Cell Motility and the Cytoskeleton 66: 1017–1029 (2009)

Review Article

The Heel and Toe of the Cell's Foot: A Multifaceted Approach for Understanding the Structure and Dynamics of Focal Adhesions

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Focal adhesions (FAs) are large clusters of transmembrane receptors of the integrin family and a multitude of associated cytoplasmic ''plaque'' proteins, which connect the extracellular matrix-bound receptors with the actin cytoskeleton. The formation of nearly stationary FAs defines a boundary between the dense and highly dynamic actin network in lamellipodium and the sparser and more diverse cytoskeletal organization in the lamella proper, creating a template for the organization of the entire actin network. The major ''mechanical'' and ''sensory'' functions of FAs; namely, the nucleation and regulation of the contractile, myosin-II-containing stress fibers and the mechanosensing of external surfaces depend, to a major extent, on the dynamics of molecular components within FAs. A central element in FA regulation concerns the positive feedback loop, based on the most intriguing feature of FAs; that is, their dependence on mechanical tension developing by the growing stress fibers. FAs grow in response to such tension, and rapidly disassemble upon its relaxation. In this article, we address the mechanistic relationships between the process of FA development, maturation and dissociation and the dynamic molecular events, which take place in different regions of the FA, primarily in the distal end of this structure (the ''toe'') and the proximal ''heel,'' and discuss the central role of local mechanical forces in orchestrating the complex interplay between FAs and the actin system. Cell Motil. Cytoskeleton 66: 1017–1029, 2009.

Key words: adhesion-mediated mechanosensitivity; nascent adhesions; lamellipodia; stress fibers; myosin II; speckle microscopy; FRAP

Contract grant sponsors: Israel Science Foundation; the Minerva Foundation; the Maurice Janin Fund; NIH (Cell Migration Consortium); Contract grant number: U54GM64346; Contract grant sponsor: NanoMedicine Consortium; Contract grant number: GM-54508.

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Received 10 March 2009; Accepted 22 June 2009

Published online 13 July 2009 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cm.20410

INTRODUCTION

Focal adhesions (FAs, also known as ''focal contacts'') were first visualized in live cells in culture, using interference reflection microscopy [Curtis, 1964], by Abercrombie and Dunn [1975]; Izzard and Lochner [1976]. These discrete areas, located at the cell's ventral surface and measuring several square micrometers, lie within 10–15 nm of the substratum. When first discovered, these apparent adhesion sites attracted immediate attention, since they appeared to be located at the termini of ''stress fibers,'' prominent bundles of actin filaments [Heath and Dunn, 1978; Wehland et al., 1979], suggesting mechanical continuity between the cell's contractile machinery and the extracellular matrix (ECM). The notion of such mechanical continuity was also suggested by earlier, electron microscopy studies of vertical sections of fibroblasts, which revealed electron-dense plaques, associated with bundles of microfilaments and apparently attached to the substrate [Abercrombie et al., 1971].

The ''molecular era'' of FAs began in the late 1970s and early 1980s, with the discovery of their first molecular component; namely, vinculin [Geiger, 1979; Burridge and Feramisco, 1980] and pp60src [Rohrschneider, 1980], work that was highlighted in a mini-review in Nature, entitled, ''Hot Foot'' [Lloyd, 1980]. At that time, it was already clear that FAs play a major role in the dynamic crosstalk between the actin cytoskeleton and the ECM in the course of cell movement, cell adhesion, and matrix remodeling. The complex molecular composition of FAs, their intricate structure, and their dynamic organization were, however, obviously underestimated in those early years. Three decades later, we can state that though remarkable progress has been made in identifying novel FA components and dynamic characteristics, our knowledge and understanding of FAs remain far from saturation, keeping the ''foot'' still pretty ''hot.''

Recent bioinformatic approaches revealed that FAs contain more than 80 types of proteins (commonly referred to as ''plaque proteins''), located at the interface between the transmembrane adhesion receptors (mainly $\alpha5\beta1$ and $\alpha v\beta3$ integrins, and probably also syndecans [Morgan et al., 2007] and the actin cytoskeleton [Zaidel-Bar et al., 2007]). Among these proteins, there are a few molecules that directly connect integrin with actin (mainly talin, along with tensin, filamin and α-actinin), as well as multiple indirect linkers and numerous bona fide signaling proteins, including different kinases and phosphatases, their many targets, exchange factors for small G-proteins, and the like [Zaidel-Bar et al., 2007; Geiger et al., 2009]. Bioinformatic analyses of existing experimental data on the interactions between FA components enable identification of functional circuits within this complex molecular network [Zaidel-Bar et al., 2007]. In fact, the molecular machinery located at FAs generates a variety of signals that not only affect the fates of the adhesion itself and the associated actin cytoskeleton, but also cell growth, differentiation, and survival [Reddig and Juliano, 2005; Assoian and Klein, 2008; LaFlamme et al., 2008; Legate et al., 2009]. Thus, individual FAs appear to act as an ''intelligent system'' capable of responding to multiple sensory cues, integrating them in time and space, and responding to the input signals, both locally and globally.

However, unlike man-made ''intelligent machines,'' the most immediate and apparent FA-mediated response involves the modulation of FA size, location and, most likely, composition. Indeed, time-lapse movies of FAs indicated that these structures are dynamic and versatile, and can grow, shrink, and sometimes translocate along the substratum [Zamir et al., 2000; Ballestrem et al., 2001; Rid et al., 2005; Holt et al., 2008; see also Zaidel-Bar et al., 2005] (Fig. 1). These continuous changes in FA structure are apparently necessary, if FAs are to accommodate themselves to the ever-changing mechanical stress under which they operate, and to changes in the underlying matrix, particularly prominent during cell migration. Local changes in FAs also have long-range effects on the remodeling and mechanical activities of the actin cytoskeleton. For example, they locally promote actin polymerization [Turnacioglu et al., 1998; Gupton et al., 2007; Hirata et al., 2008], a process which is required for the assembly of straight actomyosin bundles (stress fibers) and their end-on anchorage in the FA plaque [Hotulainen and Lappalainen, 2006; Endlich et al., 2007]. In fact, stress fibers have never been observed in cells lacking mature FAs, suggesting that FAs are an integral part of these cytoskeletal structures, analogous to the relationship between the soles of our feet, and the musculature of the leg.

The input cues affecting FAs are numerous, and can be both external or internal. Notable among them are mechanical signals—typically, forces generated by the actomyosin machinery within the cell, as well as mechanical cues transmitted via the extracellular environment (e.g., the stretching or rigidity of the ECM). How the FA mechanosensory machinery interprets these input cues and transforms them into output events is not yet clear, but general behavioral characteristics of these structures have begun to emerge.

THE BIRTH, MATURATION, AND DEATH OF FOCAL ADHESIONS

The commonly held view that FAs evolve from initial ''punctate,'' ''dot-like,'' ''nascent'' ''focal complexes'' appearing continuously at the cell's leading

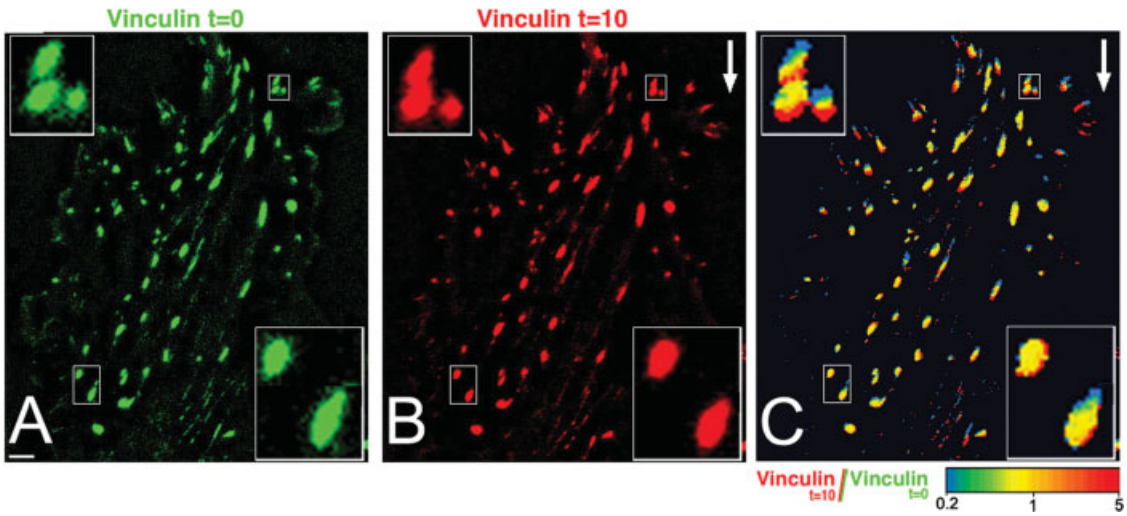

Fig. 1. Shear stress-induced growth of "upstream focal adhesions" and reduction in the size of "downstream focal adhesions." Cultured endothelial cells expressing CFP-vinculin were monitored at 2-min intervals before and after application of flow (20 dyn/cm^2, direction is indicated by white arrow). Frames from such a movie, taken 10 min apart (**A** and **B**), and an intensity ratio image of the two time points (**C**) are shown. Scale bar (in A): 5 μm. Selected groups of focal adhesions (in small rectangles) are shown at high magnification in the insets. In the ratio image (C), the colors represent the intensity ratio values in a spectral scale. Extension of focal adhesions appears red, whereas loss of focal adhesion area appears blue. Note that focal adhesions grow centripetally (toe-to-heel), and shrink in the opposite direction [Adapted from Zaidel-Bar et al., 2005].

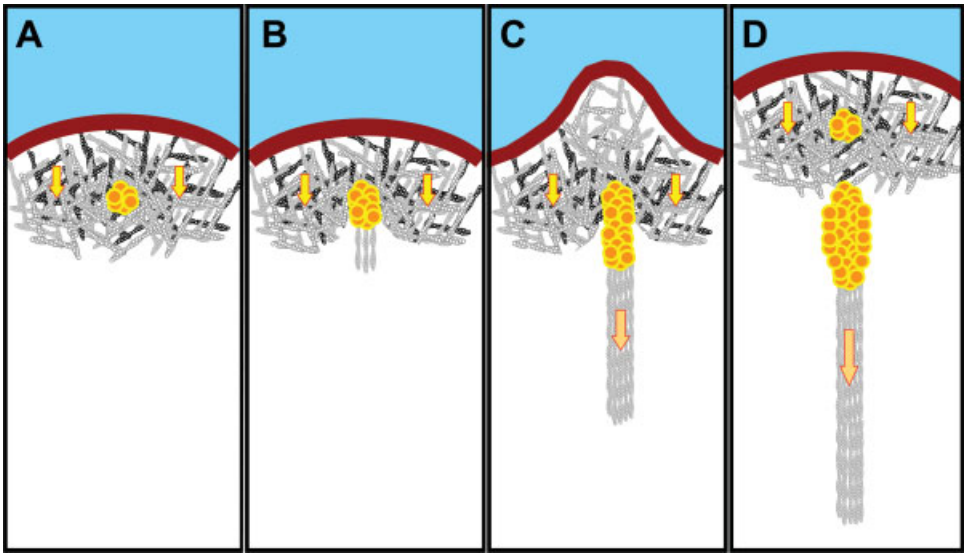

Fig. 2. A cartoon that summarizes the main stages of FA formation and maturation, and the simultaneous advancement of the boundary between the lamellipodium and the lamella domains. The substrate is marked blue; the plasma membrane at the cell edge shown as a brown thick line. The cell is viewed from its "ventral" substrate-attached aspect. Nascent and mature FAs of different sizes are shown as clusters of red-yellow circular "subunits." The lamellipodium is filled with a dense, branched network of actin filaments; yellow arrows symbolize the centripetal actin flow characteristic of that area. The stress fibers are shown as actin filament bundles; cross-linking proteins and myosin-II are not shown. The forces generated by the actomyosin contraction in the stress fibers are indicated by the orange arrows. (**A**) Formation of nascent adhesion underneath the branched actin network in the lamellipodium. (**B**) Early stage of FA maturation, formation of the precursor of a stress fiber (by filament nucleation and crosslinking) and appearance of a new border between the lamellipodium and the lamella. (**C**) Formation of a contractile stress fiber and force-dependent growth of FAs. Simultaneously, bulging of the lamellipodial protrusion just opposite the growing FA occurs. (**D**) The lamellipodial network moves forward due to FA-triggered disassembly and the assembly at the tip. The mature FA and the associated stress fiber continue to grow in the lamella, while a new, nascent adhesion appears in the lamellipodium. The sequence of events is based on Refs. Alexandrova et al. [2008]; Choi et al. [2008]; Vicente-Manzanares et al. [2009].

edge, into mature structures is supported by many studies [Bershadsky et al., 1985; Clark et al., 1998; Rottner et al., 1999; Alexandrova et al., 2008; Choi et al., 2008]; see also Fig. 2. The so-called ''leading edge'' of moving or spreading cells can be subdivided into peripheral lamellipodium (a 2- to 4-μm-wide ribbon immediately adjacent to the cell border and filled with a very dense, branched network of actin filaments), and the more internal ''lamella proper,'' containing a sparser network composed of circumferential and radial actin filament bundles and individual, unbranched actin filaments, along with intermediate filaments and microtubules [Svitkina et al., 1984; Svitkina and Borisy, 1999; Small et al., 2002b; Ponti et al., 2004; Danuser, 2005]. In both the lamellipodium and the lamella proper, the bulk of the cellular material is continuously moving backward, but the velocity and, perhaps, driving forces underlying this centripetal flow differ noticeably [Ponti et al., 2004; Alexandrova et al., 2008]. In lamellipodia, the flow is always two- to five-fold faster than in the lamella, and is generated by Arp2/3 complex-dependent actin polymerization [Ponti et al., 2004, 2005; Vallotton et al., 2004; Alexandrova et al., 2008]. (In fact, some observations would suggest that the centripetal movement of the upper layer of the lamellipodial network is also myosin-II-dependent [Giannone et al., 2007]). The slow flow in the lamella proper depends entirely on contractile forces, primarily generated by myosin-II [Ponti et al., 2004; Alexandrova et al., 2008]. In many cell types, the boundary between the lamellipodium and the lamella is a site where FA maturation apparently takes place [Hu et al., 2007; Alexandrova et al., 2008; Choi et al., 2008].

Initial, or ''nascent'' FAs mainly form underneath the lamellipodium (Fig. 2). They appear as submicron-sized vinculin- or paxillin-positive dots, sometimes traveling for short distances with the actin flow, but usually, they remain immobile [Alexandrova et al., 2008; Choi et al., 2008]. The fast centripetal flow in the lamellipodium plays a critical role in the initiation of FA formation, since mild treatment with cytochalasin D, which halts the flow, leads to the rapid disappearance of these structures [Alexandrova et al., 2008; Choi et al., 2008]. The mechanism underlying the association of the lamellipodial actin meshwork with these early adhesions is not clear, though some data suggest that a direct link may be formed between the Arp2/3 complex, and either vinculin [DeMali et al., 2002] or focal adhesion kinase (FAK) [Serrels et al., 2007]. Both in lamellipodia and filopodia (finger-like cell projections capable of establishing small, transient integrin-mediated contacts with the ECM), the location of integrin molecules in an active conformation [Calderwood, 2004; Banno and Ginsberg, 2008] strongly correlates with sites of actin polymerization [Galbraith et al., 2007].

Nascent FAs (or focal complexes) are short-lived structures (on the order of seconds) that either disappear or rapidly grow centripetally, undergoing transition into elongated, mature FAs. Several important features typically underlie such growth and maturation (Fig. 2). First, the entire process is strictly myosin-II-dependent [Riveline et al., 2001; Alexandrova et al., 2008]; the myosin-IIA isoform seems to play a major role [Choi et al., 2008]. Second, the FA always grows in a centripetal direction, from the initial adhesion toward the cell center, corresponding with the centripetal direction of the actin flow [Alexandrova et al., 2008]. Third, FA maturation is accompanied by formation of an actomyosin bundle associated with the growing proximal end of the FA, and also oriented centripetally [Hotulainen and Lappalainen, 2006; Endlich et al., 2007].

Thus, transition from a nascent to a mature FA creates a symmetry break in the FA structure: instead of a seemingly isotropic, dot-like focal complex, an elongated polar structure with a distal tip (''toe''), and proximal end (''heel'') associated with the growing actin bundle, emerges. The changes in protein composition or phosphorylation underlying this reorganization remain to be elucidated, but one interesting feature is well-documented: the plaque protein zyxin is never recruited to nascent adhesions, but rather binds to FAs upon their maturation [Zaidel-Bar et al., 2003].

The F-actin cross-linker α-actinin, along with myosin-II, are among the first proteins to appear in the actin filament bundle growing from the ''heel'' portion of the maturing FA [Hotulainen and Lappalainen, 2006; Choi et al., 2008]. Notably, these two proteins occupy distinct, even mutually exclusive, domains along the actin bundle in skeletal muscle, smooth muscle, and striated stress fibers [Draeger et al., 1990; Sanger et al., 2002; Naumanen et al., 2008]. At early stages of maturation, these proteins are thought to crosslink the actin filaments into a coherent bundle [Choi et al., 2008; Vicente-Manzanares et al., 2009]. Later on, myosin-IIA produces contractile forces that become major regulators of FA dynamics and growth (see following section). The directionality of such growth coincides with the direction of retrograde actomyosin flow, also driven essentially by myosin IIA [Cai et al., 2006]. Moreover, several bona fide components of the FAs are carried along the centripetal flow, continuously moving within the FAs in the direction of the flow [Guo and Wang, 2007; Hu et al., 2007].

As it matures, the growing FA defines a new boundary between the lamellipodium and the lamella [Alexandrova et al., 2008]. This boundary apparently connects neighboring young FAs by arcs, concave in the centripetal direction (Fig. 2); thus, the formation of new

FAs shifts the boundary forward. Older, apparently more mature adhesions remain, and sometimes continue to grow, within the lamella.

Once their maturation is essentially complete, FAs within the lamella domain stop growing, remain stationary for up to tens of minutes, and gradually disappear. In migrating cells, stationary FAs usually undergo disassembly when, due to the forward movement of the entire cell, they find themselves at the rear part of the lamella [Rid et al., 2005]. The high density of microtubules and microtubule tips found in this region is believed to be a major factor promoting FA disassembly [Kaverina et al., 1999; Broussard et al., 2008]. Indeed, in cells with disrupted microtubules or inhibited kinesin motor activity, FAs and stress fibers are larger and more stable [Bershadsky et al., 1996; Liu et al., 1998; Krylyshkina et al., 2002].

Though the physiological mechanism underlying FA disassembly is not completely understood, it is well-established that FA disassembly can be triggered by any treatment interfering with myosin-IIA-driven contractility both in the presence and in the absence of microtubules [Bershadsky et al., 1996; Helfman et al., 1999]. A plausible hypothesis is that FA disassembly might involve local downregulation of the myosin-II-driven pulling forces. If this is the case, microtubules may serve as (local) regulators of myosin-II contractility, consistent with numerous previous observations [Elbaum et al., 1999; Small et al., 2002a] and with more recent findings of their inhibitory role in the regulation of the RhoA exchange factor, RhoGEF-H1 [Krendel et al., 2002; Chang et al., 2008]. However, other mechanisms, such as local proteolysis by the protease calpain resident in FAs [Bhatt et al., 2002] or enhanced, microtubule-dependent endocytosis activated by the FAK-dynamin pathway [Burridge, 2005; Ezratty et al., 2005] may also participate in this process. How all of these factors work in concert is still unknown.

It is interesting that in well-controlled situations such as FA disassembly following myosin-II inhibition, the disassembly proceeds in a vectorial manner—from heel to toe. Thus, FAs grow from toe to heel and shrink from heel to toe, so that the heel (proximal part) of the FA should be more dynamic than the distal, toe part. This is consistent with recent, direct measurements of FA dynamics [Wolfenson et al., 2009] (and see below). However, the possibility that, in addition to the disassembly from heel to toe, disassembly at the distal toe end could also occur, cannot be excluded. In particular, it has often been observed that some FA translocate, as a whole, in the centripetal direction [Zamir et al., 2000; Ballestrem et al., 2001]. Such translocations can occur due to expansion of FA at the heel area, and disassembly at the toe (a ''treadmilling'' mechanism). On the other hand, true centripetal ''sliding'' of large FAs (namely, translocation of the FA, as a coherent unit, relative to the substrate) often occurring at the rear of the cell, is most probably the result of partial or complete detachment of such adhesions from the substrate, or the centripetal ''dragging'' of loosely bound components of the ECM [Broussard et al., 2008].

ADHESION-DEPENDENT MECHANOSENSITIVITY: PHENOMENOLOGY AND ELEMENTARY MECHANISMS

A variety of myosin-II inhibitors efficiently interfere with the maturation, growth, and maintenance of mature FAs, but not with the formation of nascent adhesions (focal complexes). These include agents inhibiting myosin-II light chain phosphorylation by suppressing Rho, Rho kinase (ROCK), and myosin light chain kinase [Volberg et al., 1994; Chrzanowska-Wodnicka and Burridge, 1996; Geiger and Bershadsky, 2001; Riveline et al., 2001; Alexandrova et al., 2008], as well as the low molecular-weight inhibitor blebbistatin [Gupton and Waterman-Storer, 2006; Even-Ram et al., 2007] and the regulatory protein caldesmon [Helfman et al., 1999; Grosheva et al., 2006], which block the actin-activated myosin-II ATPase by inhibiting productive binding of myosin-II to actin [Kovacs et al., 2004; Alahyan et al., 2006; Zhao et al., 2008]. Experiments with RNAi-mediated knockdown of myosin-II isoforms revealed that myosin-IIA plays a major role in FA growth and maintenance, at least in cultured fibroblasts and epithelial cells [Sandquist et al., 2006; Even-Ram et al., 2007; Vicente-Manzanares et al., 2007]. Myosin-IIB in these cells is normally responsible for the formation of FAs at the rear of the cell, and therefore in the establishment of cell polarity in 2D cultures [Lo et al., 2004; Vicente-Manzanares et al., 2008]; in 3D conditions, the distribution of functions between myosins IIA and IIB may differ [Meshel et al., 2005; Ahmed et al., 2007]. The localization and possibly functions of myosin IIA and IIB isoforms depend on their short C-terminal tail region [Sandquist and Means, 2008]. The role of the third myosin-II isoform, myosin-IIC, remains to be elucidated. Surprisingly, another related myosin, myosin 18A (MYO18A) was recently shown to function in concert with myosin IIA and to be required for the proper assembly of lamellar actomyosin bundles [Tan et al., 2008].

Besides myosin-driven contractility, actin polymerization can also create mechanical forces [Marcy et al., 2004; Prass et al., 2006; Footer et al., 2007], which may play a role in the formation of nascent adhesions under-

neath lamellipodia and filopodia [Galbraith et al., 2007; Alexandrova et al., 2008; Choi et al., 2008].

The notion of FA mechanosensitivity gained support from experiments involving the more or less direct application of mechanical stimuli to these structures. Local interventions involving the stretching of the cell edge by micromanipulation [Riveline et al., 2001], or by application of local stretching forces to either the elastic substrate adjoining the cell edge [Kaverina et al., 2002], or to the PDMS micro-pillars to which the cell is attached [Sniadecki et al., 2007], all seem to result in the apparent growth of FAs experiencing the pulling force. The incorporation of new FA components from solution into the FAs can be triggered even by the stretching of Triton X100-treated demembranated cytoskeletons [Sawada and Sheetz, 2002]. The effect of stretching on FA subunit incorporation was also evident in cells with inhibited actomyosin contractility [Riveline et al., 2001]. Altogether, these and other findings suggest that FAs are, by nature, mechanosensory units responding to stretching forces by growth, and to relaxation by disassembly. On average, the force required to maintain the FA is about 5 nN per square micrometer [Balaban et al., 2001; Bershadsky et al., 2003], though some adhesions at the leading edge can experience stronger forces [Beningo et al., 2001].

Several novel pathways that could, in part, be responsible for the reinforcement of FAs were recently discovered. Among FA components, talin seems to form the most important link between the cytoplasmic portion of β-integrin and the actin filament [Brown et al., 2002; Jiang et al., 2003; Ziegler et al., 2008]; it is also required for integrin activation [Calderwood, 2004; Wegener et al., 2007] and FA formation [Zhang et al., 2008]. Besides actin and β-integrin, talin binds another FA component, vinculin. There are several sites on the talin rod that bind the vinculin head [Ziegler et al., 2008]. The majority of these sites are cryptic (buried inside the rod), but can be opened by unfolding the talin molecule, as was proposed in structural studies [Papagrigoriou et al., 2004; Fillingham et al., 2005; Gingras et al., 2006], verified by steered molecular dynamic simulations [Lee et al., 2007, 2008; Hytonen and Vogel, 2008], and finally directly demonstrated in experiments involving the stretching of talin rods in vitro [del Rio et al., 2009]. Notably, binding of the vinculin head to talin promotes binding of the vinculin tail to the actin filament [Bois et al., 2006]. Thus, one scenario for the force-dependent reinforcement of a talin-mediated integrin-actin link could be based on the force-mediated binding of the extra vinculin molecules to the talin rod and, consequently, formation of additional, vinculin-mediated talin-actin bonds. It is not yet clear whether this reinforcement mechanism is only involved at the initial stages of FA formation, or if it is operative throughout the FA life cycle.

Other components of FAs were also shown to demonstrate force-dependent conformational changes (unfolding), thereby serving as individual mechanosensory components in the FA molecular network [Vogel, 2006; Schwartz, 2009]. Among such components is the signaling protein p130Cas which, upon stretching, exposes the Src-phosphorylation site [Sawada et al., 2006]. Molecular dynamic simulation shows that stretching forces applied to an integrin molecule could facilitate its transition from an inactive to an active conformation [Puklin-Faucher et al., 2006]. Indeed, in a recent experimental study [Friedland et al., 2009], myosin-II-dependent conformational transition in α5β1 integrin, enhancing its interaction with the ECM protein fibronectin, was detected. Finally, stretch-induced unfolding of fibronectin itself may also play a role in adhesion mechanosensitivity [Vogel, 2006; Smith et al., 2007]. Theoretical considerations imply the involvement of other mechanisms (e.g., a "thermodynamic" model, not requiring stretch-induced unfolding [Shemesh et al., 2005]), possibly underlying the force-induced assembly processes occurring in FAs (summarized in Bershadsky et al. [2006]). An in-depth examination of these issues is beyond the scope of this review.

ASYMMETRIC MOLECULAR DYNAMICS OF FOCAL ADHESIONS

Recent studies involving fluorescence correlation spectroscopy (FCS), fluorescent speckle microscopy, and fluorescence recovery after photobleaching (FRAP) revealed intriguing facets of FA dynamics. Direct measurements (mostly using FRAP) indicated that FAs are molecularly dynamic sites in which molecules from within the structure exchange with molecules from the cytoplasm. These measurements also indicated significant variations in the exchange rates among the different FA plaque proteins, suggesting differences in the repertoire of binding sites specific to each protein [Ballestrem et al., 2001; Edlund et al., 2001; Chandrasekar et al., 2005; Hamadi et al., 2005]. One study [Lele et al., 2006], for example, has demonstrated that the molecular binding kinetics of zyxin are sensitive to mechanical forces. The authors used several methods, including treatment with cytochalasin D, selective ROCK inhibition, or laser incision of stress fibers, to reduce or dissipate cell tension, to show that the rate at which zyxin unbound from FAs increases, under such conditions. These results are in line with zyxin's role as a typical constituent of mature FAs [Zaidel-Bar et al., 2003] and a component of mechanosensing machinery [Hirata et al., 2008; Colombelli et al., 2009], since, as mentioned

above, the maturation of focal complexes into FAs depends upon the tension exerted on the adhesion proper.

Fluorescent speckle microscopy was also used to analyze the dynamic interactions of several resident adhesion proteins within FAs [Hu et al., 2007; Gardel et al., 2008; Ji et al., 2008]. Studies by Hu et al. [2007] revealed that motions of different FA components correlate, to varying degrees, different degrees with motions of F-actin speckles, suggesting a hierarchical system of transmission of actin-driven forces through FAs. Assuming that the temporal variations of the F-actin flow gradients depend on the friction forces between F-actin and FAs/ECM, Ji et al. [2008] were able to calculate these adhesion forces and correlate them with the degrees of coupling between the motions of F-actin and vinculin speckles. It appeared that the predicted adhesion forces were higher when the motion of vinculin speckles was incompletely coupled to the F-actin flow, than when the motions of vinculin and F-actin speckles were fully coupled [Ji et al., 2008]. This suggests that vinculin is, indeed, a component of a slippage clutch that transmits forces from F-actin to the FAs/ECM.

The study by [Gardel et al., 2008] represents the first attempt to correlate actin dynamics with the directly measured mechanical forces, transduced from the actin cytoskeleton to the ECM via FAs. The authors employed quantitative fluorescent speckle microscopy to assess actin dynamics and high-resolution traction force microscopy to monitor cell-generated traction forces. They then superimposed the map of actin flow velocities onto the map of cell-generated traction forces, as well as onto FA distribution, and plotted the traction forces against the velocity of actin flow through the FAs. Consistent with previous observations, they noticed that the flow velocity drops when FAs are formed. Furthermore, they discovered a biphasic (''upside-down V-shaped'') dependence of the cell-mediated traction force on the actin flow velocity. Both low and high velocities corresponded to low traction force, while at intermediate velocities, the observed traction force was maximal. Analysis of such plots specifically for FAs revealed that the F-actin flow speed is inversely related to stress during FA assembly and growth, but is directly related to force during FA weakening. The authors conclude that FA-transduced traction force on the ECM responds in varying ways to F-actin motion, depending on the state of FA assembly and maturation. It is interesting that such behavior was observed in cells even under conditions of myosin-II inhibition, or upon expression of constitutively active Rac and Rho. Thus, the authors claim that a biphasic response to actin flow velocity is an intrinsic feature of FA complexes [Gardel et al., 2008].

In a series of FCS microscopy studies, Digman et al. [2008, 2009a,b] explored the dependence of the molecular dynamics of several FA resident proteins, on the assembly/disassembly stage of the adhesion. In these articles, the authors employed several novel correlation microscopy-based methods, including temporal image correlation spectroscopy and raster image correlation spectroscopy, along with particle number and brightness (N&B) analyses, and photon-counting histograms. These techniques enabled the detection of molecular complexes within FAs, and measurement of their dynamics in different regions of the adhesion, as well as comparison of adhesions at different stages of their life cycle. In these studies, the authors used several FA proteins to demonstrate that the binding equilibrium that occurs during adhesion assembly is characterized by monomers binding to immobile structures, whereas in disassembling adhesions or regions of adhesions, the equilibrium is characterized by the release of large protein aggregates. In addition, the treadmilling mechanism by which FAs perform their sliding movement involves the addition of monomers at one end, and removal of relatively large protein aggregates/complexes from the other. Once released from the FA, these complexes disassemble rapidly in the cytoplasm.

In a recent study, we combined FRAP studies with in silico simulations and mathematical modeling to characterize the dynamics of FA proteins in FAs proper, in nonadherent regions, and in the cytoplasm [Wolfenson et al., 2009]. Our findings demonstrated differences in the exchange rates of FA plaque proteins (paxillin and vinculin) between the two ends (heel and toe) not only in sliding adhesions, but also in FAs at steady state. At the heel, or proximal end, closest to the attached actin bundle, the majority of the paxillin and vinculin populations ($\sim$80%) underwent exchange at relatively high rates, while at the toe, or distal end, further from the actin attachment, the exchange rate was very slow; most of the plaque protein population remained immobile, and did not exchange (Fig. 3). β_3-integrin, a transmembrane protein, was laterally mobile outside FAs, but exhibited very low mobility, accompanied by a large immobile fraction, within FAs, indicating that the exchange of plaque proteins does not occur through movement of molecular complexes connected to integrin, but rather through the actual exchange of proteins between the FA and the cytoplasm.

Together with earlier observations suggesting a polarized distribution of actin within FAs, with the actin bundles mainly contacting the proximal end (heel) and, to a far lesser extent, the distal end (toe) [Zamir et al., 2000] (and Fig. 3), these findings imply that the forces exerted by the actin stress fibers may differ at the two ends, resulting in more rapid exchange in the region with

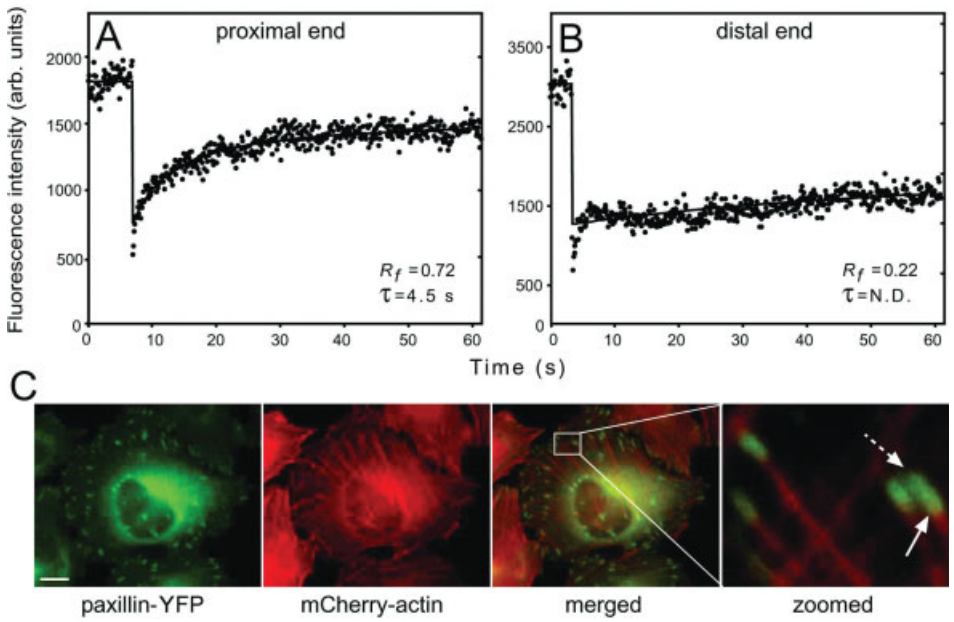

Fig. 3. Paxillin and vinculin display different dynamics at the FA proximal and distal ends. FRAP experiments were carried out, focusing the beam on the two FA ends. (**A**) A typical FRAP curve of paxillin-YFP at the proximal (heel) FA end (60-s timescale). (**B**) A typical curve at the distal (toe) FA end (60-s timescale). Fast recovery by diffusion exists, but the ensuing exchange is very slow. (**C**) Paxillin colocalizes with actin at the FA proximal end. HeLa-JW cells coexpressing paxillin-YFP and mCherry-actin were visualized by fluorescence microscopy. Colocalization was visible at the proximal edge (solid arrow), but not at the distal edge (dashed arrow). Scale bar: 10 μm. [Adapted from Wolfenson et al., 2009].

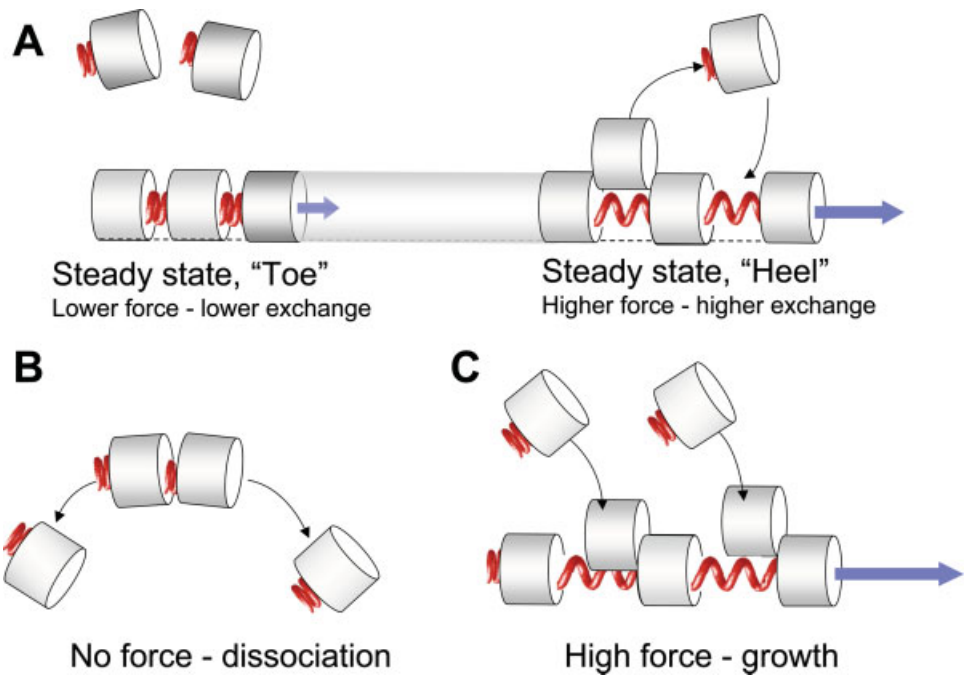

Fig. 4. (**A**) A diagram illustrating a hypothetical mechanism of asymmetric dynamics of FAs. Proteins or protein complexes corresponding to building blocks (subunits) of FA (gray cylinders) bind to each other, forming "elastic" bonds (red springs), which expand upon stretching and permit incorporation of new subunits in between them (this topology of subunit organization, similar to that proposed by Shemesh et al. [2005], is obviously an over-simplification). We hypothesize that the pulling forces experienced by the subunits (blue arrows) are higher at the "heel" region (immediately adjacent to the stress fiber), and lower at the distal, "toe" region. Accordingly, the incorporation of the new subunits and thus the subunit exchange rate at steady state are higher at the "heel," than at the "toe," in agreement with the experimental data [Wolfenson et al., 2009]. (**B**) Under conditions of complete relaxation (no force), the incorporation of new subunits is completely blocked, and the FA undergoes gradual disassembly. (**C**) Application of strong pulling force (i.e., higher that the steady-state force, marked by a blue arrow) promotes the incorporation of new FA subunits, and hence stimulates FA growth.

higher actin density. This suggestion is in line with models designed to explain the regulation of FA dynamics by the actomyosin machinery [Shemesh et al., 2005; Besser and Safran, 2006].

Another novel observation in the Wolfenson et al. [2009] study was the identification of a juxtamembrane region surrounding FAs, which displays a gradient of FA proteins with respect to both concentration and dynamics (the greater the distance from the adhesion, the more rapid the dynamics). This region may act as an intermediate layer between the cytoplasm and the membrane-associated FAs, dynamically trapping FA components to create a high local density of plaque subunits. This, in turn, would enable the rapid recruitment of plaque proteins into the FA, as may be required for FA growth in response to local, rapidly applied stress.

CONCLUSIONS, AND MORE OPEN QUESTIONS

At a nearby gym, the cycling trainer Kobi Appelbaum made the following "motivational statement": "Muscle understands only one language—force! But it is not the force per se that matters; rather, the cell's response to it." This review addresses this precise issue of adhesion mechanosensitivity, focusing on multiple features of the response to force, ranging from the effects on mechanosensitive components of integrin adhesions, force-responsive multiprotein complexes, to intact FAs, and integrated networks of FAs and the associated contractile cytoskeleton. The challenge lies in understanding the dynamic inter-relationships between all of these hierarchical states. Understanding such relationships requires a detailed characterization of the inner molecular architecture of FAs, which has only now begun to emerge, based on photo-activated light microscopy [Betzig et al., 2006; Shroff et al., 2007, 2008a,b] and cryo-electron tomography (O. Medalia and colleagues, unpublished data). These findings indicate that FAs contain substructure(s) characterized by arrays of "plaque" protein complexes.

Of particular interest is a unique feature of FAs; namely, their tendency to develop a toe-to-heel polarity. This polarity manifests itself in the tendency of FAs to grow, under tension, centripetally ("toe-to-heel") and to fade, upon mechanical relaxation, in the opposite direction ("heel-to-toe"). It would appear that FA organization is primarily driven by mechanical forces acting somewhere along the interface between the cytoskeleton and the ECM-attached membrane.

Some of these force-dependent features are presented in a highly schematic and hypothetical manner in Fig. 4. We suggest that the mechanical forces applied by the actomyosin system to the FA plaque are higher in the heel region and lower in the toe region, thereby differentially distorting the plaque components to which it is anchored. We further propose that the higher tension at the heel region enables an active exchange process to take place. Although the lower tension at the toe region is sufficient to keep the plaque components in place, it is insufficient to drive their active exchange. Accordingly, an increase in the force (and stretching of the plaque) beyond steady-state levels can induce FA growth, whereas relaxation below that of the "toe levels" might be insufficient for maintaining the basic FA structure, thus leading to its disassembly (Fig. 4). The subunit topology hypothesized in this Figure is just one example of many possible architectures (compare with models [Shemesh et al., 2005; Bershadsky et al., 2006; Besser and Safran, 2006]), and should be considered in that light.

The paradigm of differential force-dependent regulation of FA dynamics fits local measurements of the dynamic reorganization of plaque components [Wolfenson et al., 2009]. It could also account for such supramolecular processes as FA growth, shrinking, and centripetal "migration," most likely due to a treadmilling process, whereby the "heel" is extending and the "toe" is dissociating. At this stage, however, evidence for the suggested model is rather circumstantial, and the underlying mechanisms are still poorly understood. Furthermore, the identities of presumably multiple mechanosensitive components of FAs remain obscure. Are these molecules directly involved in diverse adhesion-driven processes, such as the regulation of the cytoskeletal assembly, differential force transduction, and even signaling activities? What is the relationship between the topological and spatial organization of FA subunits and their mechanosensing activities? How is the complex information collected at FAs about the chemical and physical properties of the cell's environment integrated and interpreted, thereby triggering long-range responses? The growing interest in the structure and function of FAs, coupled with the development of advanced molecular perturbation techniques as well as sensitive tools for measuring cellular forces at high spatial and temporal resolution, and the persistent attempts to model the physical properties of FAs, will likely result in a new, more comprehensive view of the mechanisms underlying FA-mediated sensing.

ACKNOWLEDGMENTS

The authors are grateful to Barbara Morgenstern for editorial assistance. Y.H. holds the Zalman Weinberg Chair in Cell Biology, A.D.B. holds the Joseph Moss Professorial Chair in Biomedical Research, and B.G. holds the Erwin Neter Professorial Chair in Cell and Tumor Biology.

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