Actomyosin-generated tension controls the molecular kinetics of focal adhesions

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Summary

Focal adhesions (FAs) have key roles in the interaction of cells with the extracellular matrix (ECM) and in adhesion-mediated signaling. These dynamic, multi-protein structures sense the ECM both chemically and physically, and respond to external and internal forces by changing their size and signaling activity. However, this mechanosensitivity is still poorly understood at the molecular level. Here, we present direct evidence that actomyosin contractility regulates the molecular kinetics of FAs. We show that the molecular turnover of proteins within FAs is primarily regulated by their dissociation rate constant (k_{off}), which is sensitive to changes in forces applied to the FA. We measured the early changes in k_{off} values for three FA proteins (vinculin, paxillin and zyxin) upon inhibition of actomyosin-generated forces using two methods – high temporal resolution FRAP and direct measurement of FA protein dissociation in permeabilized cells. When myosin II contractility was inhibited, the k_{off} values for all three proteins changed rapidly, in a highly protein-specific manner: dissociation of vinculin from FAs was facilitated, whereas dissociation of paxillin and zyxin was attenuated. We hypothesize that these early kinetic changes initiate FA disassembly by affecting the molecular turnover of FAs and altering their composition.

Key words: Focal adhesions, Actomyosin contractility, Mechanosensitivity

Introduction

The notion that focal adhesions (FAs) are force-sensing structures was proposed over a decade ago, on the basis of experiments with agents that perturb the mechanical properties of cytoskeletal actomyosin (Volberg et al., 1994; Chrzanowska-Wodnicka and Burridge, 1996). Indeed, all known inhibitors of myosin II light chain phosphorylation, as well as the drug blebbistatin, which interferes with actin-dependent myosin II ATPase activity (Kovacs et al., 2004), were shown to prevent the formation of mature FAs, and induce rapid disassembly of existing ones (Geiger et al., 2009; Wolfenson et al., 2009a). The myosin IIA isoform was later shown to have a primary role in the growth and maintenance of FAs (Sandquist et al., 2006; Even-Ram et al., 2007; Vicente-Manzanares et al., 2007). The mechanosensory function of FAs was corroborated by experiments in which FA growth was induced by local mechanical stimulation (Riveline et al., 2001; Sniadecki et al., 2007). Notably, the incorporation of some protein components into FAs can even be triggered by stretching detergent-demembranated cytoskeletons (Sawada and Sheetz, 2002).

In the search for molecular mechanisms underlying FA mechanosensitivity, several pathways were recently discovered. Binding of vinculin to talin was shown to be strongly enhanced by stretching the talin rod, an act that unfolds cryptic vinculin-binding sites (del Rio et al., 2009). Moreover, stretching forces applied to an integrin molecule enhanced its interactions with extracellular matrix (ECM) proteins in a 'catch-bond' fashion (Friedland et al., 2009; Kong et al., 2009). Signaling components of FAs, such as p130Cas, were also shown to display mechanosensory characteristics (Sawada et al., 2006). Recently, it was shown that recruitment of vinculin to FAs is mediated by myosin II contractility-induced paxillin phosphorylation by focal adhesion kinase (FAK) (Pasapera et al., 2010). Several other mechanisms were also suggested, based on theoretical models of FA organization (Nicolas et al., 2004; Bruinsma, 2005; Shemesh et al., 2005; Besser and Safran, 2006). Morphological monitoring of FA precursors (nascent FAs or focal complexes) has shown them to be short-lived adhesions that can either disassemble or grow into FAs, a process that is apparently driven by mechanical stress (Riveline et al., 2001; Vicente-Manzanares et al., 2007).

In addition to monitoring FAs at the structural level, FA dynamics were also studied at the molecular level: FRAP studies have shown that FA proteins exchange with cytoplasmic proteins at varying rates (Ballestrem et al., 2001; Hamadi et al., 2005; Lele et al., 2006; Wolfenson et al., 2009b); fluorescence correlation microscopy demonstrated that the binding or unbinding of FA proteins depends on FA assembly or disassembly status, location in the cell and apparent translocation (Digman et al., 2008; Digman et al., 2009); additional studies gave a measure of the integrin–actin linkage (Brown et al., 2006) and of the coupling between the centripetal flow of FA proteins and actin, through FAs (Hu et al., 2007; Gardel et al., 2008; Ji et al., 2008).

Taken together, the above data indicate that FAs are mechanosensitive, dynamic structures that undergo structural reorganization on a time scale of minutes to hours, and that molecular turnover of their plaque components occurs within seconds. However, the mechanism(s) whereby mechanical force affects FA structure and, in particular, the effects of force on the turnover of individual proteins within FAs, remain enigmatic.

Here we show, in both live and permeabilized cells, that actomyosin-generated forces regulate the molecular kinetics of vinculin, paxillin and zyxin. Interestingly, inhibition of actomyosin tension using the drug blebbistatin had distinct, often opposite

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effects on the three proteins: paxillin dissociation from FAs was attenuated by blebbistatin-mediated relaxation, zyxin displayed a similar (albeit weaker) response, whereas vinculin dissociation was facilitated by this treatment. At a later stage, inhibition of actomyosin contractility by blebbistatin led to the gradual disassembly of FAs. This effect was also differential, as the three proteins exited FAs at different onset times, and at different rates. Based on these findings, we suggest that changes in the molecular kinetics of FA components following inhibition of actomyosin-based contractility ultimately lead to changes in FA molecular composition and to gradual FA disassembly.

Results

Inhibition of myosin II contractility leads to long-term FA disassembly at varying rates, depending on the FA protein

To determine the effects of the relaxation of mechanical stress on FA organization, we analyzed time-lapse movies for changes induced by blebbistatin in the local intensities of YFP-tagged FA plaque proteins in HeLa cells. We focused on paxillin, vinculin and zyxin because of their diverse turnover rates within FAs (Lele et al., 2006; Wolfenson et al., 2009b). For paxillin and vinculin, the observed differences in turnover rates are particularly intriguing, because they were shown to directly interact with each other (Turner and Miller, 1994; Salgia et al., 1995; Mazaki et al., 1997). Also, zyxin and vinculin have been shown to be highly mechanosensitive (Zaidel-Bar et al., 2003; Mierke et al., 2008), but the dependence of paxillin organization on mechanical stress has not been characterized.

In line with previous studies (Nayal et al., 2006; Zaidel-Bar et al., 2007a), blebbistatin induced major changes in the distribution of all three proteins, manifested by partial disruption of large FAs and accumulation of small FAs along the cell periphery (Fig. 1A). Quantitative analysis of large FAs located at cell lamella indicated that the rate of intensity loss differed for each protein (Fig. 1B). Paxillin displayed a delayed response to blebbistatin, which was only apparent 8–10 minutes after applying the drug, and its levels within FAs decreased gradually to ~20% of the initial intensity over 60 minutes. Vinculin levels in FAs began to decline earlier (after ~2 minutes) and at a faster rate, stabilizing at 15–20% of the initial intensity within 35–40 minutes of blebbistatin treatment. Changes in zyxin levels were apparent at ~1 minute after addition of the drug, followed by a rapid decrease in its FA levels, which stabilized at ~10% of the initial intensity after 20 minutes. Notably, the FA-associated fluorescence of the three proteins in untreated cells (no blebbistatin) remained relatively stable over time, showing only small, transient fluctuations (Fig. 1C and supplementary material Fig. S1). These results indicate that different plaque proteins exit FAs at varying rates, following blebbistatin treatment, with typical half-times ranging from 10–15 minutes for zyxin and vinculin, to ~40 minutes for paxillin. Notably, this time frame is considerably longer than the exchange rates of the respective proteins, as determined by FRAP (see below). Specifically, the values of the characteristic fluorescence recovery time for the molecular exchange process (τ_{ex}, the time required to attain half of the recoverable fluorescence by exchange in a FRAP experiment), measured for the same proteins in HeLa cells in the absence of blebbistatin, were 9.1 seconds for paxillin, 40.1 seconds for vinculin and 2.8 seconds for zyxin (see below). These values are about 260, 20 and 220 times less than the corresponding half times for exit of these proteins from FAs following blebbistatin treatment. Performing the opposite experiment, using the serine/threonine

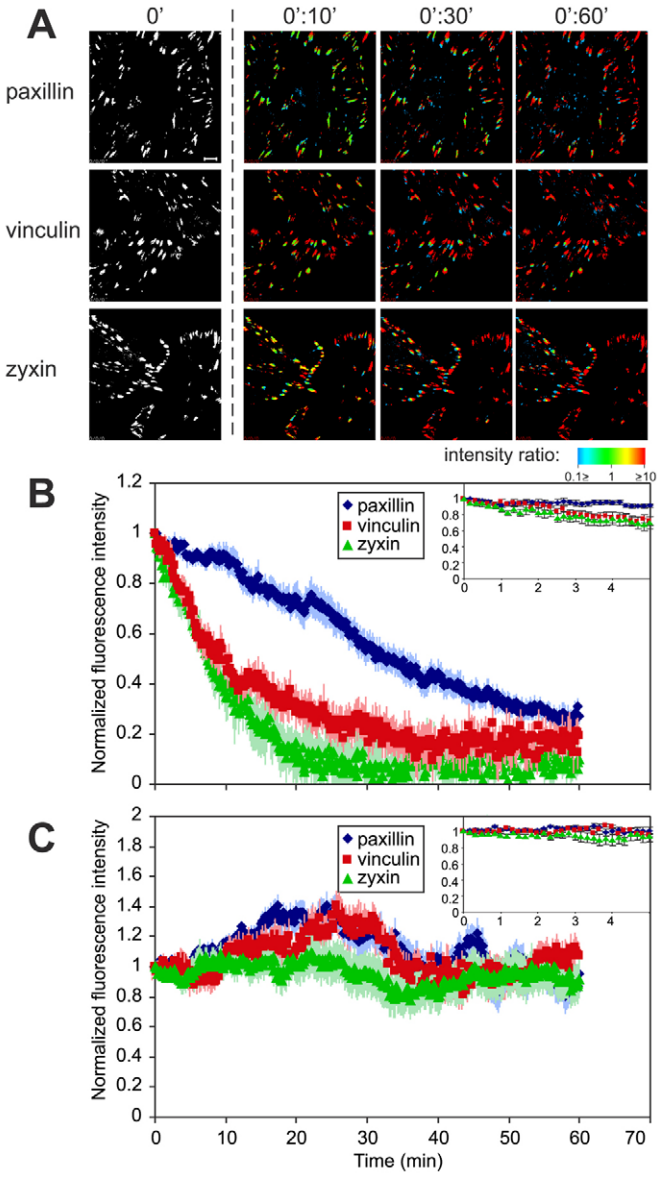

Fig. 1. Effect of blebbistatin on FA reorganization in HeLa cells expressing YFP-tagged paxillin, vinculin or zyxin. Frames from time-lapse movies were analyzed by temporal ratio imaging. (**A**) Ratio between the intensity at time point 0 (first column) and time points 10, 30 and 60 minutes. Scale bar: 5 µm. Red shift denotes structures with decreased intensity; blue shift denotes an increase in intensity; green and yellow hues mark unchanged pixels. Whereas paxillin showed a delayed reaction to blebbistatin, vinculin and zyxin responded much faster (compare time points 0':10' and 0':30'). (**B**,**C**) Mean relative FA fluorescence (± s.e.m., in lighter hues) over time, as derived from the time-lapse movies (n=10–15). FAs in cells treated with blebbistatin disassemble over time, resulting in very small FAs (B), whereas FAs in untreated cells remain stable over time, and their mean relative fluorescence levels fluctuate around 100% (C).

phosphatase inhibitor calyculin A (5 nM) for 10 minutes to increase actomyosin contractility (Ishihara et al., 1989; Henson et al., 2003), resulted in a gradual increase in FA area, without influencing the intensity of paxillin–YFP (Fig. 2A). Interestingly, in the 10 minutes after addition of calyculin A, there were no noticeable differences in paxillin–YFP FRAP when the laser beam was focused on the

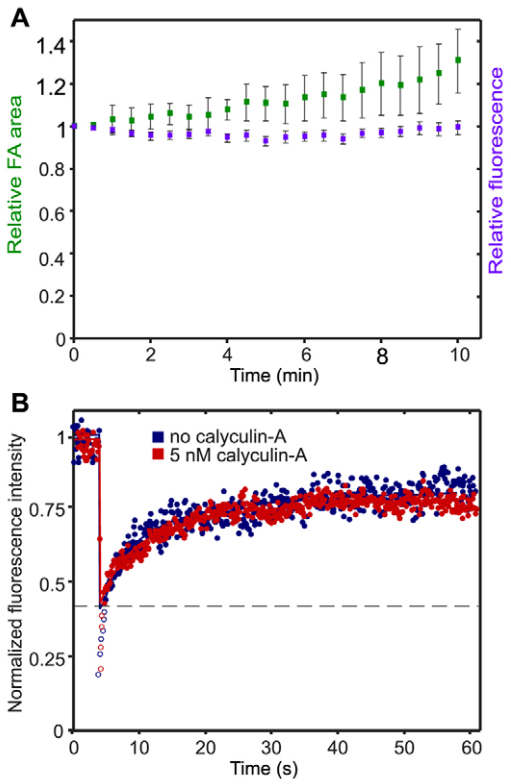

Fig. 2. Effects of calyculin A treatment on FA area, fluorescence intensity and FRAP in HeLa cells expressing paxillin–YFP. (**A**) Live-cell imaging was used to monitor the relative FA area (green) and fluorescence intensity (purple) in response to calyculin A treatment. Images were taken every 30 seconds, and the resulting data were analyzed using ImageJ. During the 10 minute incubation with calyculin A, a gradual increase in FA area was noticed, without influencing paxillin–YFP intensity. Values are means ± s.e.m. (**B**) Typical FRAP curves of YFP-tagged paxillin before (blue) and after (red) treatment with calyculin A. Solid lines denote the best fit of a least squares regression analysis. Empty data points below the dashed line correspond to recovery by diffusion; the slower phase represents proteins that recover by exchange (Wolfenson et al., 2009b). No significant change in the exchange rate of paxillin–YFP was noted within the 10 minute interval following calyculin A treatment.

center of FAs (to avoid regions of FA growth; Fig. 2B). These results suggest that there is an apparent plateau in the dependence of the exchange rate on force, so that the added contraction in the calyculin-A-treated cells does not affect the exchange kinetics.

Relaxation of actomyosin-generated tension rapidly changes the rate of molecular turnover of different FA components in a protein-specific manner

The discrepancy between the rates of net loss of FA proteins and their normal exchange rates, seen when myosin-II contractility was inhibited, raises an intriguing question: what is the relationship between the molecular kinetics of FA proteins and their overall structural reorganization, when mechanical force is reduced? To address this issue, we took advantage of the fact that the local intensity of each of the FA proteins did not decline substantially during the initial 5 minutes after addition of blebbistatin (Fig. 1). Within this time frame, no major alterations in actin filament organization were noticed (data not shown). However, the reduction in traction force was almost immediate, as could be deduced by observing wrinkles produced by HeLa cells plated onto flexible silicone rubber substrate (Harris et al., 1980); blebbistatin treatment led to the disappearance of such wrinkles in less than 20 seconds (Fig. 3A). Also, all three proteins showed similar kinetics of reduction in FA area within 8 minutes of blebbistatin treatment, and differences only appeared later (Fig. 3B). The reduction in FA area typically occurred at the distal edge of the FA (the edge pointing towards the cell periphery), and was sometimes accompanied by inward translocation of the FAs, in line with previous studies (Zamir et al., 2000; Ballestrem et al., 2001). Notably, it was shown that the turnover rate of FA plaque proteins at this edge is much slower than that seen at the center of FAs (Webb et al., 2003; Wolfenson et al., 2009b).

In view of these findings, we chose to focus on the initial 5 minutes after the addition of blebbistatin, and conduct FRAP experiments in the center of FAs (to avoid regions of FA reduction or translocation) within this timeframe. Notably, we have recently shown (Wolfenson et al., 2009b) that FA plaque proteins exist in four dynamic states at steady state: a fast-diffusing cytoplasmic pool; a juxtamembrane cytoplasmic fraction surrounding FAs and displaying attenuated diffusion; an FA-associated fraction undergoing exchange with the juxtamembrane fraction; and an immobile FA-bound fraction. To explore the mechanosensitivity of these kinetic processes, we now performed FRAP studies on live cells before or within 5 minutes of blebbistatin treatment. These measurements indicated that force reduction by blebbistatin had no effect on the cytoplasmic-diffusing and juxtamembrane-diffusing fractions (supplementary material Fig. S2), but did alter the kinetics of the exchanging and immobile fractions of all three proteins inside FAs (Fig. 4). For paxillin and zyxin, the effects of blebbistatin were similar, manifested by a reduction in both the mobile fraction (R_f) and the exchange rates of the exchanging population, as determined by fitting the FRAP data to the analytical expression for FRAP by diffusion in the juxtamembrane region, followed by exchange (see the Materials and Methods) (Wolfenson et al., 2009b). For vinculin, however, treatment with blebbistatin increased both the exchange rate and R_f (Fig. 4). Similar effects on the three proteins were observed within ~1–2 minutes of treatment with the Rho kinase inhibitor Y-27632 (25 μM), which is known to reduce cell contraction (supplementary material Fig. S3), supporting the notion that the effects observed are controlled by mechanical force. A similar effect on paxillin recovery was also seen when the same experiments were performed on nontransformed cells (REF52 rat embryo fibroblasts). Here, force reduction by blebbistatin (measured 1–5 minutes after addition of the drug) induced an increase in τ_{ex} of paxillin–YFP, from 10.5 seconds to 133 seconds. These findings indicate that the force-reduction effect is general, and not limited to transformed cells. Taken together, the results presented here indicate that changes in turnover kinetics following force reduction differ, among individual FA-associated proteins.

Reduction of actomyosin-generated forces regulates the dissociation of plaque proteins from FAs

In principle, modulation of the exchange rate of specific proteins from FAs could involve a change in either k_{off} or k_{on} (the dissociation and association rate constants, respectively), or in both. Using blebbistatin and detergent-permeabilized cells, we were able to gain direct information on the force dependence of the processes that control the exchange rates of paxillin, vinculin and zyxin in FAs. To this end, we directly measured the dissociation rates of these FA proteins in Triton X-100-permeabilized cells, in which

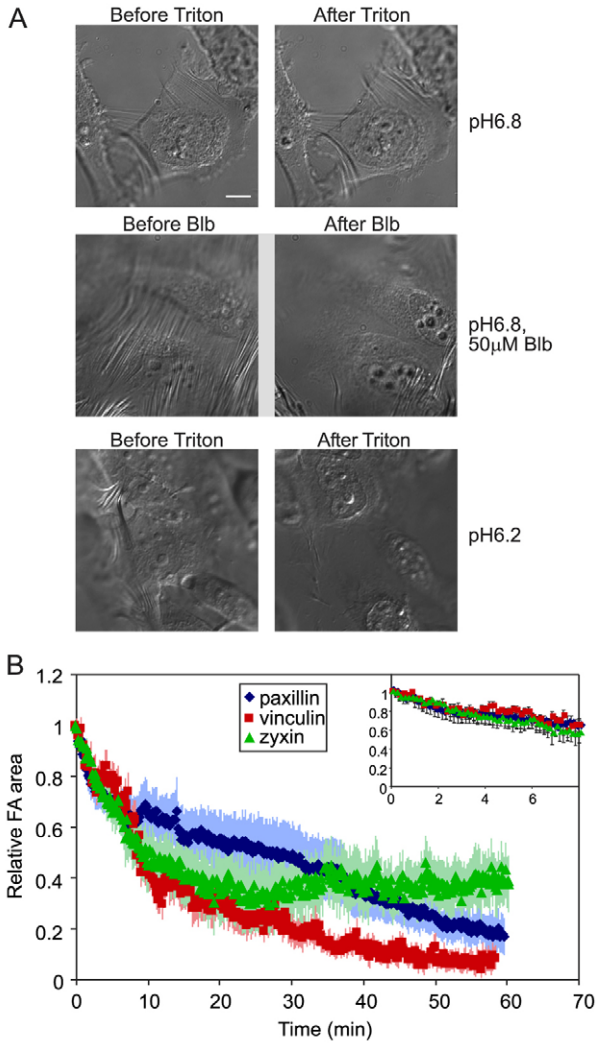

Fig. 3. Effects of force reduction on cell contraction and FA area. (**A**) Phase-contrast images of HeLa cells plated on coverslips pre-coated with elastic silicon substrata. Contraction of the cells induces wrinkling of the silicon surface (Harris et al., 1980). Once the wrinkles are created by the cells, they usually persist for several hours. Top panels, HeLa cells in medium, before (left) and after (right) addition of Triton X-100 (0.1% final concentration in 20 mM HEPES buffer, pH 6.8, for 10 minutes). Virtually no change in the wrinkles is evident following permeabilization of the cells, suggesting that the cellular tension is unaltered under these conditions. Middle panels, cells before (left) and after addition of blebbistatin (Blb) to a final concentration of 50 μM (right). Note that the wrinkles vanish almost completely. Bottom panels, cells before (left) and after (right) permeabilization with Triton X-100 in 50 mM MES buffer, pH 6.2 (tension-relaxing conditions) (Metzger and Moss, 1990). The wrinkles disappear within 1–2 minutes of permeabilization at the lower pH. (**B**) Effect of blebbistatin on FA area in HeLa cells expressing YFP-tagged paxillin, vinculin or zyxin. Mean relative FA areas (± s.e.m., in lighter hues) over time were derived from time-lapse movies (n=10–15) of cells treated with blebbistatin. For all three proteins, the total FA area decreased essentially instantaneously following blebbistatin treatment, reaching a value of ~60% of the initial areas within 8 minutes. From that point onwards, the rates at which the area decreased differed among the three proteins: for paxillin, the FA area decreased gradually to 20% of the initial area after 60 minutes; for vinculin, the FA area continued to drop at a similar rate for an additional 4 minutes, and then gradually decreased to 10% of the initial area after 60 minutes; for zyxin, the FA area continued to drop at a similar rate for an additional 4 minutes, but unlike vinculin, it then stabilized at 40% of the initial area, within 60 minutes after treatment with blebbistatin.

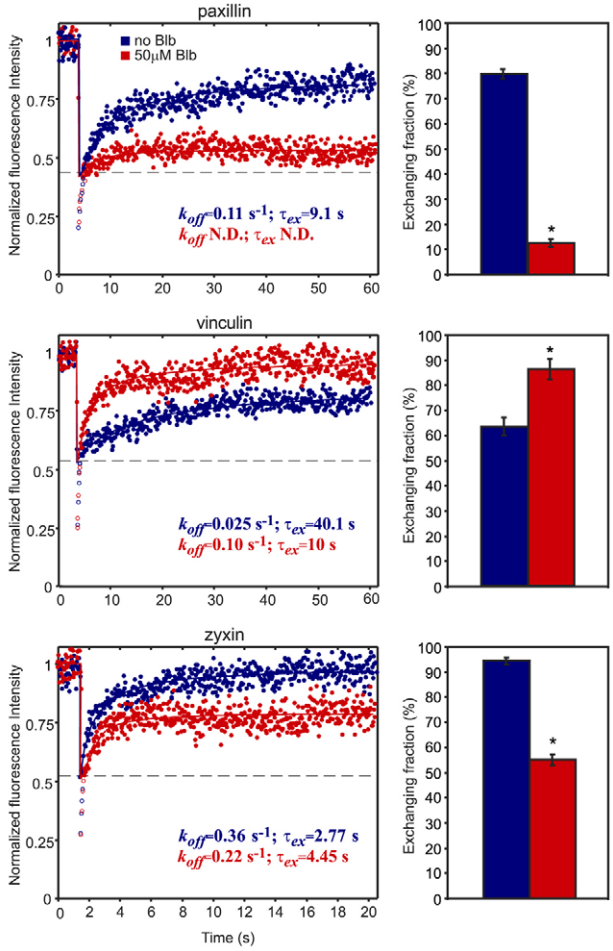

Fig. 4. Effect of blebbistatin treatment on FRAP in FAs. Typical FRAP curves (left panels) of YFP-tagged paxillin, vinculin or zyxin, with (red) or without blebbistatin (blue). Solid lines denote the best fit of a least squares regression analysis. Average τ_{ex} and k_{off} values for each protein are shown (n=20–30). Empty data points below the dashed line correspond to recovery by diffusion; the slower phase represents proteins that recover by exchange (Wolfenson et al., 2009b). Mean ± s.e.m. (n=20–30) of the fractions undergoing exchange are shown on the right (*P<10^{-4}; Student's t-test). For paxillin and zyxin, blebbistatin induced a decrease in the exchange rates, accompanied by lower exchanging fractions. For vinculin, blebbistatin induced the opposite effect, increasing both the exchange rate and the exchanging fraction.

actomyosin contractility was either maintained or inhibited. When permeabilized, cytoplasmic proteins are diluted to near infinity as they diffuse into the extracellular medium, dramatically reducing the cytoplasmic concentrations of plaque proteins, and effectively preventing their re-association with FAs. Thus, in these permeabilized cells, association with FAs is negligible, and dissociation from FAs prevails. We permeabilized the cells in HEPES buffer (pH 6.8) containing Triton X-100, which provides conditions under which the cells preserve their contractile properties, as evidenced by their capacity to maintain the deformation of the flexible substrate to which they adhere (Fig. 3A). Under these conditions, the permeabilized cells lost ~80% of FA-associated paxillin (Fig. 5). Incubating the cells with blebbistatin before permeabilization, or permeabilizing the cells in MES buffer, pH 6.2 (tension-relaxing conditions) (Metzger and Moss, 1990),

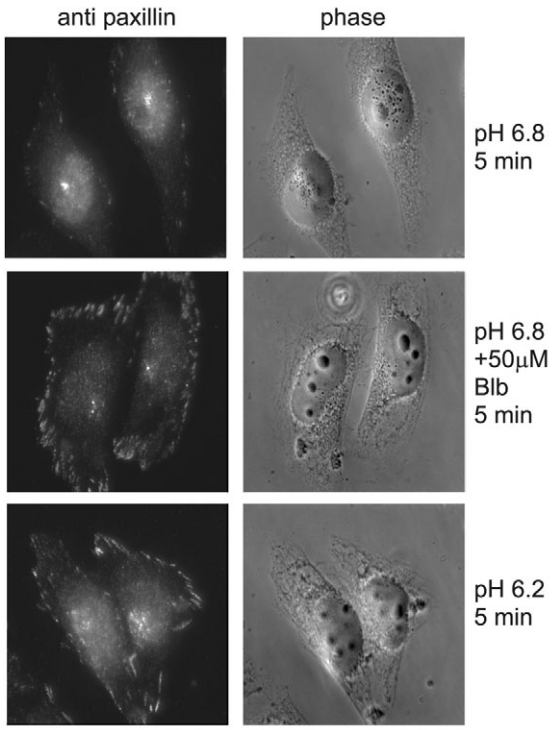

Fig. 5. Effect of reduction of actomyosin-generated forces on paxillin dissociation from FAs. Fluorescent (left) or phase-contrast images (right) of HeLa cells plated on FN-coated coverslips. Cells were incubated (5 minutes) in either HEPES buffer, pH 6.8, supplemented with Triton X-100, with or without 1 minute pre-incubation with blebbistatin, or in MES buffer, pH 6.2, supplemented with Triton X-100. Cells were then fixed and immunolabeled for paxillin. At pH 6.8, in the absence of blebbistatin, almost no FAs were detected. However, substantial amounts of FAs were identified in cells pre-incubated with blebbistatin at pH 6.8, or incubated at pH 6.2.

induced instantaneous relaxation of the cells (Fig. 3A), and dramatically reduced the loss of paxillin from FAs (Fig. 5). These results suggest that relaxation of actomyosin-generated tension attenuates paxillin dissociation from FAs.

We next adapted the permeabilized cell models for real-time monitoring of the effects of mechanical force on the k_{off} of paxillin, vinculin and zyxin. As shown in Fig. 6, all three proteins followed first-order dissociation kinetics from FAs, enabling us to extract the k_{off} values from the half-time of the fluorescence loss curves ($t_{1/2}$). Notably, for all three proteins, these k_{off} values were essentially identical to those derived from the FRAP experiments (compare Figs 4 and 6). Analogous studies of cells treated with 50 μM blebbistatin for 1 minute before permeabilization were in excellent agreement with the FRAP data. Thus, k_{off} for paxillin and zyxin in the presence of blebbistatin decreased from 0.1 second^{-1} to 0.011 second^{-1} and from 0.35 second^{-1} to 0.22 second^{-1}, respectively. However, k_{off} for vinculin increased from 0.027 second^{-1} to 0.078 second^{-1}. Permeabilizing the cells at pH 6.2 reduced k_{off} for paxillin and zyxin, and increased k_{off} for vinculin (data not shown), which is similar to the effects induced by blebbistatin. These results corroborate the findings from the FRAP experiments, and show that the molecular turnover of proteins within FAs is primarily regulated by k_{off} (see below), which, in turn, is sensitive to changes in the forces applied to the FA.

Discussion

The current study provides the first direct evidence for the involvement of mechanical force in regulating the molecular kinetics of FA components. In an earlier report (Lele et al., 2006), FRAP was used for monitoring the effects of force on FA dynamics; however, the limited temporal resolution in that study did not allow for proper analysis of the initial phase of the FRAP curves. Furthermore, the possible differential effects on different FA plaque proteins were not investigated. The assumption in that study was that recovery in the cytoplasm is instantaneous; as shown in our studies, this assumption is not valid for the juxtamembrane cytoplasm above FAs, where FA plaque proteins display attenuated diffusion (Wolfenson et al., 2009b). Moreover, the studies presented herein provide the first direct measurement of k_{off} from FAs using the detergent-mediated dissociation assay, and enable comparison between this biochemical assay and FRAP measurements conducted on live cells. Our use of permeabilized cell models, coupled with an ability to conduct FRAP measurements at a temporal resolution high enough to record the initial phase of the FRAP curves, and to fit the experimental data to a model of diffusion plus exchange, shed new light on the mechanical regulation of FA molecular kinetics.

Dissociation of plaque proteins from FAs is the rate-limiting step in their molecular turnover

One of the assumptions in the proposed model in our previous study (Wolfenson et al., 2009b) was that protein exchange within FAs at steady state obey first-order kinetics, where $k_{off}C_m=k_{on}C_c$ [C_m and C_c represent protein concentrations in the membrane and the cytoplasm, respectively (Wolfenson et al., 2009b)]. Here, we present direct evidence that the dissociation of paxillin, vinculin and zyxin from FAs is indeed a first-order process (Fig. 6). Moreover, the dissociation rates measured in the permeabilized cell models were essentially identical to those obtained by fitting the FRAP data to the analytical expression based on the above model. Given the fact that in the permeabilized cells, the only process effectively measured is dissociation from FAs, we suggest that the dissociation of FA plaque proteins from the membrane is the rate-limiting step in the exchange process. Thus, in order for a cytoplasmic molecule to bind to a FA, a bound molecule has to first dissociate from its binding partner(s) on the membrane. Otherwise, a second-order process, in which association is the rate-limiting step, would occur (i.e. proteins entering the FA would cause the dissociation of pre-bound proteins). This latter mechanism is possible, but rather unlikely, because two independent methods for k_{off} determination (FRAP and loss of fluorescence from FAs in permeabilized cells) yielded essentially the same values, although in the latter method, re-association was negligible as a result of dilution of the dissociating protein into the medium. Although one cannot fully exclude the possibility that treatment with Triton X-100 might have some differential effects on the kinetics of individual FA components, the fact that we obtained essentially the same k_{off} values for all three proteins tested as those measured by FRAP in intact cells, strongly suggests that if such effects exist, they do not significantly interfere with our measurements. It should be noted that association of proteins with FAs does occur in the unperturbed cells measured in the FRAP experiments; however, because dissociation is the rate-limiting step, and the cytoplasmic reservoir of plaque proteins is large enough so that association with FAs does not alter their effective concentrations in the cytoplasm, the product of $k_{on}C_c$ can be regarded as a constant.

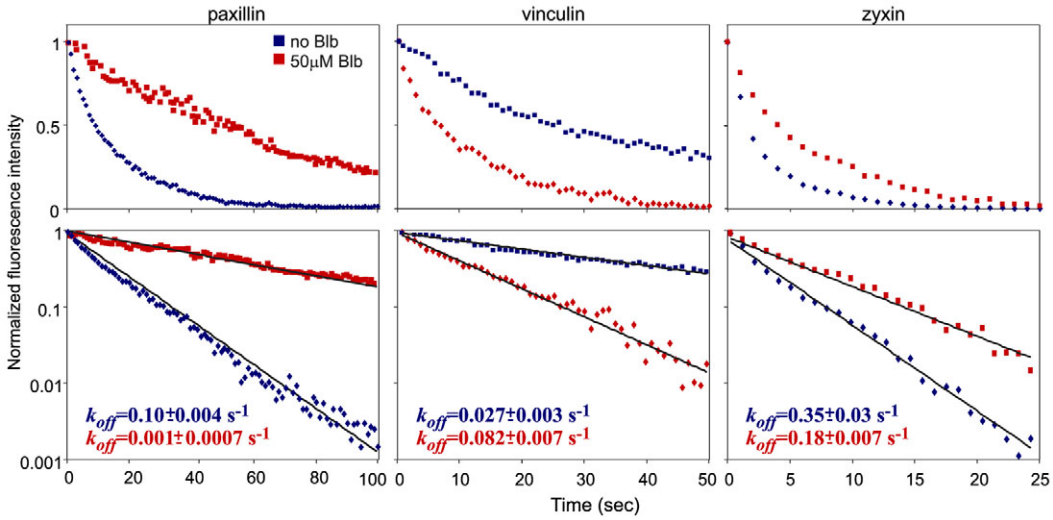

Fig. 6. Typical curves of fluorescence loss from FAs after treatment with Triton X-100. Time-lapse movies of HeLa cells expressing YFP-tagged paxillin, vinculin or zyxin, treated with Triton X-100 at pH 6.8, with or without pre-incubation with blebbistatin, were analyzed for loss of fluorescence from FAs. The same data for each protein are presented on linear (top) and logarithmic scales (bottom). Blebbistatin reduced the fluorescence loss rates for paxillin and zyxin, but enhanced the loss rate for vinculin. k_{off} ± s.e.m. values (calculated using $k_{off}=\ln(2)/t_{1/2}$ for a first-order process; see the Materials and Methods) are presented for untreated (blue) or blebbistatin-treated cells (red) (n=15–20). Note the similarities between the k_{off} values presented here, and those in Fig. 4.

Model for the observed differential effects of actomyosin contractility on the turnover of specific proteins within FAs

Quantification of blebbistatin-induced relaxation on FAs showed that it affected the three plaque proteins in differing ways: paxillin dissociation from FAs was strongly attenuated and zyxin was similarly affected, although less dramatically; however, dissociation of vinculin from FAs was facilitated by blebbistatin. These results indicate that inhibition of actomyosin contractility does not merely enhance long-term FA disassembly, but has specific effects on the exchange kinetics of different FA components. To illustrate this principle, we propose a hypothetical model that could account for the observed differential effects of actomyosin relaxation on the turnover of distinct FA proteins. Conceptually, this model suggests that the FA multiprotein scaffold is subject to mechanical forces generated by the contractile machinery of the cell. These forces can deform the scaffold (which is probably elastic), thereby changing the distribution of binding sites, and hence, the apparent affinity of different FA components (e.g. paxillin, vinculin and zyxin). An intriguing feature is that reduction of actomyosin-generated stress might reduce k_{off} for some components (paxillin, zyxin) but increase k_{off} for others (vinculin). A schematic representation of such a hypothetical model is shown in Fig. 7. This model suggests that the availability of binding sites for specific components is differentially regulated following force-induced extension, or upon relaxation of the elastic FA-associated scaffold. Specifically, for paxillin and zyxin, force reduction resulted in decreased dissociation rates, while at the same time, a long-term FA disassembly process was observed. This indicates that even though dissociation is the rate-limiting step in the process of molecular turnover, it is not the only determining factor in long-term FA disassembly; rather, effects on the association rates of plaque proteins with FAs might have a major role later. This notion is in line with the model presented here, because changes in the availability of binding sites might affect both k_{on} and k_{off}. Interestingly, zyxin was shown to translocate from FAs to stress fibers upon application of force, whereas paxillin did not (Yoshigi et al., 2005), suggesting either an increase in the affinity of zyxin to actin under stretching forces, or greater availability of zyxin in the cytoplasm, as a result of its dissociation from FAs. Our findings indicate that under force relaxation, k_{off} for zyxin is reduced (Figs 4, 6) and zyxin levels in FAs are rapidly diminished (within ~1 minute; unlike paxillin, which showed a delayed response; see Fig.

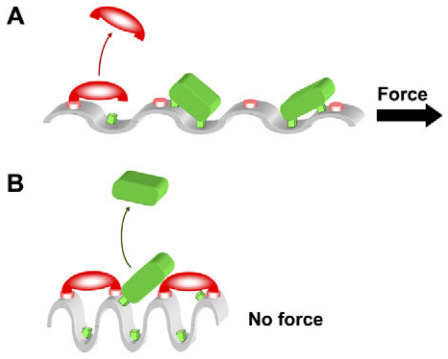

Fig. 7. A hypothetical scheme depicting the dynamics of FA proteins at steady state and immediately after relaxation of the force applied to the FA. FAs are represented by an elastic scaffold to which molecules are transiently bound, and exchange with similar molecules outside the FA (red and green symbols). (**A**) When an FA experiences a threshold pulling force (black arrow), the conformation of the binding modules and their mutual organization permit the steady-state level of exchange of FA components (which differ between proteins). (**B**) When the pulling force drops, the conformation and organization of the modules change, due to contraction of previously stretched internal elements. As a result, dissociation of some proteins from the modules is facilitated (green symbols), whereas others become more strongly tethered, and their dissociation is inhibited (red symbols). These changes in dissociation rates create an imbalance in the module composition, which eventually leads to FA disassembly (data not shown).

1). These observations suggest that the reduction in the association rate of zyxin is more drastic than in its dissociation rate, in line with former observations (Yoshigi et al., 2005). Apparently, this is not the case for paxillin, because the initial rate of loss of paxillin fluorescence from FAs after blebbistatin treatment (Fig. 1) is relatively low. Testing of the model presented here will require extensive characterization of the molecular and physical properties of the FA scaffold, as well as the hierarchy of molecular interactions that regulate the recruitment of specific components from the cytoplasm.

In this regard, the differences noted between the response of paxillin and vinculin to force reduction might at first seem surprising, because the two are known to directly bind each other at FAs. However, we suggest that under these conditions, other pathways could have a role in determining the exchange kinetics of these proteins. These include interactions with other components of the integrin adhesome (Zaidel-Bar et al., 2007b), conformational changes in proteins that could affect their interactions (Grashoff et al., 2010; del Rio et al., 2009), or post-translational modifications that might regulate the binding and dissociation of the proteins (Nayal et al., 2006; Zaidel-Bar et al., 2007a). Interestingly, it was recently shown that treatment with blebbistatin for 1 hour results in disruption of the paxillin–vinculin interaction, because of the reduced phosphorylation levels of paxillin (as a result of decreased FAK recruitment to FAs) (Pasapera et al., 2010). We propose that such long-term, signaling-dependent effects are important for regulation of the composition of FAs, whereas the immediate short-term effects on the turnover of plaque proteins in response to force reduction might provide the underlying mechanism for the initiation of FA disassembly.

The results of the current study, and in particular, the fact that the turnover of individual FA plaque proteins is differentially regulated by mechanical force, imply that changes in stress at FAs ultimately lead to changes in their molecular composition. This might be attributed to the variable rates of dissociation and onset of response, following alterations in local stress (Fig. 1). This notion is in line with the measured effect of actomyosin relaxation on FA molecular composition (Zamir et al., 2008). Moreover, it is conceivable that under conditions of long-term stress reduction, alterations in composition underlie the changes in FA area and stress fiber induction. Notably, however, such global changes occur at rates that are 1–2 orders of magnitude slower than the molecular exchange rate of the corresponding proteins (compare the timescales in Figs 1, 4 and 6). These new findings on FA dynamics present a novel perspective on FA organization at steady state. At this stage, this perspective is necessarily incomplete, because out of the vast repertoire of FA proteins, we focused here on only three, and a detailed study of other key proteins is still missing. In addition, the link between mechanosensitivity at the molecular and structural levels should be studied thoroughly, perhaps by observing its effects on these levels under different known force regimes with quantitative measurement of the force generated by the actomyosin machinery.

Materials and Methods

Reagents, plasmids and buffers

MES, HEPES, PBS, HBSS, polyethylene glycol (PEG) 8000, EGTA and fibronectin (FN) were supplied by Sigma. TransIT-LT1 transfection reagent was purchased from Mirus. Blebbistatin was purchased from Calbiochem. The buffers used for experiments involving live cell permeabilization consisted of HBSS supplemented with 2% PEG8000, 1 μM EGTA, either with 20 mM HEPES, pH 6.8 or 50 mM MES, pH 6.2. In all experiments, a concentration of 50 μM blebbistatin was used.

Cell lines and transfection

The HeLa-JW cell line expressing paxillin–YFP was previously described (Paran et al., 2006). For experiments involving transient expression (vinculin–YFP, zyxin–YFP), cells were transfected with TransIT-LT1 transfection reagent. All cell lines were cultured in DMEM, supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM glutamine. In all experiments, cells were plated on 20 μg/ml fibronectin-coated coverslips. All cell culture reagents were supplied by Biological Industries.

FRAP measurements

FRAP studies were conducted on live cells expressing distinct YFP-tagged FA proteins. The cells were taken for FRAP experiments 24 hours after plating on glass coverslips coated with 20 μg/ml fibronectin. Measurements were taken in HBSS supplemented with 20 mM HEPES, pH 7.2, at 37°C. An argon ion laser beam (Innova 70C; Coherent) was focused through a fluorescence microscope (AxioImager D.1; Carl Zeiss MicroImaging) to a Gaussian spot of 0.77±0.01 μm (plan-apochromat 63×/1.4 NA oil-immersion objective). After a brief measurement at monitoring intensity (528 nm, 1 μW), a 5 mW pulse (~10 ms) was used to bleach 50–75% of the fluorescence in the spot. The time course of fluorescence recovery was tracked by the attenuated monitoring beam. In Fig. 4, the k_{off} and R_f values were extracted (except in the case of paxillin in blebbistatin-treated cells, where k_{off} could not be determined owing to inability to fit such shallow curves) from the averaged FRAP curves by nonlinear regression analysis, fitting to the analytical expression for a combination of lateral diffusion and exchange (Wolfenson et al., 2009b). In short, this analytical expression was derived to fit FRAP curves in which fluorescence recovery is composed of two mechanisms: (1) diffusion in the cytoplasm; and (2) exchange of membrane molecules with cytoplasmic molecules. We previously showed that in the case of FAs, the rates of the two processes are well separated, and it is possible to approximate the data as two populations, one recovering by relatively fast diffusion and the other by slow exchange. This enables extraction of the mobile fraction (R_f) and the rate constant for dissociation from FAs (k_{off}) (Wolfenson et al., 2009b). In supplementary material Fig. S2, the characteristic diffusion time, τ_D, was extracted from fitting the FRAP curves to the analytical expression only for diffusion (Axelrod et al., 1976).

Immunolabeling, imaging and analysis

For immunofluorescence labeling (Fig. 5), cells were incubated for 5 minutes either with HBSS/HEPES buffer, pH 6.8, supplemented with 2% PEG8000, 1 μM EGTA and 0.1% Triton X-100, with or without pre-incubation for 1 minute with 50 μM blebbistatin; or, alternatively, incubated with HBSS/MES buffer, pH 6.2, supplemented with 0.1% Triton X-100. Under these conditions, the cells maintain their general structure, despite the rapid extraction of cytoplasmic content (Crowley and Horwitz, 1995). After fixation with 4% paraformaldehyde, cells were labeled with 5 μg/ml mouse anti-paxillin (BD Transduction Laboratories), followed by 3 μg/ml Cy3-goat anti–mouse IgG (Jackson Immunoresearch Laboratories, Inc.). For time-lapse movies (representative frames shown in Fig. 1), cells were imaged at 37°C, using a TILL iMIC digital microscope (TILL Photonics) equipped with a plan-apochromat 100×/1.45 NA oil-immersion objective (Olympus). The imaging set-up consisted of an epi-illumination Polychrome V monochromator set at 514 nm for YFP excitation, a 520–550 nm band-pass filter for emitted light (Chroma Technology) and an Andor iXon 888 EMCCD camera (Andor Technology). Live imaging was controlled by Live Acquisition software (TILL Photonics). Temporal ratio imaging analysis following treatment with 50 μM blebbistatin (Fig. 1) was performed as previously described (Zamir et al., 1999). In general, dividing the intensity values of each pixel at time point 0 by the intensity in the same pixel 10, 30 and 60 minutes later, created the temporal ratio images, which are presented in color scale according to the look-up table. For measurements of fluorescence loss from FAs after treatment with Triton X-100, cells were imaged at a rate of 1 Hz for 10–15 frames. Triton X-100 was then added to a final concentration of 0.1% (with or without pre-incubation with 50 μM blebbistatin for 1 minute), and imaging continued until no differences in fluorescence intensity over time could be detected. Quantification of fluorescence intensity in FAs over time (Figs 1, 2, 6 and supplementary material Fig. S1) and of changes in FA area (Figs 2, 3) was performed using ImageJ software (NIH), after correcting for fluorescence bleaching over time, using data from untreated cells. Quantification of fluorescence loss from FAs (Fig. 6) began immediately after the fluorescence from the surrounding cytoplasm disappeared, due to leakage from the permeabilized cells. Mapping the fluorescence loss from FA data on logarithmic graphs enabled us to establish that this process follows first-order kinetics (Fig. 6); we could then extract k_{off} for each protein, using $k_{off}=\ln(2)/t_{1/2}$ (for a first-order process).

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