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Mechanical Forces Alter Zyxin Unbinding Kinetics Within Focal Adhesions of Living Cells

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The formation of focal adhesions that mediate alterations of cell shape and movement is controlled by a mechanochemical mechanism in which cytoskeletal tensional forces drive changes in molecular assembly; however, little is known about the molecular biophysical basis of this response. Here, we describe a method to measure the unbinding rate constant k_{OFF} of individual GFP-labeled focal adhesion molecules in living cells by modifying the fluorescence recovery after photobleaching (FRAP) technique and combining it with mathematical modeling. Using this method, we show that decreasing cellular traction forces on focal adhesions by three different techniques—chemical inhibition of cytoskeletal tension generation, laser incision of an associated actin stress fiber, or use of compliant extracellular matrices—increases the k_{OFF} of the focal adhesion protein zyxin. In contrast, the k_{OFF} of another adhesion protein, vinculin, remains unchanged after tension dissipation. Mathematical models also demonstrate that these force-dependent increases in zyxin's k_{OFF} that occur over seconds are sufficient to quantitatively predict large-scale focal adhesion disassembly that occurs physiologically over many minutes. These findings demonstrate that the molecular binding kinetics of some, but not all, focal adhesion proteins are sensitive to mechanical force, and suggest that force-dependent changes in this biophysical parameter may govern the supramolecular events that underlie focal adhesion remodeling in living cells. J. Cell. Physiol. 207: 187–194, 2006.

Cell adhesion, spreading, and motility are mediated by the formation and remodeling of specialized extracellular matrix anchoring complexes known as focal adhesions (Balaban et al., 2001; Zamir and Geiger, 2001). Focal adhesion assembly is controlled by a mechanochemical mechanism: mechanical forces that are transferred to the cell surface integrin receptors from the extracellular matrix (Wang et al., 1993) or from the internal contractile cytoskeleton (Schmidt et al., 1993) produce changes in the assembly or disassembly of integrin-associated linker proteins, such as vinculin (Geiger et al., 1980) and zyxin (Crawford and Beckerle, 1991), that form the cytoskeletal backbone of the focal adhesion. Mechanical forces may alter biochemistry and influence focal adhesion assembly by producing changes in the three-dimensional conformation of individual molecules that change their chemical potential (Ingber, 1997; Geiger and Bershadsky, 2002; Sawada and Sheetz, 2002) or expose cryptic binding sites (e.g., as shown for force-induced fibronectin assembly (Zhong et al., 1998; Baneyx et al., 2002; Vogel and Baneyx, 2003). Mechanical forces also may directly alter molecular binding kinetics by changing bond dissociation energy landscapes, as demonstrated in vitro (Merkel et al., 1999). In fact, steered molecular dynamics simulations suggest that the focal adhesion targeting (FAT) domain of focal adhesion kinase (FAK) may change conformation in response to mechanical force, and result in an increase in its binding affinity for paxillin (Kamm and Kaazempur-Mofrad, 2004). However, due to lack of experimental methods that can directly measure the binding rate constants of proteins in a spatially-localized and tension-dependent manner, it remains unclear whether mechanical forces alter molecular binding affinities of focal adhesion proteins in living cells.

Because focal adhesions play a central role in cellular control and mechanotransduction, the dynamics of their assembly have been analyzed by measuring changes of focal adhesion size over time. 'Apparent' rate constants of focal adhesion proteins have been calculated by fitting an exponential curve to the changes in the total fluorescence intensity of molecules in the focal adhesion measured over several minutes in response to various perturbations, including inhibition of cytoskeletal tension generation (Webb et al., 2004). However, these apparent rate constants do not reflect the protein's molecular-scale affinity for its binding partners in the adhesion. For example, slow changes of focal adhesion assembly may result from removal and addition of molecular components due to protease degradation, enzymatic phosphorylation-dephosphorylation events, or alterations in the number of available binding sites for a given molecule, even if intrinsic molecular kinetic parameters do not change. Thus, while the time course of focal adhesion formation or disappearance measured over minutes may be a useful metric to compare relative

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changes of assembly or disassembly, apparent rate constants measured using these data only represent gross properties; they are not interpretable in terms of changes in the native binding affinities of a given protein at the molecular biophysical level.

Thus, to investigate if mechanical force can affect molecular binding kinetics of focal adhesion proteins, we developed a new approach based on fluorescence recovery after photobleaching (FRAP) to measure the unbinding rate constant k_{OFF} of individual GFP-labeled focal adhesion proteins in living cells. We then used this technique to examine whether two focal adhesion proteins that are known to be involved in mechanosensation—zyxin and vinculin (Ezzell et al., 1997; Alenghat et al., 2000; Goldmann and Ingber, 2002; Zaidel-Bar et al., 2003)—alter their molecular binding kinetics when the level of tension applied to integrin receptors is altered by chemically inhibiting cytoskeletal tension generation, physically disrupting a single actin stress fiber using a femtosecond laser, or altering extracellular matrix compliance. Our results show that the k_{OFF} of zyxin varies inversely with mechanical force whereas the k_{OFF} of another focal adhesion protein, vinculin, is insensitive to force. Moreover, the changes of zyxin's k_{OFF} that occur over seconds were found to be sufficient to explain large-scale changes of focal assembly that take place over many minutes.

MATERIALS AND METHODS

Cell culture

Bovine adrenal capillary endothelial cells (passage 10–13) cultured as described (Parker et al., 2002) were transfected with EGFP-vinculin or EGFP-zyxin (kindly provided by Dr. Benjamin Geiger and Dr. Matthew Pettroll, respectively) using Effectene (Qiagen, Chatsworth, CA), and then plated on uncoated glass-bottomed dishes (MatTek) in DMEM (GIBCO/Invitrogen, Carlsbad, CA) containing 10% bovine calf serum (Hyclone), 10mM HEPES, pH 7.4 (JRH Biosciences, Lenexa, KS), L-glutamine (0.292 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 ng/ml fibroblast growth factor (Sigma-Aldrich, St. Louis, MO). Microscopy studies were carried out in bicarbonate-free optically clear medium containing Hank's balanced salts (Parker et al., 2002) containing $CaCl_2$ (1.26 mM), $MgSO_4$ (0.81 mM), KCl (5.36 mM), KH_2PO_4 (0.44 mM), NaCl (137 mM), Na_2HPO_4 (0.3 mM), D-glucose (5.55 mM), L-glutamine (2.0 mM), sodium pyruvate (1.0 mM), HEPES (20.0 mM)] supplemented with L-glutamine (2.0 mM), HEPES (20.0 mM), MEM essential and non-essential amino acids (Sigma), and 10% bovine calf serum. Flexible polyacrylamide gel substrates were prepared on glass coverslips and coated with fibronectin (670 ng/cm^2), as previously described (Polte et al., 2004); endothelial cells (20,000 cells/cm^2) were cultured on these substrates overnight before imaging. Substrates with Young's moduli of 1, 50, and 300 kPa were prepared by varying bisacrylamide to polyacrylamide ratios, as previously described (Polte et al., 2004; Peyton and Putnam, 2005).

Immunofluorescence microscopy

Immunostaining studies carried out as described (Parker et al., 2002) utilized mouse monoclonal anti-human vinculin (clone hVIN-1, Sigma-Aldrich) and polyclonal rabbit anti-human zyxin (Abcam) antibodies in para-formaldehyde-fixed cells permeabilized with 0.2% Triton X-100. Fluorescent goat anti-mouse and goat anti-rabbit conjugated with Alexa Fluor 488 and Alexa Fluor 594 fluorescent dyes (Molecular Probes/Invitrogen, Carlsbad, CA) were used as secondary antibodies. Confocal imaging was done with a Leica TCS SP2 microscope, using a 63×1.4 NA objective.

FRAP and laser surgery experiments

FRAP and laser nanosurgery experiments were performed on the Zeiss LSM 510 META/NLO microscope using a 63×0.95 NA IR corrected water immersion lens. The 488 nm line of an Argon/2 multiple-lined single-photon laser source (10% of full power) was used for GFP excitation; 100% of the 488 nm line was used for photobleaching with 10 iterations corresponding to less than a millisecond. Measured recovery curves were normalized to the amount of photobleached protein and fit to $1 - e^{-k_{OFF}t}$ with the method of least squares (MATLAB function lsqcurvefit) to calculate k_{OFF} (Lele et al., 2004) and see supplementary information. To test equality of means for the k_{OFF} values measured under different conditions, statistical analyses were performed using the Student's t-test in MATLAB (function test 2). The sample size for all t-tests was more than 30 (except for the FRAP-nanosurgery experiments, where the sample size was 19). Experimental studies confirmed that diffusing molecules do not contribute significantly to the recovery curves we measured (Fig. 2b). Any photobleaching of diffusing molecules that does occur, but is below the limit of experimental detection, will recover in less than 100 msec (see Supplementary Information), a rate which is two orders of magnitude smaller than the recovery time scales exhibited by both zyxin and vinculin. Single stress fiber incision with the nanoscissor was accomplished by focusing energy from a pulsed Ti:Sapphire laser at 100% transmission (Chameleon, Coherent) over a 0.5 square μm area within the stress fiber for 15 iterations (~170 μsec) through the objective lens at a wavelength of 740 nm, nominal laser head power of 1.5 W, pulse duration of 140 fsec, and a repetition rate of 790 MHz(see movie in Supplemental Information). Images were collected using the Zeiss LSM 510 software (version 3.2). All experiments on microscopes were performed at 37°C using a temperature-controlled stage.

RESULTS

To explore the relationship between force and focal adhesion disassembly, we first dissipated tension in cultured capillary endothelial cells by disrupting the actin cytoskeleton using cytochalasin D (Goddette and Frieden, 1986). Subsequent immunostaining confirmed that zyxin, a cytoplasmic focal adhesion protein, almost completely disappeared from focal adhesions within 15 min after cytochalasin addition, whereas another cytoskeletal linker protein, vinculin, remained present within the same focal adhesion sites in these cells (Fig. 1a). Identical results were obtained when cytoskeletal tension was dissipated without disrupting microfilament integrity by treating the cells with Y27632 (Fig. 1b), which inhibits Rho-associated kinase (ROCK) (Mammoto et al., 2004; Shen et al., 2005), confirming previous observations of differential dynamics of zyxin compared to vinculin in focal adhesions (Rottner et al., 2001). Although zyxin dissociated more rapidly, vinculin also disappeared from cell-substrate adhesion sites after 30 min of treatment with Y27632 (Fig. 1b).

Adhesion plaque disassembly in response to tension dissipation occurs over several minutes (Fig. 1) yet individual proteins exchange between focal adhesions and cytoplasmic pools over a period of seconds, as measured by FRAP (von Wichert et al., 2003). Since this fast exchange is governed by the association and dissociation kinetics of these proteins with their respective binding partners in the adhesion sites, we reasoned that measuring the k_{OFF} values of zyxin and vinculin over the time course of force dissipation might offer insight into the biophysical basis for the differential effects of tension dissipation on their disassembly dynamics. However, FRAP methods used in the past to study adhesion protein exchange could not be used to directly estimate k_{OFF} because the photobleached regions of the cell were much larger than individual focal adhesions (Ballestrem et al., 2001; Tsuruta et al., 2002). Under these conditions, the recovery curve that is measured is influenced by both diffusion and molecular binding-unbinding rates because a large amount of

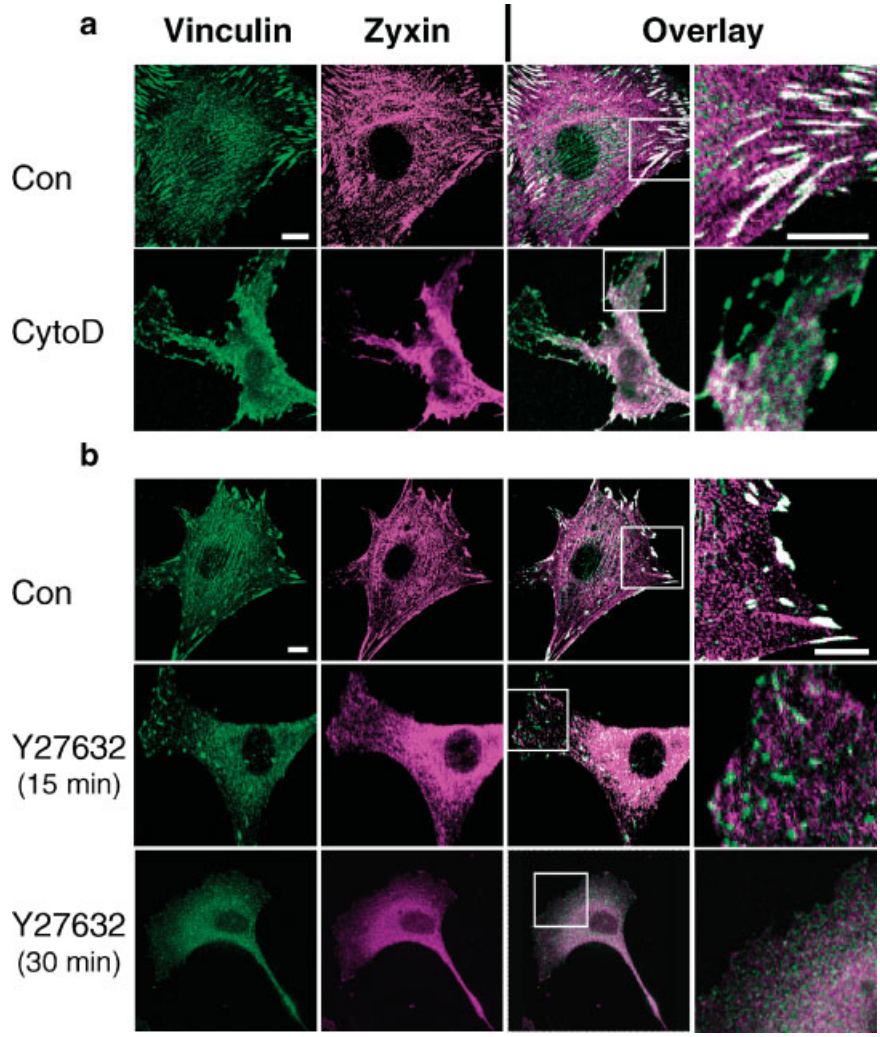

Fig. 1. Rapid disappearance of zyxin relative to vinculin upon force dissipation. **a**: Distribution of vinculin (green) and zyxin (magenta) within doubly-stained control endothelial cells (Con) compared with cells treated with cytochalasin D (CytoD; 1 μg/ml) for 15 min to disrupt actin microfilaments and dissipate cell tension. Overlay images shows the double-staining pattern in the same cell, with the region delineated by the white square shown at higher magnification at the right; focal adhesions that retain both zyxin and vinculin appear white. **b**: Similar staining of control cells compared with cells treated with Y27632 (6.7 μM) for 15 or 30 min to inhibit tension generation in the cell without physically disrupting the cytoskeleton (scale bar, 10 μm). Note that zyxin is lost more quickly (by 15 min) from focal adhesions than vinculin when cell tension is inhibited by either technique.

freely diffusing protein within the nearby cytoplasm is also photobleached. We reasoned that if the photobleaching of freely diffusing cytoplasmic protein can be minimized or eliminated, then the recovery curve will reflect only intrinsic molecular binding-unbinding kinetics.

We therefore photobleached GFP-zyxin and vinculin molecules present within focal adhesions of capillary endothelial cells using a confocal laser spot size (0.75 μm^2 area) smaller than a single focal adhesion (Fig. 2a) with a short photobleaching time (<3 μsec). This was experimentally confirmed by demonstrating that when the laser beam was focused to bleach adjacent volumes in the cytoplasm in the same focal plane, there was negligible bleaching of freely diffusing GFP-zyxin molecules (Fig. 2b). Based on these results, we were able to assume that the concentration of freely diffusing molecules is effectively constant (in time and space) during the course of the FRAP experiment. In this 'well-mixed' condition, where the diffusing molecular concentration is spatially homogeneous and temporally constant, the normalized recovery curve depends only on the unbinding constant k_{OFF} (Kaufman and Jain, 1990; Lele et al., 2004; Sprague et al., 2004), and it is independent of the binding constant k_{ON}. Although this relationship seems counterintuitive, it follows from mathematical arguments and is well established in the literature (Kaufman and Jain, 1990; Lele et al., 2004; Sprague et al., 2004) (see also Supplementary Information). The k_{OFF} we measure using this approach is an 'effective' k_{OFF} because it is representative of unbinding interactions of proteins that may interact with multiple different partners. Nevertheless, it is a direct read-out of changes in molecular binding kinetics that occur over seconds, unlike measurements of 'apparent' k_{OFF} that are calculated by measuring overall adhesion disassembly over a period of many minutes as in past studies on focal adhesion assembly (Webb et al., 2004).

Analysis of endothelial cells expressing GFP-labeled forms of zyxin and vinculin in untreated cells using this modified FRAP approach revealed that the fluorescence recovery times of both focal adhesion proteins were on the scale of seconds (Table 1), consistent with published results for vinculin and paxillin (von Wichert et al.,

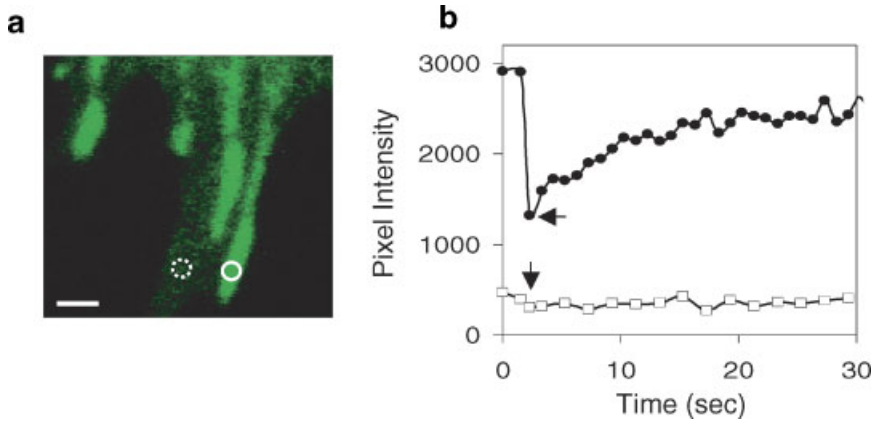

Fig. 2. Minimal contribution of freely diffusing proteins to the fluorescence recovery curve. **a**: FRAP was carried out simultaneously in two different adjacent spots (1 μm diameter): one in the cytoplasm (dashed circle) and the other in the focal adhesion (solid circle) (scale bar, 2 μm). **b**: Fluorescence recovery curves measured in the focal adhesion (solid circles) and cytoplasm (open squares) over a period of 30 sec; arrow indicates time at which the samples were photobleached. Note that the cytoplasmic pool does not exhibit any photobleaching due to the high rate of diffusion of soluble protein, and hence does not significantly contribute to the recovery curve under these experimental conditions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2003). Vinculin exhibited a biphasic recovery due to the presence of two kinetically distinct vinculin populations in focal adhesions (Table 1 and Supplementary Information Fig. S1). While the existence of two populations is unusual, the GFP-vinculin fusion protein that we used localized normally to focal adhesions and behaved similarly to endogenous vinculin protein in response to treatment with Y27632. Hence, we believe the observation is physiological and may represent two distinct time scales of interaction with different binding partners.

Importantly, the rapid recovery times (less than a minute) we measured for zyxin and vinculin was an order of magnitude smaller than the time required for focal adhesion disassembly in response to treatment with cytoskeletal modifiers, which was 15–30 min for zyxin and vinculin, respectively (Fig. 1); thus a pseudo-steady state exists on the time scale over which the FRAP analysis is carried out. We therefore were able to use FRAP during different short time windows after cytoskeletal tension was dissipated using Y27632 to directly determine associated force-dependent changes in the effective k_{OFF} of these focal adhesion proteins.

Decreasing the force exerted on the focal adhesions using Y27632 resulted in faster fluorescence recovery of zyxin into the photobleached spot relative to control cells (Fig. 3). This was associated with a continuous time-dependent increase in the k_{OFF} of zyxin (Fig. 4a,b), with the average value of k_{OFF} increasing nearly 2.5-fold (Table 1). Surprisingly, similar experiments with vinculin revealed that the values of k_{OFF} corresponding to both of its two dynamically distinct subpopulations remained unchanged after treatment with Y27632 (Table 1). Thus, the molecular binding kinetics of some, but not all, focal adhesion proteins are selectively sensitive to changes in cytoskeletal tension.

We then investigated if the k_{OFF} of zyxin determined with FRAP at various times after Y27632 treatment (Fig. 4a,b) was sufficient to predict the overall 15 min time course of disappearance of zyxin-containing focal adhesions observed in response to Y27632 treatment (Fig. 1). The concentration of zyxin in the focal adhesion relative to the initial concentration (before Y27632 treatment) was predicted by integrating the differential equation $dy/dt = 1 - k_{OFF}(1 + \beta t)y;\ y(0) = 1/k_{Off,I}$ where $y = C_{bound}/(k_{ON}SC_{free})$, t is the time of Y27632 treatment, C_{bound} is the concentration of bound zyxin in focal adhesions, C_{free} is the concentration of freely diffusing zyxin in the cytoplasm (which should not significantly change after drug treatment over this short time course), k_{ON} and $k_{OFF,I}$ are the effective, initial binding, and unbinding constants of zyxin in untreated cells, S is the binding probability (see Supplementary Information), and β is a parameter that describes the measured dependence of k_{OFF} on the time of exposure to Y27632 (Fig. 4b). Numerical integration of this equation with MATLAB revealed that the observed time-dependent increase of k_{OFF} is sufficient to explain the disappearance of zyxin from focal adhesions we observed over a period of 15 min (Fig. 4c), without requiring any change of k_{ON} or binding probability. Because our FRAP experiments were designed in such a way that the recovery curve only gave us the value of

TABLE 1. Molecular kinetic parameters measured for zyxin and vinculin using FRAP

	Zyxin				Vinculln	
	Control	Y27632	cut SF		Control	Y27632
$t_{1/2}$ (sec)	7.09 ± 0.43	3.75 ± 0.42	4.70 ± 0.75	k_{OFF1} (1/sec)	0.97 ± 0.1	1.02 ± 0.12
k_{OFF} (1/sec)	0.10 ± 0.01	0.25 ± 0.04	0.15 ± 0.03	k_{OFF2} (1/sec)	0.06 ± 0.01	0.07 ± 0.01
				$\frac{C_1}{C_1+C_2}$	0.40 ± 0.03	0.46 ± 0.05

The half-time for fluorescence recovery ($t_{1/2}$) for zyxin was calculated as $t_{1/2} = -\ln(0.5)/k_{OFF}$; the effective unbinding rate constant (k_{OFF}) was calculated by curve fitting with the method of least squares, as described in the Methods and Supplementary Methods. The differences in k_{OFF} for zyxin between control versus Y27632-treated cells, and between control versus cells containing one cut stress fiber (cut SF) were statistically significant ($P < 0.002$ and $P < 0.02$, respectively). There was no significant change in the unbinding rate constants of either the fast or slow recovering fractions of vinculin (k_{OFF1} and k_{OFF2}) in control versus Y27632-treated cells or in the proportion of the rapid recovering fraction of vinculin ($\frac{C_1}{C_1+C_2}$).

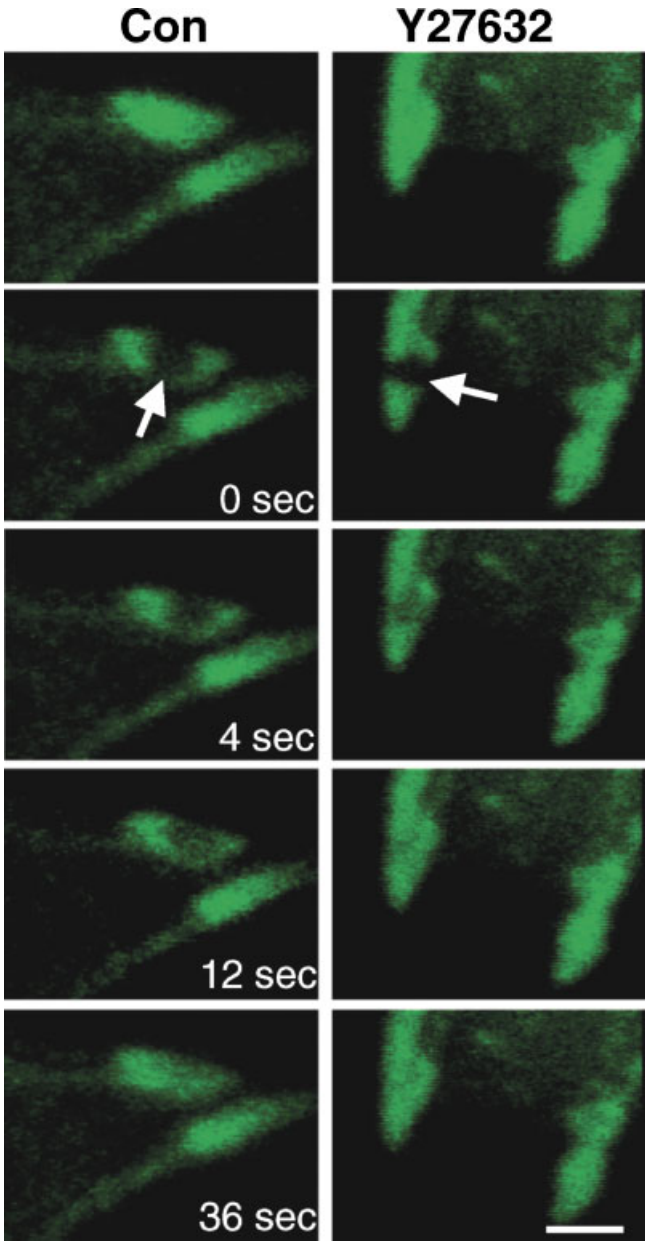

Fig. 3. FRAP analysis of GFP zyxin recovery within individual photobleached focal adhesions. Representative images of a FRAP experiment in control versus Y27632-treated cells showing that force dissipation accelerates zyxin recovery. Arrows indicate photobleached spots within individual focal adhesions that are analyzed over a period of 36 sec follow photobleaching (bar, 2 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

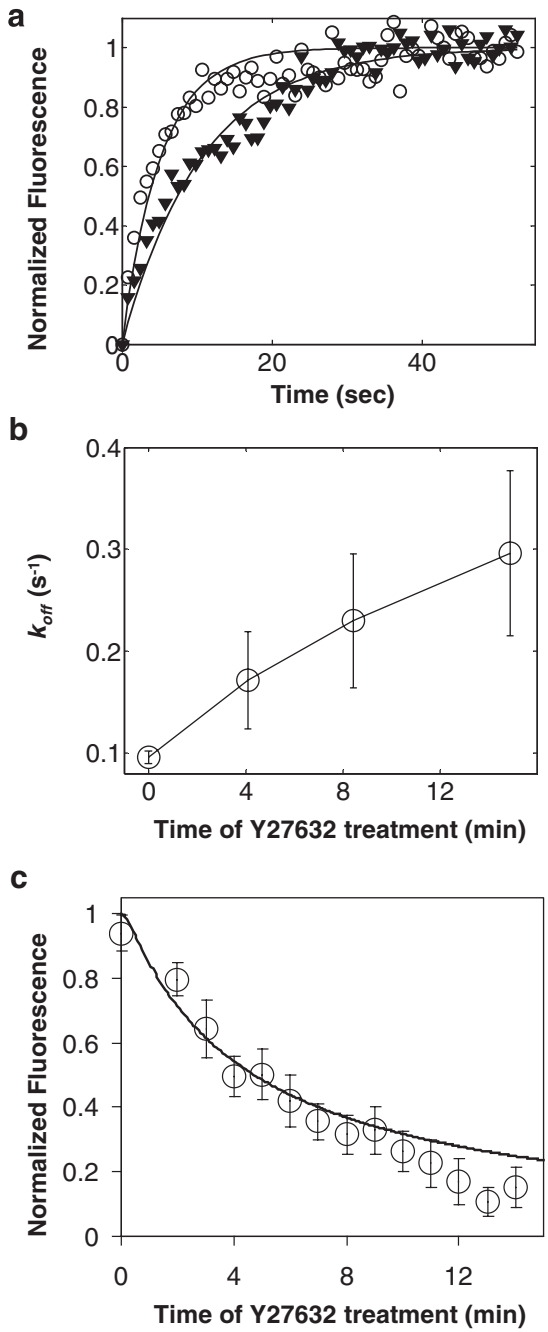

Fig. 4. Force dissipation increases the effective unbinding rate constant of zyxin. **a**: Recovery curve for zyxin in control (open circles) versus Y27632-treated cells (closed triangles) from the experiment shown in Figure 3; solid lines are curves fit to the data using the method of least squares (see Mathematical model in Supplementary Methods). **b**: Measurements of the k_{OFF} of zyxin during the time of exposure to Y27632 (error bars indicate SEM). The k_{OFF} measured in each of the Y27632-treated cells was significantly increased relative to the k_{OFF} measured in control cells ($P < 0.01$). **c**: Loss of zyxin staining from focal adhesions following Y27632 treatment measured over 15 min (open circles) compared with the numerical prediction (solid line) based on a mathematical model that incorporated the changes in the k_{OFF} of zyxin determined using FRAP. The close match (coefficient of determination $R^2 = 0.90$) between the prediction and data demonstrates that the measured increase of k_{OFF} upon Y27632 exposure is sufficient to explain the long-term dynamics of zyxin in focal adhesions.

k_{OFF}, we cannot rule out the possibility that these other properties might change during adhesion disassembly. However, our modeling results show that the changes of k_{OFF} we measured are alone sufficient to explain these effects.

To determine whether the effects on the k_{OFF} of zyxin in cells treated with Y27632 resulted directly from tension dissipation, rather than associated changes in signal transduction (e.g., alterations in other downstream components of the Rho signaling pathway), we physically released cytoskeletal tension from individual focal adhesions by severing an adjacent actin stress fiber using a femtosecond laser (Shen et al., 2005; Supatto et al., 2005). By focusing femtosecond, nanojoule laser

pulses to within a nanometer-scale volume, this method completely vaporizes material in a single living cell with a resolution as small as 200 nm without compromising cell viability (Strahs and Berns, 1979; Koonce et al., 1982; Botvinick et al., 2004; Heisterkamp et al., 2005; Shen et al., 2005). Additionally, this effect is localized so that the effects of ablation are not felt at regions only a few hundreds of nanometers away from the ablated site (Botvinick et al., 2004; Heisterkamp et al., 2005; Shen et al., 2005). We were able to use this method because GFP-zyxin also localizes to some stress fiber (Sadler et al., 1992; Li and Trueb, 2001) bundles as well as focal adhesions (Beckerle, 1997; Reinhard et al., 1999), and thus permits stress fiber visualization before and after laser ablation in our cells (Fig. 5a,b; see movie in Supplementary Information).

Direct release of cytoskeletal tension using this method to cut a single stress fiber resulted in a statistically significant increase in the k_{OFF} of zyxin (Table 1) in the focal adhesions on which it inserted at its distal ends, tens of microns away from the ablation site (Fig. 5b and movie). In contrast, no change in zyxin unbinding kinetics was detected within other distant focal adhesions that remained associated with intact stress fibers in the same cells. Moreover, the change of k_{OFF} measured in response to force dissipation caused by cutting the tensed stress fiber was similar to that produced by Y27632 treatment (Table 1), and this change was detected as fast as we could carry out the FRAP analysis (within 1 min after stress fiber disruption). Thus, the unbinding rate constant of zyxin is sensitive to the level of contractile force exerted on the focal adhesion by adjacent stress fibers, and not due to cell-wide changes of biochemistry induced by tension dissipation.

To investigate if similar effects on unbinding rate constants of adhesion proteins could be triggered by external physiological cues rather than manipulation of intracellular cytoskeletal structure or contractility, we measured the unbinding rate constant of zyxin in cells cultured on flexible polyacrylamide gels of varying stiffness coated with the extracellular matrix protein fibronectin (Fig. 6). Cells cultured on increasingly flexible substrates exert progressively less traction force on their matrix adhesions reflecting a global reduction in cytoskeletal tension (Beningo et al., 2002; Engler et al., 2004; Polte et al., 2004). On the most compliant substrate (Young's modulus = 1 kPa), cells did not form discernible adhesions, however, the k_{OFF} of zyxin in cells cultured on a substrate with intermediate flexibility (50 kPa) was approximately two times higher than that in cells grown on rigid glass substrates coated with the same matrix protein (Fig. 6). Interestingly, the k_{OFF} of zyxin in cells on the least flexible substrate (300 kPa) was only slightly higher than that observed in cells on glass, which has a stiffness that is more than ten times higher (~3,500 kPa (Peyton and Putnam, 2005)).

DISCUSSION

Mechanical forces can influence focal adhesion assembly-disassembly dynamics (Balaban et al., 2001; Riveline et al., 2001) and have differential effects on different focal adhesions proteins (Rottner et al., 2001). However, the molecular biophysical basis for these effects remains unknown. To analyze this mechanism, we modified the FRAP technique so that we could measure force-dependent changes in the unbinding rate constant of the GFP-labeled focal adhesion proteins, zyxin and vinculin, in living cells. In this method, we reduce the size of the photobleached area so that it is significantly smaller than an individual focal adhesion, thus allowing us to measure the unbinding rate constant k_{OFF} of focal adhesion proteins in living cells. Using this approach, we found that the k_{OFF} of zyxin, but not vinculin, is sensitive to mechanical force. These effects are likely to be caused directly by changes in tension in the cytoskeleton, rather than associated changes of cellular biochemistry, because the three different experimental

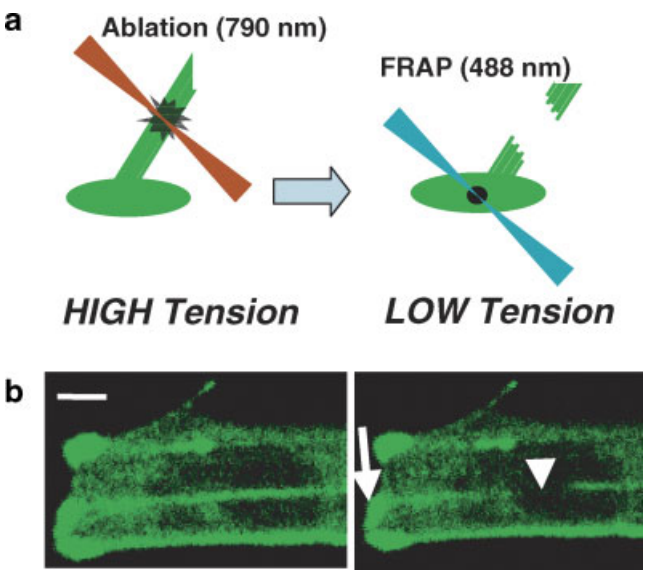

Fig. 5. Effects of laser incision of stress fibers on zyxin recovery measured with FRAP (**a**) schematic of the combined nanosurgery-FRAP experiment: a femtosecond laser (790 nm) is focused on small nanometer wide region of a stress fiber that attaches to a focal adhesion. Physical disruption of the stress fiber by laser ablation relaxes tension on the GFP-zyxin-containing adhesion which is then immediately assayed with FRAP using a 488 nm laser (Heisterkamp et al., 2005). **b**: Exposure of a small (1 μm^2) region of single zyxin-labeled stress fiber to femtosecond laser pulses in the region indicated by the arrowhead resulted in immediate physical disruption of the stress fiber and retraction of its cut ends back towards their sites of insertion on the basal focal adhesions (compare right vs. left images). FRAP analysis was then used to analyze zyxin kinetics in the adjacent focal adhesion (thin arrow) on which the stress fiber terminates (bar, 5 μm), results are presented in Table 1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

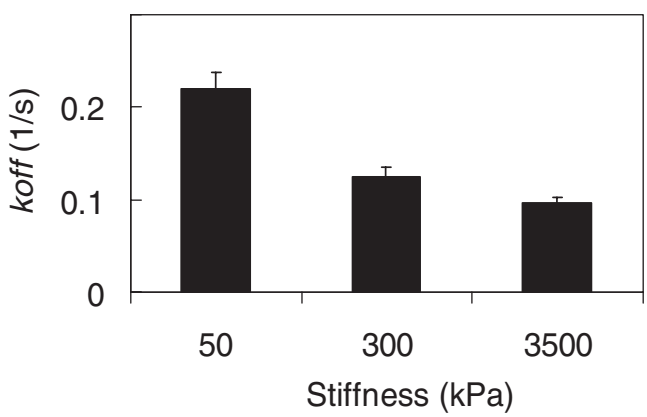

Fig. 6. Effect of matrix compliance on zyxin recovery measured with FRAP measurements of the k_{OFF} of zyxin in cells cultured on fibronectin-coated polyacrylamide gel substrates with different Young's moduli (data points are mean ± SEM). The k_{OFF} values for zyxin within cells cultured on each of the polyacrylamide gels were significantly different from each other ($P < 0.000006$), and they were all significantly increased relative to the zyxin k_{OFF} measured in cells plated on glass ($P < 0.01$).

conditions used to influence cell tension—ROCK inhibition with Y27632, laser ablation of individual stress fibers, and culturing cells on compliant subtrates—produced similar effects even though they act by distinct mechanisms. Moreover, the changes in zxyin's k_{OFF} produced using laser nanosurgery were rapid (detectable within 1 min) and spatially limited to the focal adhesions to which the ablated stress fiber inserted. In addition, we were able to predict the time course of disappearance of zyxin from adhesions measured over several minutes following tension dissipation using a mathematical model that incorporated the force-dependent changes in the k_{OFF} of zyxin we determined experimentally. Thus, the effects of tension dissipation on the k_{OFF} of zyxin are sufficient to explain the effects of force dissipation on the disassembly dynamics of zxyin-containing focal adhesions observed in both the present and past studies.

Although mechanical forces have been shown to alter ion fluxes by changing the kinetic behavior of certain 'stress-sensitive' ion channels on the cell membrane (Sukharev and Corey, 2004), these data provide the first direct experimental evidence of a specific, force-dependent change of molecular binding kinetics of a focal adhesion protein within living cells. This effect is likely to be physiologically relevant given that zyxin's k_{OFF} also changed in cells on culture substrates of different stiffness, and that vascular cells normally 'match' the compliance of their extracellular matrix by altering their internal contractile state (Wang et al., 2002; Yeung et al., 2005). The rapid dissociation of zyxin from focal adhesions also may be important biologically because when cyclic mechanical stretch is applied to vascular smooth muscle cells, zyxin can translocate into the nucleus and activate gene transcription (Cattaruzza et al., 2004).

Importantly, there is distinction between the molecular unbinding rate constant of a protein and its apparent disassembly kinetics measured in living cells. In the context of the focal adhesions, binding kinetics is a direct measure of the strength of interaction between the protein and its molecular binding partners. Unlike measurements of apparent disassembly kinetics that describe large-scale disassembly of the entire focal adhesion that occur over minutes, the unbinding rate constant describes events that take place over seconds and that can be measured in adhesions that are at steady state or in a state of assembly or disassembly. The relatively slow changes of apparent disassembly kinetics can be produced by multiple mechanisms. For example, the dissociation of a focal adhesion protein may be caused by the disappearance of its binding partner, as well as by a change of its binding kinetics under conditions in which the concentration of bindings sites remain constant. Our findings suggest that the apparent 'mechanosensitivity' of zyxin may arise from the latter mechanism and a strong dependence of its unbinding rate constant on tension that is applied to focal adhesions in living cells. This supports the possibility that mechanical forces cause direct conformational changes in either zyxin, or one or more of its binding partners giving rise to a change in its binding affinity to proteins in the adhesion complex.

The finding that zyxin's unbinding rate constant increases with tension dissipation suggests that its binding interactions with other focal adhesion proteins may be similar to molecular catch bonds where bond lifetimes increase with increasing force (Konstantopoulos et al., 2003). While molecular catch bonds and the converse, slip bonds (which break more easily under higher forces) have been extensively investigated in vitro and with physical models (Evans et al., 2004; Barsegov and Thirumalai, 2005; Pereverzev et al., 2005a,b), the methods developed here may actually help to directly investigate similar interactions inside living cells. It is important to note, however, that the k_{OFF} of zyxin measured using our modified FRAP method may represent an average value resulting from its interaction with various binding partners (e.g., α-actinin, VASP; (Beckerle, 1997; Holt et al., 1998; Li and Trueb, 2001)). But if zyxin binds all these partners simultaneously within a single multi-ligand complex, then the k_{OFF} is a direct measure of its unbinding kinetics with this protein assembly. In either case, the finding that addition of peptides that inhibit zyxin binding to α-actinin causes similar release of zyxin from focal adhesions without altering vinculin distribution (Drees et al., 1999) suggests that interactions between zxyin and α-actinin may be central to this process.

It is surprising that the k_{OFF} of vinculin did not change on tension dissipation, even though vinculin also mediates mechanical coupling between integrins and the cytoskeleton (Ezzell et al., 1997) and, hence, undoubtedly experiences force. However, our finding is consistent with a separate study (Sawada and Sheetz, 2002) in which vinculin binding was insensitive to mechanical stretch to triton-extracted cytoskeletons. Thus, tension dissipation must cause vinculin to disassemble from focal adhesions by a different mechanism, and this is consistent with its slower time course. This may involve force-dependent changes in the availability of other focal adhesion proteins, such as talin, that act as molecular binding partners for vinculin.

Taken together, these data indicate that mechanical forces produce changes in focal adhesion assembly by altering the kinetic behavior of only a subset of the molecules that comprise these supramolecular mechanotransduction complexes. Our results emphasize the importance of measuring molecular binding kinetics in living cells for understanding adhesion assembly and disassembly, and demonstrate the value of the improved FRAP technique we described here for gaining greater insight into the molecular biophysical basis of cellular mechanotransduction.

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