

## Cytoskeleton dynamics: Fluctuations within the network

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### Abstract

Out-of-equilibrium systems, such as the dynamics of a living cytoskeleton (CSK), are inherently noisy with fluctuations arising from the stochastic nature of the underlying biochemical and molecular events. Recently, such fluctuations within the cell were characterized by observing spontaneous nano-scale motions of an RGD-coated microbead bound to the cell surface [Bursac et al., *Nat. Mater.* 4 (2005) 557–561]. While these reported anomalous bead motions represent a molecular level reorganization (remodeling) of microstructures in contact with the bead, a precise nature of these cytoskeletal constituents and forces that drive their remodeling dynamics are largely unclear. Here, we focused upon spontaneous motions of an RGD-coated bead and, in particular, assessed to what extent these motions are attributable to (i) bulk cell movement (cell crawling), (ii) dynamics of focal adhesions, (iii) dynamics of lipid membrane, and/or (iv) dynamics of the underlying actin CSK driven by myosin motors.

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The cytoskeleton (CSK) is fundamental to many cellular processes, including proliferation, migration, and contraction [7,20,21]. To perform these functions, cells orchestrate a complex cascade of signals and molecules that lead to robust structural changes in the underlying CSK [15,21]. This ability of the CSK to disassemble, to reform, and to stabilize provides, for example, a basic framework for metastasis of a cancer cell as it explores, crawls, and invades other tissues [5,41,45]. As such, progression of cancer as well as pathogenesis of many chronic

disorders is now thought to be associated with abnormalities in the stability and dynamics of the underlying CSK [16,34,37,39].

The CSK is a network of actin filaments, microtubules, and intermediate filaments that are bound together by associated cross-linkers and driven by motor proteins [8,21,27]: the cell interior is a crowded environment [11,12] and, at the same time, is far from an equilibrium system [18]. Such complexity of the network and its out-of-equilibrium dynamics are the focus of much attention in the fields as diverse as condensed matter physics [10,17,28] as well as biophysics of the living cell [13,25,26,35,38,42]. Most recently, we have made a series of phenomenological observations in a number of different cell types and reported a functional assay that probes molecular level fluctuations within the living cell [1,3,6]. This assay is based on spontaneous nano-scale movements of an individual microbead that is coated with a peptide

*Abbreviations:* ASM, airway smooth muscle; CSK, cytoskeleton; MSD, mean square displacement; acLDL, acetylated low-density lipoprotein; PLL, poly-L-lysine; RGD, arginine–glycine–aspartic acid.

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containing the sequence Arg-Gly-Asp (RGD); such beads bind to cell surface integrin receptors [43], form focal adhesions [29,30], and become well integrated into the cytoskeletal scaffolding [13,19,29,33]. Accordingly, these bead motions may reflect ongoing remodeling events of the underlying cytoskeletal network [1,3,6], but the structural origin of such motions and forces that drive these dynamics in a living cell remain to be elucidated.

Like the CSK of many cell types, that of the airway smooth muscle (ASM) cell is a dynamic structure that is in a continuous state of remodeling [1,4,6,24,31,39]. Using the ASM cell as a model and, using multiple well-defined cell microenvironments, we provide here strong evidence that spontaneous nano-scale motions of an individual RGD-coated microbead report ongoing molecular level reorganization of the underlying actin CSK driven by myosin motors.

## Materials and methods

**Materials.** Tissue culture reagents were obtained from Sigma (St. Louis, MO). The synthetic Arg-Gly-Asp (RGD) containing peptide was purchased from American Peptide Company, Inc. (Sunnyvale, CA) and acetylated low-density lipoprotein (acLDL) was purchased from Biomedical Technologies (Stoughton, MA). The micropatterned (50  $\mu\text{m} \times 50 \mu\text{m}$ ) substrates were a generous gift from Dr. Phillip DeLuce (Boston, MA). All other reagents and drugs were obtained from Sigma with the exception of jasplakinolide, which was purchased from CalBiochem (La Holla, CA). Jasplakinolide and cytochalasin-D were prepared in sterile dimethylsulfoxide (DMSO). Histamine and N<sup>6</sup>,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (db-cAMP) were reconstituted in sterile distilled water. On the day of experiments, all drugs were diluted to final concentrations in serum-free media, yielding less than 0.1% DMSO in final volume.

**Cell culture.** Human ASM cells were provided by Dr. Reynold Panettieri (University of Pennsylvania, PA) and rat ASM cells were prepared as previously described [1,2]. Cells were grown until confluence at 37 °C in humidified air containing 5% CO<sub>2</sub> and passaged with 0.25% trypsin–0.02% EDTA solution every 10–14 days. In the present study, we used cells in passages 3–7. Unless otherwise specified, serum-deprived post-confluent cells were plated at 20,000 cells/cm<sup>2</sup> on plastic wells (96-well Removawell, Immulon II: VWR International, West Chester, PA) previously coated with type I collagen (Vitrogen 100; Cohesion, Palo Alto, CA) at 500 ng/cm<sup>2</sup>. Cells were maintained in serum-free media for 24 h at 37 °C in humidified air containing 5% CO<sub>2</sub>. These conditions have been optimized for seeding cultured cells on collagen matrix and for assessing their mechanical properties [2,13,43].

**Bead coating.** Ferrimagnetic microbeads were coated with RGD, acLDL, or PLL as described previously [9,13,33,43]. Unless otherwise noted, approximately  $5 \times 10^4$  beads were added to individual sample wells and incubated for 20 min. Unbound beads were then removed by washing cells with serum-free media.

**Characterization of spontaneous bead motions.** Under microscopic observation, we visualized spontaneous displacements of an individual microbead (approximately 40–100 beads per field-of-view) and recorded its positions at frequency of 12 frames/s for  $t_{\text{max}} \sim 330$  s as previously described [6]. Bead positions were corrected for the effects of microscope stage drift; the stage drift was estimated from changes in the mean position of all beads within a field of view [6]. We defined mean square displacement (MSD) of an individual bead as

$$\text{MSD}_b(\Delta t) = \langle (r(t + \Delta t) - r(t))^2 \rangle \quad (1)$$

where  $r(t)$  is the bead position at time  $t$ ,  $\Delta t$  is the time lag, and brackets indicate an average over many starting times  $t$  [6]. The limit of resolution in our system was in the order of  $\sim 10$  nm, but for  $\Delta t \sim 4$  s most beads had displaced a much greater distance. Accordingly, we analyzed data for time lags greater than 4 s and up to  $t_{\text{max}}$ . MSD of most beads increased with time according to a power-law relationship.

$$\text{MSD}(\Delta t) = D^*(\Delta t/\Delta t_0)^\alpha \quad (2)$$

The coefficient  $D^*$  and the exponent  $\alpha$  of an individual bead were estimated from a least-square fit of a power-law to the MSD data for  $\Delta t$  between 4 s and  $t_{\text{max}}/4$ . The upper cut-off of  $t_{\text{max}}/4$  was chosen arbitrarily to increase statistical accuracy of the estimated  $D^*$  and  $\alpha$ . We took  $\Delta t_0$  to be 1 s and expressed  $D^*$  in units of nm<sup>2</sup>.

In the present study, we quantified individual bead motions both before and after each drug treatment by MSD( $\Delta t$ ). To modulate actin polymerization, cells were treated either with actin disrupting agent cytochalasin-D (1  $\mu\text{M}$ ) for 30–60 min or with actin polymerizing agent jasplakinolide (1  $\mu\text{M}$ ) for 10 min. To modulate actomyosin interactions, cells were contracted for 5 min with histamine (100  $\mu\text{M}$ ) or relaxed for 15 min with db-cAMP (1 mM).

**Optical magnetic twisting cytometry (OMTC).** To estimate the stiffness of structures bound to the bead, we measured bead displacements under applied torque as previously described [13]. In brief, ferrimagnetic microbeads were first magnetized horizontally (parallel to the surface on which cells were plated) and then twisted in a vertically aligned homogenous magnetic field (20 G) at a frequency of 0.75 Hz. The resulting lateral bead displacements in response to the oscillatory torque were detected optically, and the ratio of specific torque to lateral bead displacements was computed and expressed as the cell stiffness in units of Pa/nm.

## Results and discussion

### Characterization of spontaneous bead motions

Spontaneous motions of each RGD-coated bead (4.5  $\mu\text{m}$  in diameter) bound to the surface of the ASM cell were random and consisted of relatively small steps (Fig. 1A); over the course of 5 min, bead trajectories amounted to only a small fraction of the bead diameter. Such trajectories, however, appeared elongated or directed, suggesting a certain degree of positive correlation between incremental bead steps.

For each bead, we characterized its spontaneous nano-scale motions by calculating mean square displacement ( $\text{MSD}_b$ ) (Eq. 1);  $\text{MSD}_b$  varied by two orders of magnitude, but  $\text{MSD}_b$  of most beads increased with time according to a power-law relationship (Fig. 1B). These motions were further characterized by fitting a power-law to individual  $\text{MSD}_b$  to estimate diffusion coefficient  $D^*$  and the exponent  $\alpha$ . The probability density of the diffusion coefficient  $D^*$ , between individual beads, showed monophasic and almost lognormal distributions with a maximum of 50 nm<sup>2</sup>, whereas that of the exponent  $\alpha$  exhibited monophasic and almost normal distributions with a maximum of 1.6 (Fig. 1C). Accordingly, ensemble average of all  $\text{MSD}_b$  (MSD) demonstrated superdiffusive behavior ( $\alpha > 1$ ), whereby the MSD increased with time as  $\sim t^{1.6}$  (Fig. 1B, inset). Taken together, unlike a simple diffusive thermal Brownian motion that increases its MSD linearly with time [23], spontaneous motions of an individual RGD-coated

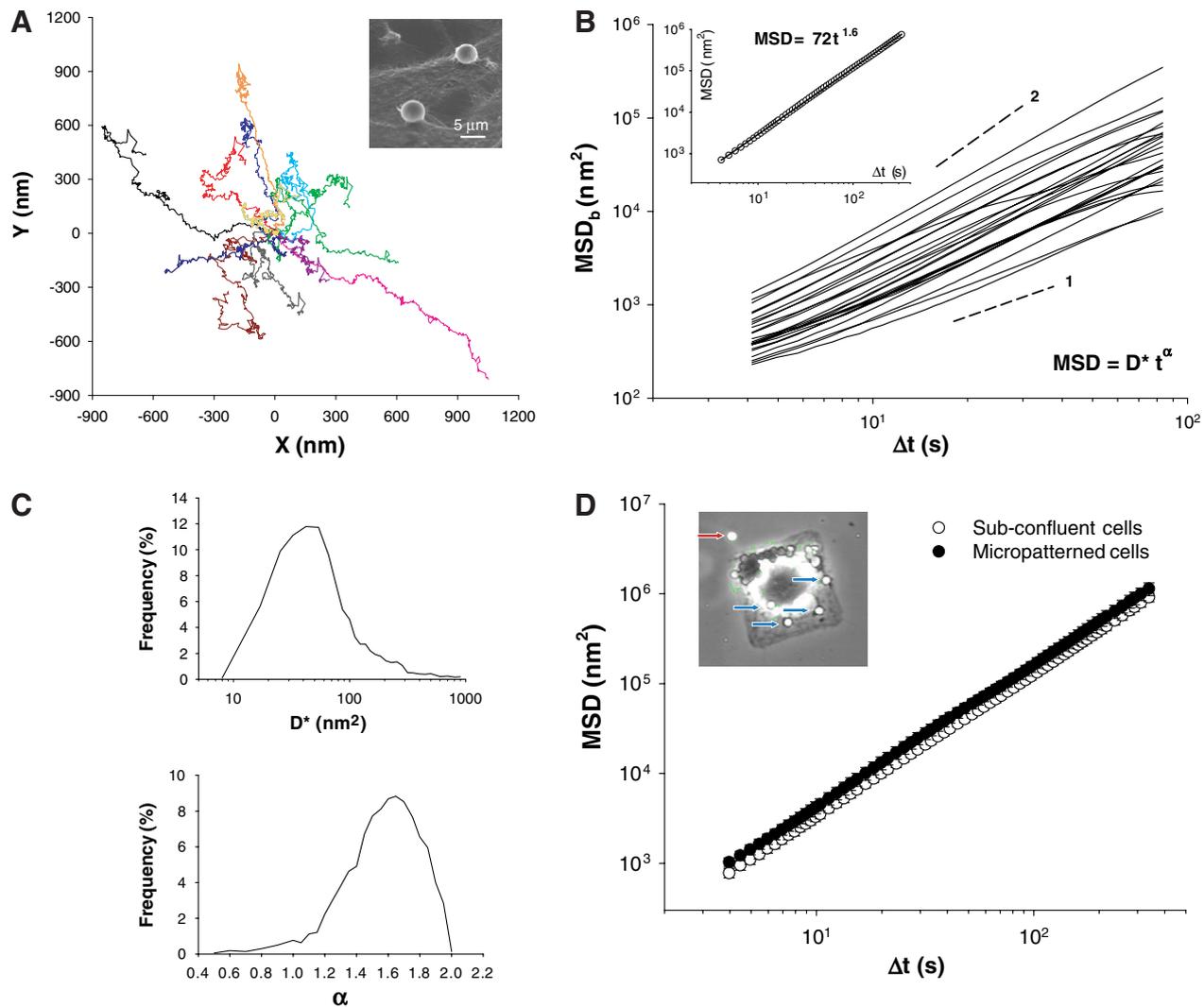


Fig. 1. Characterization of spontaneous bead motions. (A) Spontaneous motions of RGD-coated beads recorded over the course of 5 min. For clarity, only few representative tracings are depicted from the same point of origin (10× objective,  $n = 14$  beads). Inset: a scanning electron microphotograph of individual RGD-coated microbeads attached to the cell surface. (B) Mean square displacement (MSD<sub>b</sub>) calculated from Eq. 1 is shown for representative beads. Inset: ensemble average of all MSD<sub>b</sub> (MSD) increased with time as  $\sim t^{1.6}$ . (C) The histograms of diffusion coefficient  $D^*$  and exponent  $\alpha$  estimated from a least-square fit of a power-law (Eq. 2) to the MSD<sub>b</sub> data. (D) MSD of beads on subconfluent cells (○) versus cells adhered to micropatterned substrates (●). Inset: RGD-coated microbeads (blue arrows) attached to a single micropatterned ASM cell (50 μm × 50 μm). Data are expressed as means ± SE ( $n = 322$  for beads on micropatterned and  $n = 1202$  on sub-confluent cells).

bead were non-thermal in nature and, instead consistent with the notion that these anomalous motions are governed by an additional source of energy in the living cell [6].

#### Role of bulk cell movement (cell crawling)

We considered the possibility that these anomalous motions of an RGD-coated bead might be dictated by the motions of an entire cell body. To test this hypothesis, we used a micropatterned substrate on which a cell could adhere but not crawl [32]. Consistent with spontaneous bead motions on the sub-confluent cells, an RGD-coated bead attached to a serum-deprived cell seeded on a micropatterned substrate exhibited the same superdiffusive motions (Fig. 1D). Thus, these findings suggest that cell

crawling is at best a minor contributing factor for the observed anomalous bead motions.

#### Role of lipid membrane dynamics

To assess the relative contribution of cortical membrane dynamics, we used beads coated with acetylated low-density lipoproteins (acLDL); acLDL-coated beads bind to scavenger receptors thought to be floating in the cell membrane and, as such, are not linked avidly to the cytoskeletal structures deep in the cell interior [1,43]. The spontaneous motions of an acLDL-coated bead were remarkably different from motions of an RGD-coated bead (Fig. 2A). Compared with motions of an RGD-coated bead, motions of an acLDL-

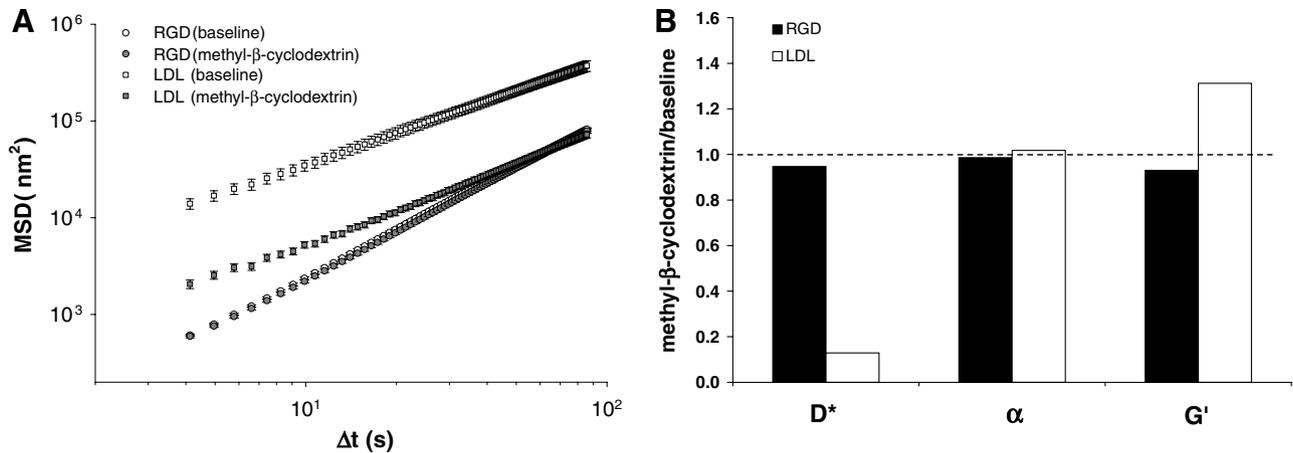


Fig. 2. Role of lipid membrane dynamics. (A) Addition of 10 mM methyl- $\beta$ -cyclodextrin for 1 h differentially affects MSD of beads coated with RGD [baseline ( $\circ$ ) and treatment ( $\bullet$ )] versus acLDL [baseline ( $\square$ ) and treatment ( $\blacksquare$ )]. Data are means  $\pm$  SE ( $n = 2054$  for RGD-coated and  $n = 887$  for acLDL-coated beads). (B) The effects of methyl- $\beta$ -cyclodextrin on  $D^*$ ,  $\alpha$ , and  $G'$  are normalized to their respective baseline values.

coated bead consisted of relatively large incremental steps (bigger  $D^*$ ) that seemed to be uncorrelated with time ( $\alpha \sim 1$ ).

In addition, acutely depleting cholesterol from the lipid membrane with methyl- $\beta$ -cyclodextrin [22] increased membrane stiffness as probed by OMTC with an acLDL-coated bead (Fig. 2B) and, at the same time, greatly reduced spontaneous lateral mobility of that same bead (Fig. 2A). Cholesterol depletion did little to CSK stiffness and spontaneous motions of an RGD-coated bead, however (Fig. 2). Accordingly, superdiffusive behavior of a RGD-coated bead seems to be insensitive to the dynamics of lipid membrane.

#### Effect of ligand coating density

Spontaneous motions of a bead (RGD versus acLDL) were also differentially affected by the amount of the ligand coating (0.1–300 ng/mg bead). Increasing coating density of RGD led to a dose-dependent increase in stiffness ( $G'$ ) as measured by OMTC and, at the same time, caused systematic changes in its spontaneous bead motions; in this concentration range (0.1–300 ng/mg bead), the diffusion coefficient  $D^*$  decreased by an order of magnitude whereas the exponent  $\alpha$  increased from nearly diffusive behavior (slope  $\sim 1.0$ ) to a highly superdiffusive behavior (slope  $\sim 1.7$ ) (Fig. 3A). In contrast, increasing coating concentration of acLDL did not yield appreciable changes in  $G'$ ,  $D^*$ , and  $\alpha$ ; in fact, these values were similar to those of the smallest amount of RGD coating (Fig. 3A).

These findings are consistent with the notion that a bead with the smallest amount of RGD coating binds to individual or just a few integrin receptors and therefore may not be firmly anchored to the deep CSK [14]. In that case, motions of such bead on the living cell may report instead dynamics of lipid membrane, at best cortical actin, similar to that measured with an acLDL-coated bead. On the other hand, the more firmly anchored a bead is to the

underlying CSK deep in the cell interior (for example, the highest amount of RGD coating), the higher stiffness ( $G'$ ) of that bead would be and, as a consequence, its spontaneous motions would exhibit smaller incremental bead steps ( $D^*$ ) while show faster and more correlated dynamics of its remodeling (higher  $\alpha$ ) (Fig. 3B).

#### Role of focal adhesion dynamics

We next considered a potential link between such anomalous bead motions and the dynamics of focal adhesions and, accordingly, quantified spontaneous motions of an individual bead coated with poly-L-lysine (PLL). A PLL-coated bead does not form focal adhesions [4,36] but, similar to an RGD-coated bead, binds tightly to the underlying CSK [9]; stiffness ( $G'$ ) as probed by OMTC with a PLL-coated bead was not appreciably different from that measured with an RGD-coated bead (Fig. 3C).

As observed with an RGD-coated bead, spontaneous motions of an individual PLL-coated bead showed the same superdiffusive behavior; the computed  $D^*$  and  $\alpha$  were not statistically different from that of an RGD-coated bead (Fig. 3C). These findings suggest that superdiffusive bead motions may report, rather than discrete changes in focal adhesions, underlying dynamics of the cytoskeletal structures deep within the cell.

#### Characterization of actin versus myosin modulations

To further quantify the relative contributions of actin and myosin motors to spontaneous bead motions, we used a panel of agencies that modulate their actions; we focused here upon actin and myosin because intermediate filaments and microtubules play little role in cell mechanics probed through an RGD-coated bead [1,40]. To modulate actin dynamics we used jasplakinolide and cytochalasin-D

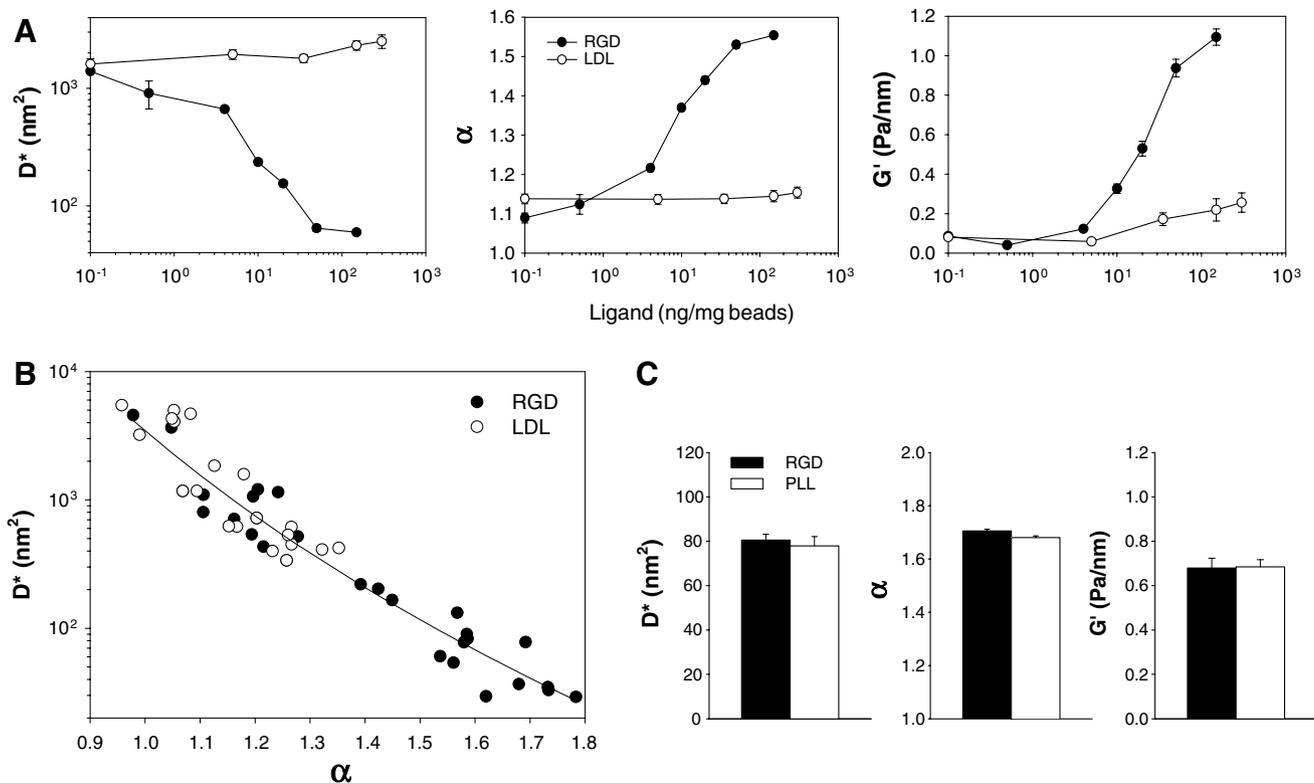


Fig. 3. Ligand bead dynamics. (A) Spontaneous ( $D^*$  and  $\alpha$ ) and forced ( $G'$ ) motions of individual microbeads (RGD versus aCLDL) show distinct characteristics with respect to the ligand coating (0.1–300 ng/mg bead). Data are means  $\pm$  SE ( $n = 700$ –2500 beads). (B) An inverse correlation between  $D^*$  and  $\alpha$ . Individual data points are ensemble averages of several hundred beads coated with different ligand concentrations and tested at different experimental days (data are taken from part A). (C)  $D^*$ ,  $\alpha$ , and  $G'$  of individual microbeads (RGD versus PLL) attached to the cell surface. Data are means  $\pm$  SE ( $n = 1044$  for RGD-coated and  $n = 1176$  for PLL-coated beads).

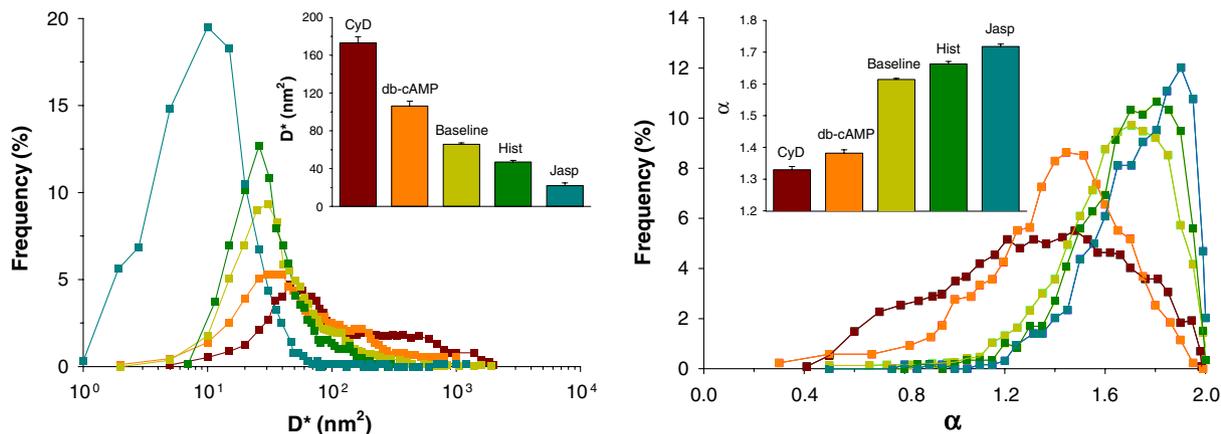


Fig. 4. Anomalous bead motions correlate with changes in tension within the CSK. Histograms of  $D^*$  and  $\alpha$  were determined both before and after addition of jasplakinolide (Jasp, 1  $\mu$ M; 10 min) and cytochalasin-D (CyD, 1  $\mu$ M, 10 min) to modulate actin dynamics, and histamine (Hist, 100  $\mu$ M; 5 min) and dibutryl-cAMP (db-cAMP, 1 mM; 15 min) to modulate myosin motors. Data are means  $\pm$  SE ( $n = 481$ –1174 beads).

whereas to modulate myosin motors we used histamine and db-cAMP.

On the one hand, decreasing actin polymerization and disrupting actin stress fibers with cytochalasin-D as well as turning off myosin motors with db-cAMP led to an increase in  $D^*$  and a decrease in  $\alpha$  (Fig. 4). On the other, increasing actin polymerization and actin stress fiber stabilization with jasplakinolide as well as turning on myosin

motors with histamine resulted in a decrease in  $D^*$  and an increase in  $\alpha$  (Fig. 4). Both actin polymerization and myosin activation are reported to increase tension within the CSK network whereas actin depolymerization and myosin inhibition decrease such tension [6,26,44]. Consistent with this notion, changes in the computed  $D^*$  and  $\alpha$  were closely correlated with changes in tension within the CSK. Taken together, these data provide strong evidence

that anomalous motions of an RGD-coated bead on the cell surface are largely attributable to discrete molecular level remodeling events of the underlying actin CSK driven by myosin motors.

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