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Acta Biomaterialia 1 (2005) 281–293
Acta BIOMATERIALIA
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Exploring the molecular basis for mechanosensation, signal transduction, and cytoskeletal remodeling

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Received 6 December 2004; accepted 15 February 2005

Abstract

Living cells respond to mechanical stimulation in a variety of ways that affect nearly every aspect of their function. Such responses can range from changes in cell morphology to activation of signaling cascades and changes in cell phenotype. Although the biochemical signaling pathways activated by mechanical stimulus have been extensively studied, little is known of the basic mechanisms by which mechanical force is transduced into a biochemical signal, or how the cell changes its behavior or properties in response to external or internal stresses. One hypothesis is that forces transmitted via individual proteins either at the site of cell adhesion to its surroundings or within the stress-bearing members of the cytoskeleton cause conformational changes that alter their binding affinity to other intracellular molecules. This altered equilibrium state can subsequently either initiate a biochemical signaling cascade or produce more immediate and local structural changes. To understand the phenomena related to mechanotransduction, the mechanics and chemistry of single molecules that form the signal transduction pathways must be examined. This paper presents a range of case studies that seek to explore the molecular basis of mechanical signal sensation and transduction, with particular attention to their macroscopic manifestation in the cell properties, e.g. in focal adhesion remodeling due to local application of force or changes in cytoskeletal rheology and remodeling due to cellular deformation.

Keywords: Mechanotransduction; Molecular mechanics and chemistry; Protein conformations; Cytoskeletal rheology and remodeling

1. Introduction

It is well known that living cells can sense mechanical stimuli. Forces applied to a cell or physical cues from the extracellular environment can elicit a wide range of biochemical responses that affect the cell's phenotype in health and disease (see for example reviews in Refs. [1–4]). Despite the wide relevance and central importance of mechanically-induced cellular response, the mechanisms for sensation and transduction of mechanical stimuli into biochemical signals, termed mechanotransduction, are still largely unknown. Various mechanisms have been proposed to explain this phenomenon and include: changes in membrane fluidity that act to increase receptor mobility and lead to enhanced receptor clustering and signal initiation [5,6]; stretch-activated ion channels [7]; mechanical disruption of microtubules [8]; and forced deformations within the nucleus [9]. Constrained autocrine signaling is yet another mechanism whereby the strength of autocrine signaling is regulated by changes in the volume of extracellular compartments into which the receptor ligands are shed [10]. Changing this volume by mechanical deformation of the tissues can increase the level of autocrine signaling. Finally,

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1742-7061/$ - see front matter © 2005 Published by Elsevier Ltd. on behalf of Acta Materialia Inc.
doi:10.1016/j.actbio.2005.02.008

others have proposed conformational changes in intracellular proteins along the force transmission pathway, connecting the extracellular matrix with the cytoskeleton through focal adhesions, as the main mechanotransduction mechanism [6,11,12]. In particular, the hypothesis that links mechanotransduction phenomena to mechanically-induced alterations in the molecular conformation of proteins has been gaining increased support. For example, certain proteins that reside in 'closed' conformation can be mechanically triggered to reveal their cryptic binding clefts. Similarly, small conformational changes may also change binding affinity or enzyme activity, e.g. when protein binding occurs through hydrophobic site interactions, a conformational change could modify this function and potentially disrupt it totally. Still other examples exist now that lend evidence in support of force-induced changes in binding characteristics, such as seen by the reduced binding of RNA polymerase to DNA filaments stretched by optical tweezers [13] and the enhanced bundling of filaments following exposure of cryptic binding sites on fibronectin [14].

Force transmission from the extracellular matrix to the cell interior occurs through a chain of proteins, called focal adhesion sites, that are comprised of an integrin-extracellular matrix protein bond (primarily vitronectin and fibronectin), integrin-associated proteins on the intracellular side (paxillin, talin, vinculin, etc.), and proteins linking the focal adhesion complex to the cytoskeleton (see Fig. 1). Stresses transmitted through adhesion receptors and distributed throughout the cell could cause conformational changes in individual force transmitting proteins, any of which would be a candidate for force transduction into a biochemical signal. The process by which changes in protein conformation give rise to protein clustering at a focal adhesion or initiate intracellular signaling, however, remains largely unknown [15].

External stresses imposed on the cell are transmitted through the cytoskeleton to remote locations within the cell. To understand these stress distributions requires knowledge of cytoskeletal rheology, as governed by the structural proteins, actin filaments, microtubules, and intermediate filaments. For example, a simplified picture can be painted of the cytoskeletal rheology that is limited to actin filaments and actin cross-linking proteins living in a dynamic equilibrium. These cross-links constantly form and unbind at rates that are largely influenced by the forces borne by the individual molecules. Cytoskeletal rheology would then be determined at the molecular scale by the mechanics and binding kinetics of the actin cross-linking proteins as well as by the actin matrix itself [16].

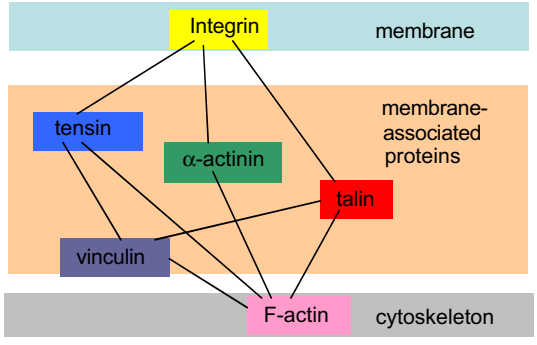

Fig. 1. A simplified view of the complex and interconnected pathways connecting extracellular domain to the cytoskeletal matrix within the confines of a focal adhesion.

To understand the phenomena related to mechanotransduction in living cells and their cytoskeletal rheology, the mechanics and chemistry of single molecules that form the biological signaling pathways that act in concert with the mechanics must be examined. This paper provides a range of case studies that seek to explore the molecular basis of mechanosensation, signal transduction, and cytoskeletal rheology and remodeling after deformation. Several examples are briefly presented that may help to introduce the reader to the different challenges that the field faces today, as well as approaches that may be used to attack these problems. Example 1, *Force-induced focal adhesion translocation: The spatial influence of force amplitude and frequency*, examines force magnitude and frequency thresholds for transducing local mechanical loads, applied via a magnetic trap, into biological signals through focal adhesion sites as marked by site translocation. Example 2, *The effect of cellular deformation on cytoskeletal rheology and remodeling*, provides evidence that mechanical force and deformation of scales comparable to those encountered by a neutrophil during transit through the microcirculation strongly impact the structure and function of the cell, as directly related to cytoskeletal rheology and post-deformation remodeling. In Example 3, *A coarse-grained model for force-induced protein deformation and kinetics*, a generic coarse-grained model is presented linking force applied to a single protein to its conformational change, expressed in terms of the mechanical properties of the protein conformational states. Finally, in Example 4, *Mechanical perturbation of the FAT–paxillin binding partnership*, molecular simulations are used to examine how force-induced changes in the molecular conformation of focal adhesion targeting domain (FAT) affect binding with its partner paxillin.

2. Case studies

2.1. Force-induced focal adhesion translocation: the spatial influence of force amplitude and frequency

2.1.1. Introduction

Focal adhesion site remodeling is used as a rapid and site-specific marker of mechanotransduction [11,17].

Single cells respond to mechanical stress with focal adhesion translocation and reinforcement via the recruitment and binding of focal adhesion proteins [17]. Furthermore, forces applied to the apical cell surface have been shown to induce local focal adhesion protein recruitment, suggesting that individual focal adhesions sense local levels of force [18]. Tyrosine phosphorylation and Src family kinases drive two signaling pathways essential for focal adhesion-associated mechanosensing [19]. Tyrosine phosphorylation assists in the recruitment and binding of focal adhesion proteins by regulating interactions among proteins that contain the Src homology 2 (SH2) [20], while Src family kinases promote focal adhesion turnover by weakening integrin-cytoskeleton connections [21]. In this study we examined force magnitude and frequency thresholds for transducing local mechanical loads into biological signals through focal adhesion sites. Further details of this study can be found in Ref. [22].

2.1.2. Methods

A highly controllable local load was applied with a magnetic trap to the apical surface of bovine aortic endothelial cells (BAEC) cultured in DMEM (Cambrex, East Rutherford, NJ) supplemented with 10% FCS and 1% penicillin/streptomycin and plated on fibronectin-coated glass culture dishes [22]. Steady loads of 0.90, 1.45, and 2.25 nN and non-reversing sinusoidal loads with a 1.45 nN mean and 2.25 nN maximum force were applied over a period of 1, 3, and 5 min. Basal surface focal adhesion translocations in response to the externally applied forces were observed with fluorescent microscopy by expressing GFP-fused paxillin, a focal adhesion protein that binds to the focal adhesion targeting (FAT) region of focal adhesion kinase (FAK). Western analysis was performed with anti-paxillin (RDI, Flanders, NJ) and anti-GFP (Sigma, St. Louis, MO) monoclonals to verify the presence of GFP-paxillin, but cytotoxicity assays were not performed. Image acquisition with an inverted light microscope (model IX-70; Olympus, Mellville, NY) involved scanning 5 μm vertically with a 0.25 μm step size and choosing the best representation of the adhesion plane. The images obtained were enhanced for improved contrast, converted to binary images, and segmented into individual focal adhesion sites for translocation analysis (Fig. 2). All cells used for analysis bound single fibronectin-coated magnetic beads and appeared adherent and spread. Translocations were quantified for the different loading conditions by tracking individual focal adhesion site centroid displacement vectors between two time points and averaging the vector magnitudes. Based on the length of these displacement vectors, quantitative analysis was used to show that the magnitude and frequency of load delivery, as well as the spatial position of focal adhesion sites with respect to the point load, affects focal adhesion activation and mechanosensing.

2.1.3. Results

Focal adhesion translocation resulting from a steady 0.90 nN load did not exhibit an appreciable difference compared to control cell translocations. Increasing the steady load to 1.45 nN, however, yielded a considerable increase in translocation. Translocation values at 1.45 and 2.25 nN were not significantly different, suggesting a threshold response level exists between 0.9 and 1.45 nN with little further change above the threshold (Fig. 2).

Focal adhesion translocation decreased as the distance from the projected point of load application increased, suggesting that forces transmit non-uniformly to the basal cell surface and that cells contain isolated mechanosensors capable of recognizing local force levels. In order to quantify this effect, translocation was examined as a function of distance from the point of load application. Comparison of 'local', defined as within a 7.5 μm distance from the point of force application [23], and 'global' (outside the local region) translocation magnitudes for a 1.45 nN steady load yielded significantly greater local translocation values (Fig. 2).

To examine the loading frequency dependence of cellular mechanosensing, experiments were performed with 0.1, 1.0, 10, and 50 Hz non-reversing sine wave forces that oscillated around a mean positive force of 1.45 nN. The mean force was equivalent to the smallest steady load found to activate focal adhesion sites. These experiments exhibited a biphasic cellular response (see Fig. 3) that may result from two separate force-induced signaling pathways. Specifically, tyrosine phosphorylation/dephosphorylation and Src family kinase activity leads to focal adhesion site turnover and downstream Rho and Rac signaling [52], while talin1 binding mediates local force-induced structural changes independent of tyrosine phosphorylation [19,24]. If magnetic trap loading differentially activates these parallel pathways depending on the frequency of loading, then the focal adhesion translocation observed could have resulted from both direct force transmission to basal surface focal adhesion sites and force-induced Src family kinase signaling. These intricacies of focal adhesion mechanotransduction, which combine direct mechanical and signaling kinase responses, are not fully understood, but may contribute to the observed biphasic frequency response.

2.1.4. Summary

Force magnitude and frequency-dependent thresholds for mechanotransduction were studied using focal adhesion dynamics activated by forces applied locally via adherent microbeads as a measure of cellular

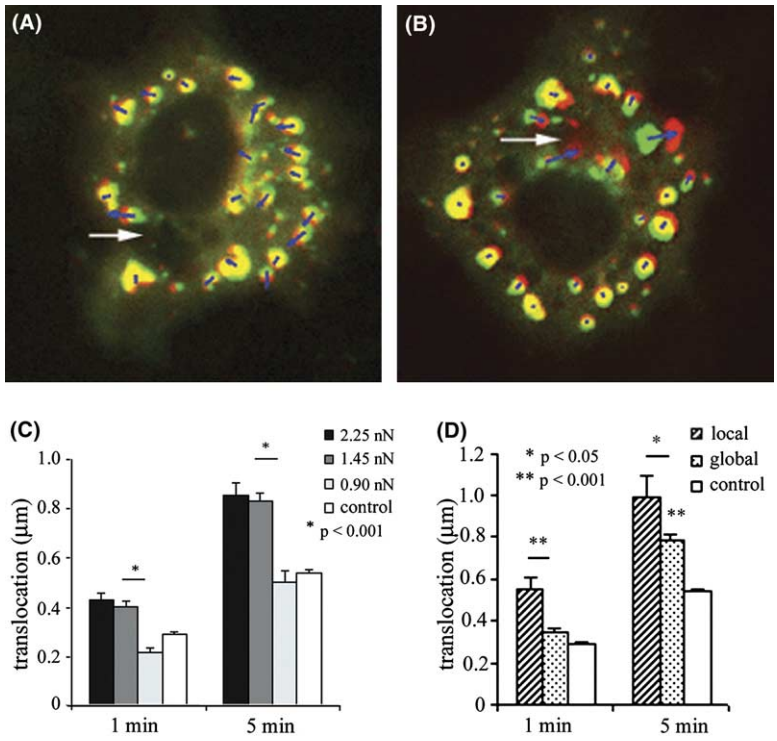

Fig. 2. Translocation vector fields, calculated by tracking the displacement of each focal adhesion site centroid, were visualized by overlaying two time points assigned different colors. Green signifies the focal adhesion site position prior to loading, whereas red represents focal adhesion site positions after 5 min of (A) 0.90 nN and (B) 2.25 nN steady loading. Yellow represents overlapping regions between the two time points. (C) A significant increase in translocation compared to un-forced baseline was found after 1.45 nN steady load application, but not 0.90 nN, suggesting that a load threshold for focal adhesion site mechanotransduction exists between 0.90 nN and 1.45 nN. An insignificant difference between 2.25 nN and 1.45 nN implies that the threshold is not graded. (D) Focal adhesion sites 'local' (<7.5 μm) to the projected point of load application undergo significantly greater translocation compared to 'global' focal adhesion sites (adapted with permission from Ref. [22]).

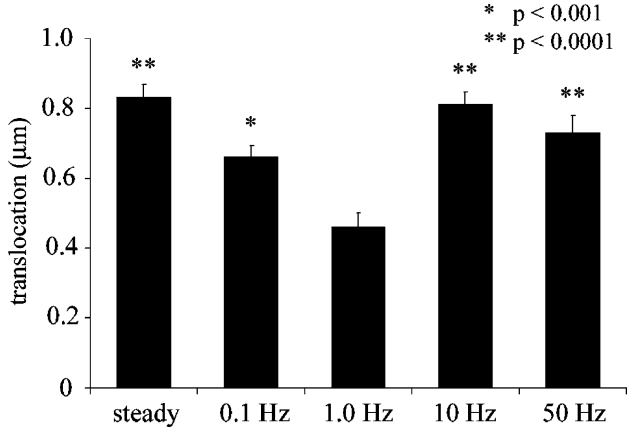

Fig. 3. Focal adhesion translocation after 5 min of load application depends on the frequency of application for loads with a 1.45 nN mean positive force level (adapted with permission from Ref. [22]).

response. Based on the translocation of focal adhesions, vascular endothelial cells exhibit a steady force magnitude threshold for mechanical activation. Below the determined threshold, forces transmit through the cell to the basal surface without producing appreciable changes in focal adhesion translocation. Moreover, it is demonstrated that the cellular response to external force depends on the magnitude, duration, and frequency of force application.

Furthermore, concentrated forces applied to the cell apex have been shown by others to transmit forces in a lumped and non-uniform fashion to basal surface focal adhesion sites [25]. Combined with our finding that individual focal adhesion sites respond differently to apex-applied forces, this supports the idea that individual basal surface focal adhesions sense local reaction forces. Two theories attempt to explain how focal adhesion sites sense local levels of force. The first recognizes that local forces reorganize neighboring structural elements, making new binding partners more readily available. Under tension, integrin density increases at focal adhesion sites in coordination with focal adhesion development [26]. The second theory hypothesizes that external forces applied directly to structural proteins alter the protein conformational state, which transform the protein from an inactive state to an active state by exposing potentially new binding sites [27]. Both theories suggest that an altered state of molecular-level equilibrium induces protein binding changes and subsequently initiates

a cascade of local biological responses. Conceivably, these changes can yield alterations in enzymatic activity of focal adhesion proteins, generating activated species that diffuse throughout the cell, thereby leading to global effects (see Fig. 4).

2.2. The effect of cellular deformation on cytoskeletal rheology and remodeling

2.2.1. Introduction

Neutrophils often encounter narrow capillary segments during their transit through the pulmonary and systemic microcirculations. Since neutrophil diameters often exceed the diameter of a pulmonary capillary, neutrophils would almost certainly have to deform in passing from arteriole to venule, particularly in the pulmonary capillary bed, where it has been estimated that a typical flow pathway encompasses 50–100 such capillary segments [28,29]. Measurements of neutrophil shape showed that neutrophils in capillaries are elongated while those in arterioles are nearly spherical, thus confirming the view that neutrophils deform when they encounter narrow capillary segments [30,31].

Despite the importance of these mechanical effects in the microcirculation, no rheological studies have yet been conducted to examine the effect on cytoskeletal rheology of subjecting neutrophils to the deformations they experience while traversing the pulmonary circulation. In this study we examined the effects of mechanical deformation on the mechanical behavior and rheological properties of neutrophils in the pulmonary capillaries.

2.2.2. Methods

Microfabrication techniques [32,33] were used to construct an in vitro poly-dimethyl-siloxane (PDMS) system (Fig. 5) with dimensions comparable to the pulmonary capillaries. PDMS being optically transparent enabled direct observation of neutrophil morphology, and simultaneously allowed us to employ the technique of multiple-particle-tracking microrheology [34] to directly measure the viscoelastic properties of the cell. Multiple-particle-tracking microrheology is able to non-invasively measure the local viscoelasticity by monitoring the Brownian motion of endogeneous granules present in the cytoplasm of the cell. Details of the particle tracking technique employed in this work can be found in Ref. [35].

2.2.3. Results

Neutrophil behavior was recorded from the start of its deformation into the channel up to the formation of the first pseudopod projection either at the leading or trailing edge of the cell (Fig. 6). It was observed that the time to pseudopod projection is inversely correlated to the rate of deformation of the neutrophil. Above the threshold stimulus, the time required for neutrophil's activation, as measured by pseudopod formation, was observed to depend upon the rate of deformation

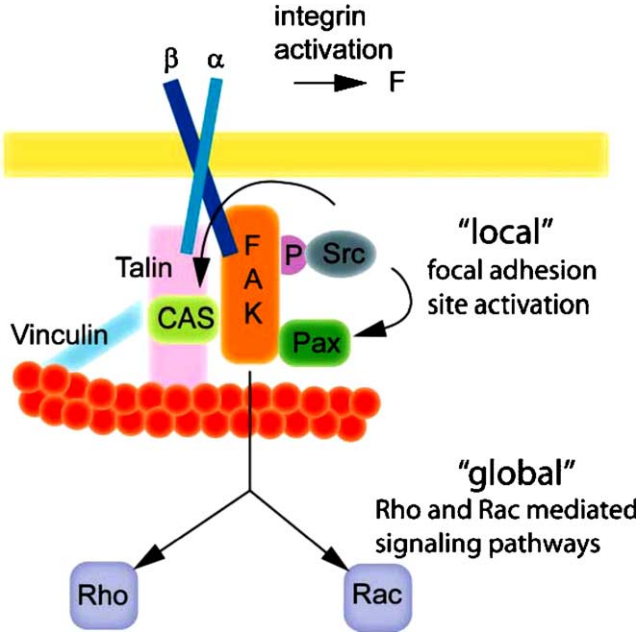

Fig. 4. Schematic representing possible mechanisms of focal adhesion activation. Local effects due to forces transmitted directly to basal surface focal adhesion sites can lead to alternations in the binding affinity of focal adhesion proteins and remodeling within the same focal adhesion complex, as well as downstream events, such as Rho and Rac signaling, that have global effects on focal adhesion sites [49].

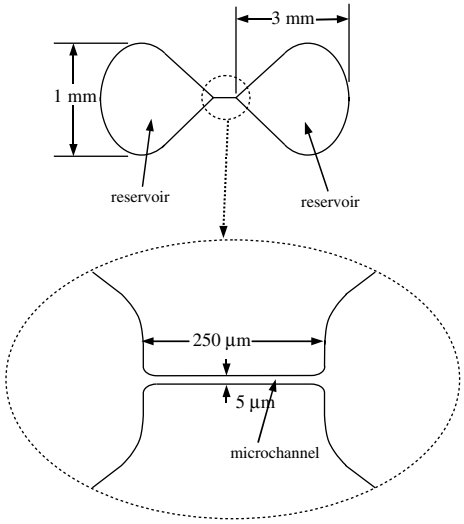

Fig. 5. Schematic showing design of the PDMS microchannel and its connecting reservoirs (top view). The microchannel section is enlarged to highlight the channel geometry, which has dimensions comparable to that of pulmonary capillaries. The microchannel height is about 1.5–2.5 μm. Diagrams are not drawn to scale.

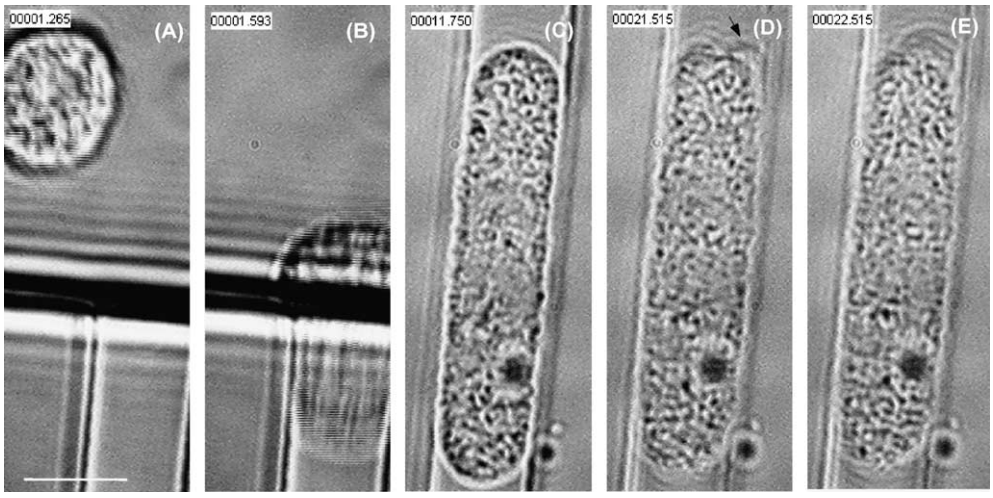

Fig. 6. Image sequence showing a neutrophil flowing towards the microchannel entrance [panel (A)], the cell undergoing deformation [panel (B)], and subsequently, the neutrophil was trapped in the channel [panel (C)]. After some time, the cell can be seen to form pseudopod projection [panels (D) and (E)]. Arrow in panel (D) points to the location at the trailing edge of the cell where pseudopod protrusion was first seen. Scale bar, 5 μm (adapted with permission from Ref. [35]).

experienced by the cell, implying the existence of a mechanosensing or signal transduction mechanism in the cell that is able to modulate the response according to the magnitude of mechanical stimulus.

To gain insight into the effects of mechanical deformation on the cytoskeletal structure of the neutrophil, intracellular granules were tracked to monitor the change in rheology of the cell in response to deformation. From these particle trajectories, mean-square displacements and the frequency-dependent elastic modulus, $G'(\omega)$, and loss modulus, $G''(\omega)$, were calculated—see Ref. [35] for details. Mechanical deformation results in a reduction in elastic moduli by 50–60%, within 10–15 s after the initial stimulus (Fig. 7). Similarly, the loss moduli drop by 35–50% in response to mechanical deformation. These data demonstrate that mechanical deformation causes either disruption or remodeling of the neutrophil cytoskeleton. In view of the decrease in *viscous and elastic moduli* of the cell, this might be due either to a sudden depolymerization of filamentous actin or rupture of cross-links bridging between actin filaments. The lack of a significant temperature effect on entrance time [35], in combination with the short time scale of the modulus changes (~10 s), leads us to favor the theory that the rapid deformation ruptures actin cross-links.

Interestingly, the magnitude of drop in the values of G' and G'' after deformation was found to be independent of the degree or rate of deformation. This is in contrast with the time to pseudopod formation which correlates with the rate of deformation. Moreover, neutrophils subjected to low deformation rates were observed to recover much of their modulus reduction within ~30 s and return nearly to their initial mechanical state. These data suggest that the extent of depolymerization or loss of actin cross-linking is similar regardless of the magnitude or rate of deformation, at least within the range of these experiments, but the initiation of actin polymerization to form pseudopods is dependent on the magnitude of force transduced. One

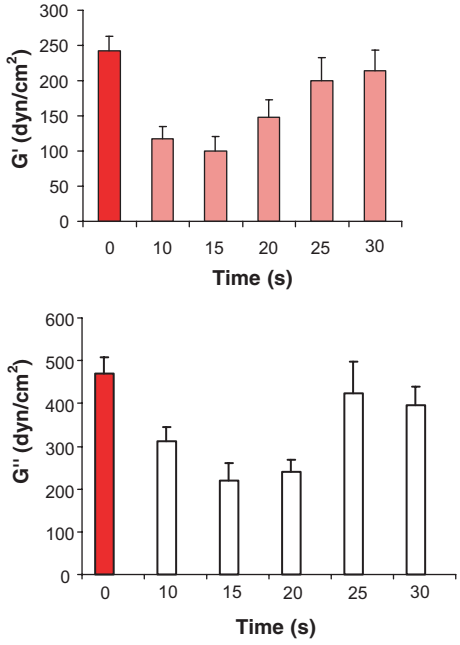

Fig. 7. Temporal change in elastic modulus, G', and loss modulus, G'', of neutrophil after mechanical deformation into a microchannel, at a lag time of 0.03 s. Graphs show changes under flow condition of $\Delta P = 10$ mm H_2O. Values at time = 0 s are obtained from passive, round neutrophils, which serves as control. Data are expressed as means ± SE (adapted with permission from Ref. [35]).

scenario consistent with these observations is that the large strain deformations effectively shear and rupture many of the actin cross-links, leaving them attached to one filament but displaced relative to their initial cross-linking site. Once the deformation stops, these cross-links can reform, but do so in the new, deformed state, returning the cell to its initial mechanical state but in a new, deformed geometry. Thus, it appears that the molecular mechanism controlling the depolymerization/actin cross-link disruption is separate from the mechanism governing pseudopod formation and viscoelastic recovery, however further experiments would be needed to confirm this.

2.2.4. Summary

This study provides direct evidence that mechanical force of a magnitude comparable to that encountered by a neutrophil during transit through the microcirculation strongly impacts the structure and function of the cell. The consequences of mechanical stimulation are substantial and immediate, occurring within seconds of stimulation. A drop in shear modulus by more than 60% is observed within seconds of entering a constriction, independent of magnitude of the stimulus. In contrast, pathways leading to migratory behavior are excited in a strain rate-dependent manner, suggesting that these two phenomena may be independently controlled. Taken together, these results suggest an important role for mechanical stimulation of neutrophils, influencing both their rheology and their migratory tendencies.

2.3. A coarse-grained model for force-induced protein deformation and kinetics

2.3.1. Introduction

A generic model for protein extension was developed based on the simple physics of diffusion under force inspired by Kramers theory for chemical reaction rates [36]. The protein was assumed to have two distinct conformational states: C_1, a relaxed state, predominant when no force is applied to the protein, and C_2, an extended state, favored by force application. In the context of mechanotransduction, the extended state can be viewed as a molecular conformation in which the protein is able to transmit signal, e.g. by binding to a molecule initiating a signaling cascade. Appearance and prevalence of C_2 is assumed to lead to transduction of the mechanical signal into a chemical one. The energy landscape for such a protein was idealized as consisting of two parabolic harmonic wells. Using this coarse-grained model, the mechanical characteristics of the protein conformations were identified that led to faster force-induced transitions to the extended state (see a similar approach in [42]).

2.3.2. Methods

Assuming that binding to a signaling molecule is a force-independent event and occurs preferentially in one conformation (relaxed or extended), the model links force applied to a protein to its propensity to initiate a signal. A simplifying case is considered of a protein having just two conformational states: C_1, dominating without force application, and C_2, an extended state favored by force. Our analysis is based on the simplest energy landscape corresponding to this situation, i.e. two harmonic wells whose minima represent the two states (see Fig. 8).

Specifically, the landscape is collapsed onto a single spatial coordinate x, corresponding to the protein extension in the direction of pulling, and is described by:

$$E(x) = \tfrac{1}{2}\kappa_1 x^2 \qquad \text{for } x < x_p$$
$$E(x) = \tfrac{1}{2}\kappa_2 (x - x_0)^2 + E_0 \qquad \text{for } x \geqslant x_p$$

with κ_1, κ_2 the stiffnesses of states C_1 and C_2, respectively; x_0 the increase in protein extension to transform C_1 into C_2; x_p the increase in protein extension between

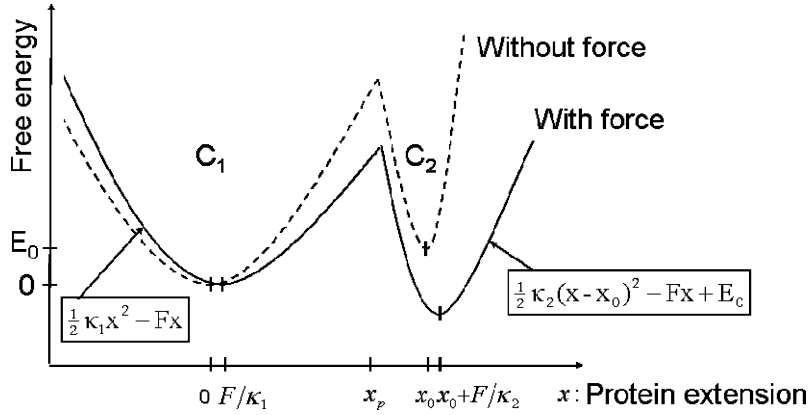

Fig. 8. Idealized protein energy landscape when extended in a direction x. The vertical axis is the protein Gibbs free energy. See text for parameter definition. The boxes contain the idealized landscape curve equations under force.

C_1 and the peak of the landscape and E_0 the difference in energy between C_1 and C_2. Therefore, even though proteins are likely to present several intermediary conformations, our model accounts only for the highest energy peak, or the last one encountered before the reactive state is attained. Both the equilibrium distribution of states as well as the rates of reaction are considered.

Obtaining the equilibrium constant between the two conformational changes is straightforward with Boltzmann's law [37], which states that the ratio of probability of states C_1 and C_2 is proportional to the exponential of their energy difference normalized by the Boltzmann factor k_BT, with k_B the Boltzmann constant and T the temperature.

Kinetic or transition rate analysis is also needed, besides the equilibrium analysis, as mechanical stimulations of cells or proteins in vivo, in experiments and in simulations, span a wide range of time scales from picoseconds (molecular dynamics simulations) to hours (cell remodeling), hence regimes likely exist for which kinetics dominates over thermodynamic equilibrium. While molecular dynamics simulations offer ways to link conformational changes of specific proteins under forces applied at specified protein locations, they require knowledge of the full atomic structure specific to the particular protein, and typically study forces large compared to those experienced in vivo in order to hasten the process due to computational constraints, hindering interpretations of protein dynamics. The present approach is complementary in that it only considers a single degree of freedom and a single transition between states.

Protein deformation typically occurs in a viscous-dominated regime [38], where motion along the reaction coordinate exhibits randomness and appears Brownian. To account for both these fluctuations and the landscape shape (not merely the transition peak energy), we extend the statistical mechanics theory of Kramers [36] by adding the effect of an external force. Movement of the protein extremity is described using the Smoluchowski equation (see Ref. [38]), a force balance on a microcanonical ensemble of particles. This allows for calculation of mean first-passage times (called 'passage times' in the remainder of the section) to travel along the energy landscape. The passage times are used along with thermodynamic equilibrium to deduce kinetic rates for forced conformational changes, as a function of the protein mechanical characteristics. Explicitly, the forward kinetic rate constant k_f for the protein to change conformation from C_1 to C_2 is calculated as $1/(t_1\varepsilon^{-1} + t_2)$, where t_1 and t_2 are passage times over the two wells, i.e. the average time necessary for the protein extremity to diffuse under force from its equilibrium state C_1 (minimum of the first well) to the transition state at the peak of the landscape (for t_1) or that necessary for the protein extremity to diffuse from the transition state to the elongated state C_2 (for t_2). ε is the transition factor as defined by Ref. [39] and is obtained from probability ratio of C_2 to C_1 at equilibrium (see derivation in Ref. [40]). The reverse kinetic constant can also be deduced similarly from backward passage times to diffuse from the elongated state C_2 to the transition state at the peak of the landscape and that necessary for the protein extremity to diffuse from the transition state back to the initial equilibrium state C_1.

The model takes into account the mechanical features of the protein (via κ_1, κ_2, x_0 and E_0), as influenced by the chemical bonds and electrostatic interactions within a single protein. Its main purpose is to mechanically characterize the behavior of a protein's force-induced deformations and kinetics.

2.3.3. Results

A force F applied to the protein in the x-direction lowers its energy $E(x)$ by the mechanical potential Fx. The free energy gain hence depends on the reaction coordinate x, and the landscape is described by $E(x) - Fx$ (see Fig. 8). Thermodynamic analysis using Boltzmann's law [37] between the states shows that conversion of the protein from 10% to 90% in extended state usually occurs over a very narrow force change of a few k_BT/x_0^2, with k_B the Boltzmann constant and T the temperature, i.e. a few pNs for states separated by distances x_0 on the order of nm. Therefore, proteins can switch from an initial to an extended conformation when subjected to a very small force change. This can be viewed in the context of forced-induced conformational changes in intracellular proteins, leading to changes in binding affinities or enzymatic activities, as has been proposed as a mechanism for mechanotransduction [3,4,41]. The methodology presented here might therefore be useful in the creation of coarse-grained models of mechanotransduction.

The extension rate k_f to obtain C_2 from C_1 was found to increase almost exponentially with force—a behavior similar to the one previously reported for bond dissociation [43]—before leveling off when larger forces have little effect on k_f (see Fig. 9). This leveling off of the extension rate with at large forces is due to the horizontal distortion of the extended state C_2 under force (i.e. the displacement under force of minima coordinates, cf. Fig. 8, which deforms the conformational states C_2 more than C_1). At large forces, distortion is large and the combined effects of a lowered energy peak for a reverse transition and the displacement of state C_2 are balanced by the increase of k_f.

2.3.4. Summary

A generic model was developed based on Kramers theory for force-induced conformational change and signal initiation. The model assumes that the protein has

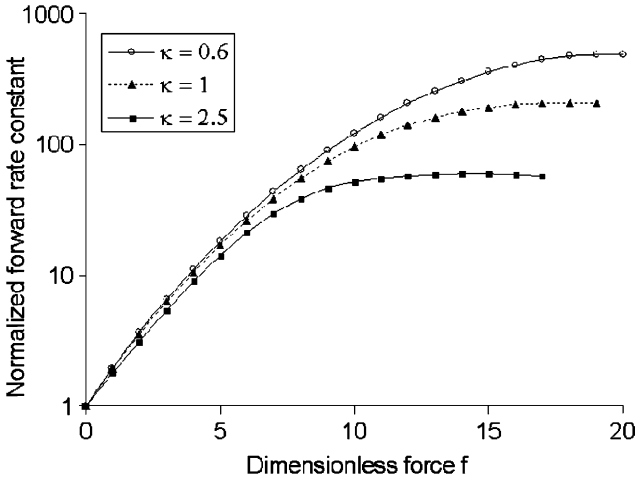

Fig. 9. Normalized extension rate k_f/k_f ($f = 0$) from C_1 to C_2 versus dimensionless force applied $f = \frac{Fx_0}{k_BT}$, normalized by same rate at zero force. k_BT is the Boltzmann factor, F the force applied, and x_0 the protein extension difference between the two protein conformational changes. Parameters are $\frac{\kappa_1 x_0^2}{k_BT} = 30$, $\frac{E_0}{k_BT} = 4$ and $\kappa = \frac{\kappa_1}{\kappa_2} = 0.6$, 1 or 2.5 (see text for parameter definition).

two distinct conformational states, and that signaling only occurs in one extended conformation. The model allows for calculation of the probability ratios of protein conformational states, and demonstrates that externally applied forces can exert a dominant influence on both equilibrium states and reaction rates.

2.4. Mechanical perturbation of the FAT–paxillin binding partnership

2.4.1. Introduction

Membrane-bound integrin receptors are linked to intracellular signaling pathways through focal adhesion kinase (FAK). FAK tends to colocalize with integrin receptors at focal adhesions (FAs) through its C-terminal focal adhesion targeting (FAT) domain [44,45]. Through recruitment and binding of intracellular proteins, FAs transduce signals between the intracellular and extracellular regions that regulate a variety of cellular processes including cell migration, proliferation, apoptosis and detachment from the extracellular matrix [45–52]. The mechanism of signaling through the cell is of interest, especially the transmission of mechanical forces and subsequent transduction into biological signals. To demonstrate how changes in a protein's conformation can affect its binding partnership, we performed steered molecular dynamics simulations that mechanically perturb the FAT structure and monitor how force-induced changes in the molecular conformation of FAT affect its binding to paxillin and conversely, how paxillin binding affects the dynamics of mechanically perturbed FAT.

2.4.2. Methods

The crystal structure of the complex shows FAT organized into a four α-helix bundle with straight anti-parallel helices and the paxillin binding domain situated in hydrophobic patches HP1 and HP2, on faces 1–4 and 2–3 of the bundle [44]. This is a good model system to analyze as α-helices and four-helix bundles are ubiquitous motifs in folded protein structures.

Starting with the crystal structure of FAT solvated with an implicit water model, either bound or unbound to paxillin (FAT–Pax or nb-FAT), FAT was unfolded by applying a constant force to its C-terminal while keeping its N-terminal fixed. Forces applied ranged from 75 pN to 200 pN. The paxillin motifs that bind to FAT are amphipatic α-helices that can bind on either of the HPs. The end-to-end distance between the N- and C-terminals was monitored with unfolding, as well as distances between the residues that formed salt bridges between FAT and paxillin.

To address more physiologically relevant conformational changes that could occur within the FAT domain, we used normal mode analysis to determine more plausible modes of force application. In normal mode analysis (NMA), the protein is modeled as a collection of masses (atoms) and springs (interactions), while the electrostatic and Van der Waals forces are ignored. NMA has proven useful for the identification of collective domain motions of proteins [50,51] where some of the lowest-frequency normal modes of several proteins have been found to correlate well with the proteins' conformational change.

2.4.3. Results

When subjected to constant-force pulling, the unfolding trajectory of FAT α-helical bundle showed several plateaus, representing the stable intermediate structures assumed by FAT where its end-to-end distance stayed relatively constant for some time before further unraveling. The occurrence of these stable intermediates depended on the force levels applied; at higher force levels, fewer intermediates existed and at high enough forces, no intermediates were observed. This corresponds to a tilt in the energy landscape by—Fx where F is the force applied and x is the reaction coordinate (end-to-end distance in this case), which removes the energy minima present at the intermediates. For FAT–Pax, the first detectable unraveling event developing within reasonable computational times occurred at a force higher than nb-FAT (85 pN cf. 75 pN), while 75 pN did not yield appreciable change in the FAT–Pax structure [52]. This indicates the stabilizing effect of paxillin bound to FAT, where its location on the hydrophobic patch of FAT increases the structural strength and stability of FAT, and therefore increases the unfolding threshold.

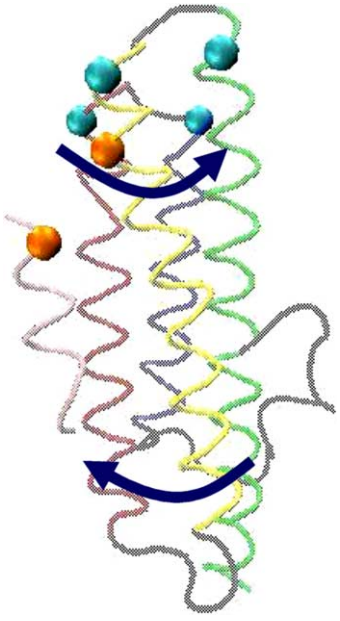

Fig. 10. Concerted twisting motion of the lowest-frequency mode obtained from normal mode analysis. FAT and paxillin are drawn with 'tube' representation going through the protein backbones. The short pink helix on the left is paxillin. The 'pulled atoms' are represented as blue Van der Waals spheres and the orange spheres indicate the atoms paired through salt bridge formation.

Using NMA, the lowest-frequency normal mode was obtained for the FAT molecular structure that corresponds to a concerted twist of the FAT bundle, as shown in Fig. 10. A twisting load was therefore applied to the FAT helical bundle mimicking the normal mode, and the resulting changes to the complex were subsequently analyzed.

With the N-terminal end of FAT held fixed, simulations were performed with a constant angular rotation rate constraint applied to the top four C-α atoms of the four helices of FAT in explicit water simulation, causing a twisting motion of the top of the bundle relative to the bottom. Each of the top four C–α atoms (pulled atoms) were 'connected' by springs to reference atoms that were rotated about the *y*-axis running through the centre of the helical bundle, parallel with the helices, up to a maximum of 200°. The pulled atoms therefore followed the reference atoms to an extent depending on the spring constant assigned to the attaching springs. A stiff spring would cause the follower atom to stay true to the reference atom but the details of the trajectory would be lost as all force will be used to pull bonds apart whereas a soft spring would cause the follower atom to lag behind the reference atom and not follow a true rotational trajectory. With the application of angular rotations of 0.5°/ps, 1°/ps and 5°/ps, and a spring constant of 69.5 pN/A as determined empirically, the force applied to each atom being pulled was found to depend on the rate of angular rotation. As seen in Fig. 11, higher rotation rates (5°/ps) produce a steadily increasing force with angle turned, that does not capture the intricacies of the rotational trajectory whereas slower rotation rates (0.5°/ps, 1°/ps) produce force trajectories that peak at instances when specific hydrogen bonds are broken.

To characterize the binding of paxillin to FAT with the application of this rotation, the distance between Glu10 on paxillin and Lys97 on FAT, two residues that form a salt bridge, was monitored. For both the lower rotation rates, initially paxillin closely followed the motion of FAT as the hydrophobic surface HP1 is still intact and binding is maintained, but as FAT is twisted by 90°, HP1 becomes distorted and binding is impaired (Fig. 12A). As FAT is further twisted, paxillin departs from the binding face (Fig. 12B), as HP1 is severely distorted.

The twisting simulation has shown that disruption of binding is also possible with this kind of motion. One possible physiological scenario would be the concentration of applied force at focal adhesions causing a twist of the top or 'free' side of the FAT bundle relative to a 'fixed' N-terminal that is connected to the rest of the FAK protein.

2.4.4. Summary

This study seeks to demonstrate that force-induced conformational change in individual proteins is a highly plausible mechanism for transduction of mechanical signals carried via alteration of binding events in the mechanosensing pathways. Force application eventually remodeled the hydrophobic groove of FAT, affecting the FAT–Pax binding partnership. Note that due to the limitations in current computational resources, force levels

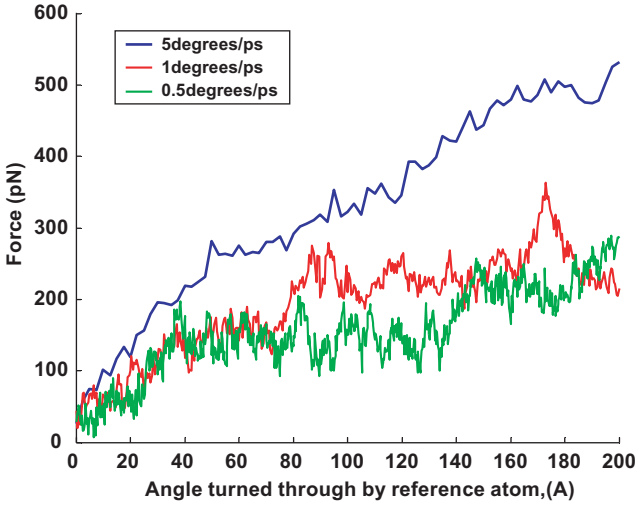

Fig. 11. Constant rotation rate steered molecular dynamics simulation of FAT-complex: comparison of forces applied at constant rotation rates of 0.5°/ps, 1°/ps, 5°/ps.

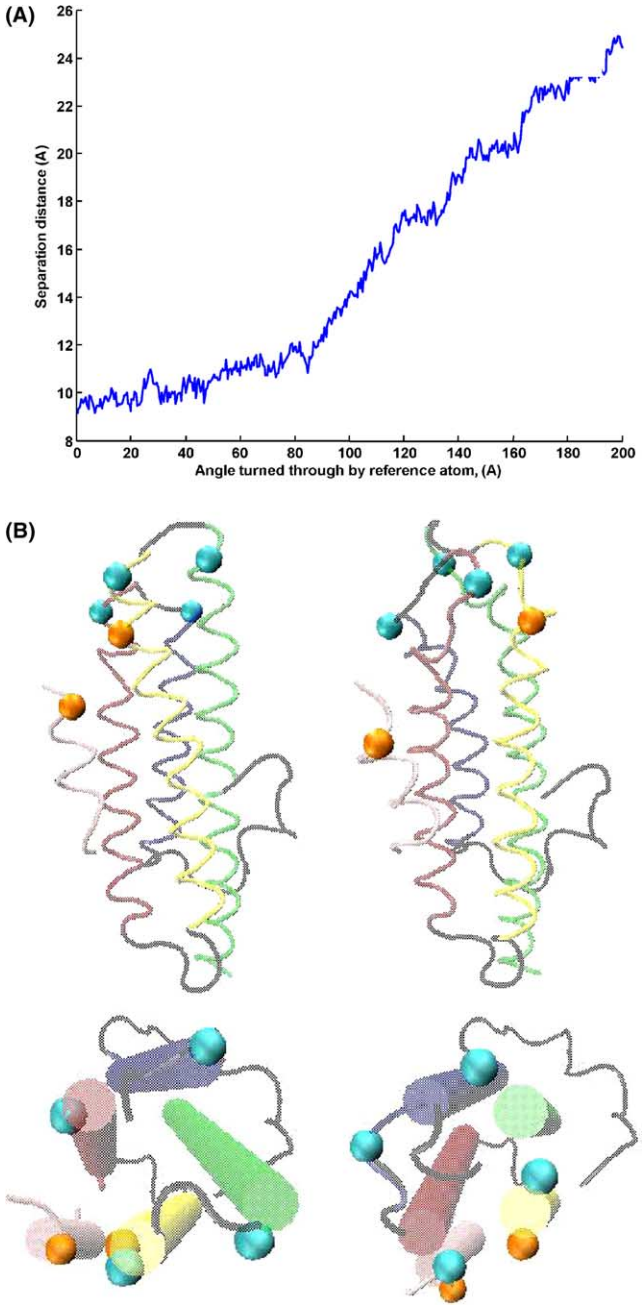

Fig. 12. (A) Separation profile of salt bridge distance with angle turned through by the reference atom. (B) Corresponding view of FAT–paxillin face-on and from the top: at the start of simulations (left) and after 90° rotation (right). The distance between the orange spheres (salt bridge) has increased and paxillin position has changed from being on the HP1 face to being cocked out at an angle as helix 4 'swings' by.

on the order of tens to hundreds of pN were required that yielded unfolding within a few nanoseconds. Physiologically, conformational change would occur within milliseconds to minutes at much lower force levels. Nevertheless, the trends observed here in the stabilization effect caused by binding of paxillin to FAT should hold qualitatively. The interested reader is referred to Ref. [52] for further details of this study.

3. Concluding remarks

Mechanotransduction is an essential function of the cell, controlling its growth, proliferation, protein synthesis, and gene expression. Extensive data exist documenting the cellular responses to external force, but less is known about how force affects biological signaling. More generally, the question of how the mechanical and biochemical pathways interact remains largely unanswered. To understand the mechanobiology of the cell requires a multi-scale/multi-physics look at how externally applied stresses on the cell are transmitted through adhesion receptors and distributed throughout the cell, leading subsequently to conformational changes that occur in individual mechanosensing proteins that in turn lead to increased enzymatic activity or altered binding affinities. This presents both a challenge and an opportunity for further research into understanding the intrinsically coupled mechanobiological phenomena that eventually determine the macroscopic behavior and function of the cell.

Acknowledgments

The middle authors contributed equally to this paper and their names are listed in alphabetical order. Support from the National Heart, Lung, and Blood Institute is gratefully acknowledged.

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