1), indicating that the tails of zyxin and VASP were physiological structures. Because RFP-zyxin generated images with low background and few clumps, the following experiments were performed mainly with RFP-zyxin.

We noticed that focal adhesions with zyxin tails were located mainly in relatively stationary NIH3T3 cells and in NRK epithelial cells, which were not as motile as NIH3T3 cells. In addition, plots of the number zyxin tails in NIH3T3 cells against the speed of migration showed an inverse relationship

Figure 2. Inverse correlation between zyxin fluxes and cell migration for NIH3T3 cells. Scatter plot of the number of zyxin tails in a cell against the speed of migration shows an inverse relationship (A). The formation of zyxin tails is amplified on square patterned substrates (B), where long tails are concentrated at the four corners of an RFP-zyxin transfected NIH3T3 fibroblast (C). Bar, 20 μm .

(Figure 2A). The dependence on cell migration was further tested by immobilizing NIH3T3 cells on patterned substrates consisting of $40 \times 40 \mu\text{m}^2$ islands of SU-8 photoresist on an otherwise nonadhesive polyacrylamide surface (Figure 2B). Constraining NIH3T3 cells on such substrates induced an increase in the percentage of cells with zyxin tails, from $<10\%$ of cells on glass or uniform SU-8 surfaces to $30\sim 40\%$ (50 cells examined) on SU-8 islands. Moreover, $86 \pm 6\%$ of the tails were located within 23° from the four corners of these square-shaped cells (Figure 2C; based on 250 tails in 10 cells), where ruffling activities and strongest traction forces were concentrated (Parker *et al.*, 2002; Wang *et al.*, 2002).

Relation of Retrograde Zyxin Flux to Flux of Actin Filaments

Staining of transfected cells with fluorescent phalloidin, and cotransfection of EGFP-actin and RFP-zyxin, showed several distinct configurations of focal adhesions in the lamella region. Some focal adhesions carried neither an actin bundle nor zyxin tail, whereas other focal adhesions were associated with a tapering tail containing both zyxin and actin filaments (Figure 3, A and B). In cells cotransfected with RFP-zyxin and EGFP-actin, all the tails showed concomitant flux of zyxin and actin (Supplemental Movie 2). The similar flux rates of 0.71 ± 0.04 and $0.75 \pm 0.02 \mu\text{m}/\text{min}$ for zyxin and actin, respectively, in NRK cells (Figure 3, C–E; $n = 9$ for each), suggested that zyxin was carried away by the flux of actin subunits. Spot photobleaching of RFP-zyxin and EGFP-actin confirmed the flux (Figure 3, F and G, and Supplemental

Figure 3. Correlation between zyxin and actin fluxes. NRK epithelial cells are double stained with anti-zyxin antibodies and fluorescent phalloidin (A), or transfected with RFP-zyxin then stained with fluorescent phalloidin (B). Three categories of focal adhesions exist, those with neither an associated actin bundle nor zyxin tail (A; arrowheads), those with a tail containing both zyxin and actin (A and B), and in some situations, those with an associated bundle of actin but no zyxin tail (A; arrows). Kymograph analysis of images of RFP-zyxin (C) and actin filaments (D) from the same bundles shows a similar flux rate ($p = 0.449$; $n = 9$ for each; E). Kymographs of RFP-zyxin (F) and EGFP-actin (G) after photobleaching show similar retrograde movements of the bleaching spot (lines in F and G). However, signals of zyxin recover at a much higher rate than actin. Error bars represent standard deviations. Bar, 20 μm.

Figure 4. Differential behavior of focal adhesion proteins. The distribution of various focal adhesion proteins is compared against RFP-zyxin expressed in NRK epithelial cells, after fixation and staining with antibodies against vinculin (A), paxillin (B), or FAK (C). Both zyxin and FAK form tails that extend away from focal adhesions, as defined by the localization of vinculin and paxillin. Bar, 20 μm.

tal Movies 3 and 4). However, photobleached RFP-zyxin spot recovered within 90 s while traveling along the flux

(Figure 3F and Supplemental Movie 3), suggesting that zyxin associated dynamically with the actin bundle and

Figure 5. Correlation between zyxin tails and fibrillar adhesions. An NIH3T3 cell is processed for double immunofluorescence against zyxin and fibronectin (A). Fibronectin shows only very limited colocalization along zyxin tails. In addition, zyxin tails often extend across multiple planes of focus from the substrate, taken at a separation of 250 nm (B). Bar, 10 μ m.

underwent exchange with a cytoplasmic pool. There were also focal adhesions with an associated actin bundle but without a tapering zyxin tail. These bundles typically showed staining of zyxin as weak, punctuate structures with no detectable flux of either actin or zyxin, and likely represent mature stress fibers.

Thus, the appearance of zyxin tails was tightly coupled with the flux of both zyxin and actin. Because RFP-zyxin has a longer wavelength and lower autofluorescence than EGFP-actin, and the expression of EGFP-actin tended to enhance robust, mature stress fibers not typically found in nontransfected cells, we used the appearance of zyxin tails as a reliable indicator for the flux of both zyxin and actin in the following experiments (referred to hereafter as focal adhesion fluxes).

Characterization of Retrograde Focal Adhesion Fluxes

We compared the immunofluorescence localization of zyxin with other focal adhesion proteins, including paxillin, vinculin, and FAK. The tail of zyxin and VASP extended far beyond focal adhesions as defined by vinculin or paxillin (Figure 4, A and B), although limited fluxes of these proteins have been detected with total internal reflection fluorescence optics (Brown *et al.*, 2006; Hu *et al.*, 2007). Interestingly, FAK extended further into the tail than vinculin or paxillin (Figure 4C). These observations may reflect differential affinities of focal adhesion proteins with the actin bundle versus the structural scaffold at focal adhesions.

To determine whether focal adhesion fluxes colocalized with fibrillar adhesions, we examined the distribution of fibronectin, a known component of fibrillar adhesions (Pankov *et al.*, 2000; Zamir *et al.*, 2000). Immunofluorescence staining indicated that fibronectin colocalized only partially

Figure 6. Response of zyxin fluxes to intracellular contractile forces and to external stretching forces. Treatment of NRK cells with 5 μ M blebbistatin for 30 min caused complete disappearance of zyxin tails (compare A and B). Inhibition of zyxin flux is shown as the disappearance of slanted lines (arrowheads) in the kymograph of images after treatment (C; arrow indicates the time of blebbistatin treatment). To determine the effect of external forces, an NIH3T3 cell plated on flexible polyacrylamide gel of 5% acrylamide/0.08% bis-acrylamide (D) is subject to pulling forces, applied with a blunt needle near the top edge of the cell. Increased zyxin flux is observed within 4 min of the application of forces (E and F, arrows). The flux decreases upon the release of pulling forces (G). Bar, 20 μ m.

with zyxin tails (Figure 5A). In addition, ventral fibronectin-containing fibrils were localized within a single optical section against the substrate (data not shown), whereas some zyxin tails extended over several optical sections (Figure 5B). Moreover, unlike fibrillar adhesions (Zamir *et al.*, 2000), zyxin tails disappeared rapidly upon the inhibition of contractile force as described below. However, EGFP tagged N-fragment of tensin, which associates with fibrillar adhesions, did localize to zyxin tails (Supplemental Movie 5), suggesting that focal adhesion fluxes may contribute to the formation of fibrillar adhesions.

Dependence of Focal Adhesion Fluxes on Cellular Contractility and Mechanical Stimulation

We noticed that focal adhesion fluxes were concentrated in the regions known to be active in generating traction forces, such as frontal region of the cell and corners of square-shaped cells (Pelham and Wang, 1999; Wang *et al.*, 2002). Therefore, mechanical forces, either generated internally by

Figure 7. Responses of zyxin fluxes to substrate rigidity. NIH3T3 cells expressing RFP-zyxin are plated on micropatterned stiff (A) or soft (B) polyacrylamide gels. Cells on stiff gels show a significantly higher average number of zyxin tails than cells plated on soft gels (C; $p < 0.001$; $n = 10$ for each). The length of the tail and the size of focal adhesions are also visibly different. Error bars represent standard deviations. Bar, 20 μm .

myosin II or applied externally through focal adhesions, may play a role in inducing the fluxes. Y-27632, an inhibitor of the Rho-dependent kinase, and blebbistatin, an inhibitor of myosin II ATPase, both caused strong inhibition of traction forces (Beningo *et al.*, 2006), and focal adhesion fluxes, while reducing zyxin tails to small dot-like structures (Figure 6, A–C, and Supplemental Movie 6). Conversely, application of pulling forces to cells plated on flexible polyacrylamide substrates stimulated the appearance of focal adhesion fluxes (Figure 6, D–G).

Previous studies showed that adherent cells not only respond to applied forces but also actively probe the rigidity of the environment (Discher *et al.*, 2005). To determine the effect of substrate rigidity on focal adhesion flux while maintaining a similar cell shape and spread size, we applied soft lithography to polyacrylamide substrates to generate extracellular cell matrix (ECM)-coated islands of different stiffness (Wang *et al.*, 2002). NIH3T3 cells showed prominent zyxin tails on islands of stiff polyacrylamide as they did on photoresist ($n = 10$ cells; Figure 7A and Supplemental Movie 7), but they formed only small focal adhesions with few zyxin tails on soft substrates ($n = 10$ cells; Figure 7, B and C).

Dependence of Focal Adhesion Fluxes on Tyrosine Phosphorylation

Given the response of tyrosine phosphorylation to mechanical signals (Pelham and Wang, 1997; Schmidt *et al.*,

1998), we asked whether tyrosine phosphorylation might be involved in regulating focal adhesion fluxes. Immunofluorescence localization showed that phosphotyrosine was concentrated only at focal adhesions irrespective of the presence of zyxin tails (Figure 8A). Consistent results were obtained with live cells expressing YFP-dSH2, a probe for sites of tyrosine phosphorylation (Kirchner *et al.*, 2003; Figure 8B and Supplemental Movie 8). Interestingly, although FAK was found both at focal adhesions and along the flux, FAK phosphorylated at Tyr397, which is known to activate its kinase activity (Schaller *et al.*, 1994), was localized predominantly at focal adhesions (Figure 8C), consistent with the localization of phosphotyrosine.

To determine the involvement of Src family tyrosine kinases in focal adhesion flux, we examined the effects of various inhibitors of tyrosine kinases. Treatment of cells with a broad-spectrum tyrosine kinase inhibitor, genistein, caused disappearance of zyxin tails ~ 30 min after treatment, without causing disappearance of focal adhesions as shown by RFP-zyxin (Figure 9, A and B, and Supplemental Movie 9), or immunofluorescence (Figure 9C). PP2, which selectively inhibits Src family kinases, also reduced zyxin flux without disassembling focal adhesions (Figure 9, C and D), whereas PP3, an inactive analogue of PP2, had no significant effect (data not shown).

Figure 8. Concentration of tyrosine phosphorylation at focal adhesions. RFP-zyxin transfected epithelial NRK cells are either stained with an antibody against phosphotyrosine (A) or cotransfected with YFP-dSH2 (B), a probe of tyrosine phosphorylation. Both show concentration of phosphotyrosine only at focal adhesions irrespective of the presence of zyxin tails. Double staining of NRK cells with monoclonal anti-zyxin antibody and polyclonal anti-FAK pY397 antibody indicates that FAK phosphorylated at Tyr397 is also localized predominantly at focal adhesions. Bar, 20 μ m.

DISCUSSION

In this report, we showed that cell migration and mechanical signals regulate the formation and flux of actin filaments anchored at focal adhesions, which also carries several focal adhesion proteins toward the cell center. The flux is active in stationary cells and it is suppressed during cell migration. In addition, mechanical stimulations, including stretching forces and substrate rigidity, promote the flux, whereas inhibitors of Src tyrosine kinases inhibit the flux. A similar flux of actin, dependent on the nucleation factor formin, was observed at nascent focal adhesions, and it was suggested as the mechanism for the formation of stress fibers (Cramer *et al.*, 1997; Hotulainen and Lappalainen, 2006).

The maintenance of a steady-state flux requires treadmilling of actin subunits, where subunits are continuously added at focal adhesions and removed at the proximal ends. The process may be regulated by the rates of actin nucleation, elongation, dissociation, and possibly myosin-dependent forces that pull on actin filaments (Medeiros *et al.*, 2006). The dependence of the flux on cell migration and mechanical signals explains why prominent stress fibers are present only in relatively immobile cells (Herman *et al.*, 1981; Tomasek *et al.*, 1982), and in cells on stiff substrates (Pelham and Wang, 1997).

The responses to cell migration and substrate compliance are likely related, because conditions that favor the focal adhesion flux share a common feature, that the actin cytoskeleton undergoes large retrograde displacement relative to the substrate anchorage site, as a result of either contrac-

Figure 9. Involvement of tyrosine kinase activity in the formation of zyxin fluxes at focal adhesions. Treatment of an RFP-zyxin transfected NRK cell with a broad-spectrum tyrosine kinase inhibitor, genistein, causes inhibition of zyxin fluxes (B), without causing significant disassembly of focal adhesions as shown by RFP-zyxin (A and B), or immunofluorescence staining (E). PP2, which selectively inhibits the Src family kinases, also reduces zyxin tails without disassembling focal adhesions (C and D). Bar, 20 μ m.

tile forces pulling against a stationary anchorage site (Figure 10D), or anterograde deformation of a flexible substrate (Figure 10C). Conversely, conditions that inhibit the flux, including migration of the cell body (Figure 10A), and large substrate deformability (Figure 10B), are likely to cause a smaller retrograde displacement of the actin cytoskeleton relative to the substrate anchorage site. Therefore, the regulation of the flux may be explained by a structural shear model (Figure 10), where focal adhesion proteins at different distances from the membrane anchorage site are displaced by different distances along the direction of migration or mechanical forces under different conditions, which may in turn regulate tyrosine phosphorylation and actin assembly, e.g., by exposing tyrosine kinase binding domains and/or activating the substrates (Sawada *et al.*, 2006).

This structural shear mechanism also explains the different degrees of correlated movements with actin flux for different focal adhesion proteins (Brown *et al.*, 2006; Hu *et al.*, 2007), and they may serve as a feedback mechanism that adjusts structural assembly, possibly coupled with the generation of contractile forces, in response to the needs of cell migration. The mechanism will provide the strongest propulsive forces for slow-moving cells on stiff substrates, which are known to induce strong resistive adhesions as well as traction forces (Guo *et al.*, 2006). A second potential function of a regulated flux at focal adhesions is to serve as a slippage clutch for cell migration (Smilenov *et al.*, 1999; Hu *et al.*, 2007). Retrograde flux may reflect disengagement of the clutch, when contractile forces are consumed in the transport of actin filaments rather than transmitted to the substrate for cell migration.

Figure 10. A structural shear model for mechanotransduction at focal adhesions. Different vertical layers of focal adhesion proteins are represented as three lines, with orange lines representing proteins near integrins and yellow lines representing proteins near the actin cytoskeleton. Cell migration (A) and substrate compliance (B) share a common feature, that the substrate anchorage site is separated by a limited horizontal (shear) distance from the interior actin cytoskeleton and associated proteins, due to the forward movement of the cell body or backward movement of the soft substrate. In contrast, larger shear separation between the substrate anchorage site and the interior actin cytoskeleton takes place when flexible substrates are dragged away from the cell (C), or when strong traction forces pull on focal adhesions anchored to stiff substrates (D). The structural shear may in turn stimulate tyrosine phosphorylation, assembly of actin bundles, and increases in myosin II-dependent contractile forces.

Focal adhesion fluxes may also be involved in the formation of fibrillar adhesions and the assembly of a fibronectin network to guide cell migration in tissues (Boucaut *et al.*, 1990; Darriber and Schwarzbauer, 2000). Although the flux does not correlate exactly with fibrillar adhesions in their localization and sensitivity to cell contractile forces (Zamir *et al.*, 2000), the colocalization of zyxin with tensin and the partial colocalization with fibronectin in NIH3T3 suggest that the flux may serve as a precursor for the formation of fibrillar adhesions in fibroblasts (Zamir *et al.*, 2000; Figure 4D), through recruitment and retrograde transport of $\alpha 5 \beta 1$ integrins along the flux followed by fibronectin binding (Pankov *et al.*, 2000). Because focal adhesion fluxes also exist in epithelial cells, which produce different extracellular matrix proteins than fibroblasts, the fluxes may play a general role in organizing the extracellular matrix. Further evaluation of the role of fluxes in three-dimensional environment may elucidate this possible function of fluxes.

An alternative function of the focal adhesion flux is to facilitate the transport of signals from focal adhesions in response to mechanical cues, as suggested by impaired responses of zyxin^{-/-} cells to ECM anchorage and mechanical stimulations (Yoshigi *et al.*, 2005; Hoffman *et al.*, 2006). Although focal adhesions have been recognized as an important source of signals with multiple downstream effects such as activation of the mitogen-activated protein kinase pathway and eventually gene expression (Giancotti and Ruoslahti, 1999), how these signals propagate is unclear. A diffusion-like mechanism seems inefficient within a crowded cytoplasmic environment, and transport along the flux of actin filaments may serve as a much more effective mechanism. Particularly relevant is the relocation of zyxin from focal adhesions into the nucleus of vascular smooth muscle cells in response to mechanical signals, where zyxin itself may serve as a transcription activator (Degenhardt and Silverstein, 2001). In addition, retrograde flux of FAK may have a profound effect on the state of phosphorylation at

focal adhesions and actin filaments, which in turn affects the maturation and turnover of focal adhesions (Webb *et al.*, 2004). Our results further suggest that the flux of focal adhesion proteins requires tyrosine phosphorylation, which, in conjunction with the small GTPases Rac and Rho, represents the primary molecular switches in response to integrin-ECM anchorage and mechanical signals (Burridge *et al.*, 1992; Schmidt *et al.*, 1998). Tyrosine phosphorylation regulates the dynamic turnover of focal adhesion proteins through a number of substrates including paxillin, p130Cas, and FAK (Carragher and Frame, 2004). In addition, Rho activities show a transient decrease followed by an increase upon integrin signaling, which may be responsible for the activation of formin that in turn nucleates the assembly of actin filaments at focal adhesions (Waller and Alberts, 2003). Understanding the regulation of focal adhesion flux is likely to shed light on a wide range of activities that involve cell migration and mechanosensing.

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