Biochemical and Biophysical Research Communications 422 (2012) 653–657

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Tandem zyxin LIM sequences do not enhance force sensitive accumulation

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ARTICLE INFO

Article history:
Received 1 May 2012
Available online 15 May 2012

Keywords:
Cell migration
Focal adhesion
Zyxin
LIM domain
Traction force
Force sensor

ABSTRACT

The ability to sense mechanical forces is vital to cell physiology. Yet, the molecular basis of mechano-signaling remains unclear. Previous studies have shown that zyxin, a focal adhesion protein, is recruited at force-bearing sites on the actin cytoskeleton and, therefore, identifying zyxin as a mechano-sensing protein candidate. Furthermore, zyxin accumulation at force-bearing sites requires the LIM domain located at the C-terminus of zyxin. The zyxin LIM domain consists of three LIM motifs, each containing two zinc-binding sites. Since individual LIM motifs do not accumulate at focal adhesions or force-bearing sites, we hypothesize that multiple zyxin LIM domains increase force sensitivity. Using a miniature force sensor and GFP-tagged LIM variants, we quantified the relationship between single, tandem dimer and trimer LIM protein localization and traction forces. While the presence of extra LIM domains affected VASP recruitment to focal adhesions, force sensitivity was not enhanced over the single LIM domain. Therefore, zyxin force sensitivity is optimal with a single LIM domain, while additional LIM domains fail to enhance force sensitivity.

1. Introduction

Physical force is an integral component of the cellular microenvironment, and serves as a signaling mechanism to complement chemically-mediated signaling. Such mechanical perturbations originate from an elastic extracellular matrix, neighboring cells, or shear stress exerted by fluid flow. Numerous types of cells have been shown to sense physical forces, e.g. stiffness of extracellular matrix, which in turn alter cell migration, stem cell differentiation and cancer cell invasion [1]. Yet, the molecular mechanisms by which cells convert mechanical signal to chemical signal remain ambiguous.

Zyxin is a focal adhesion protein with unique binding sites for Ena/VASP and α-actinin at the N-terminus and the LIM domain located at the C-terminus of the protein. Zyxin localizes at focal adhesions, actin stress fibers and cell–cell contacts. The unique feature of zyxin is that zyxin preferentially binds to force-bearing actin stress fibers. For example, when the actin stress fibers are stretched by a cantilever of an atomic force microscope [2] or prodded by a micropipette [3], zyxin rapidly accumulates at the sites of mechanical perturbation. Zyxin is thought to recognize the actin filaments under tension, possibly through severed filaments with exposed actin tips, and facilitate the repair of actin stress fibers [3]. In addition, zyxin accumulation also correlates with traction force exerted by migrating cells [4]. These results suggest that zyxin responds to both externally applied and internally generated forces, and zyxin may serve as a key component of mechano-sensing module for various cellular processes.

The ability of zyxin to accumulate at focal adhesions or force-bearing sites is solely due to the C-terminus LIM domain. This LIM domain consists of three motifs containing two zinc binding loops or zinc fingers where zinc binding is essential for the structure of the LIM domain [5]. Previous studies have demonstrated that all three zyxin LIM motifs are required for the efficient localization of zyxin at focal adhesions and force-bearing sites [4,6], suggesting that the force sensing function of zyxin requires the complete zyxin LIM domain. Interestingly, unlike zyxin, other LIM containing proteins have more than three LIM motifs [5], though how varying numbers of LIM motifs may alter protein function or force sensitivity is not known.

To further analyze the force-sensitivity of the zyxin LIM domain, we generated artificial LIM constructs that consist of multiple zyxin LIM domains. We hypothesize that increasing the number of LIM domains will increase force-sensitivity. Using a miniature force sensor, we quantified the accumulation of LIM variants at force-bearing sites and found that extra LIM domains do not enhance force sensitivity. Our results suggest that the single zyxin LIM domain has optimal force-sensitivity.

2. Materials & methods

2.1. Cell lines and reagents

MDCK GII cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Atlanta Biologicals, Lawrence-

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http://dx.doi.org/10.1016/j.bbrc.2012.05.046

ville, GA). For western blot or immuno-fluorescence analyses, the antibodies used were GFP (Invitrogen, Carlsbad, CA), VASP (BD Biosciences, San Jose, CA) and tubulin (Sigma–Aldrich, St. Louis, MO). For western blot, the signals on the nitrocellulose membrane were detected by chemiluminescence with an enhanced ECL reagent (Pierce Biotechnology).

2.2. LIM constructs

The LIM sequences were PCR amplified from the single LIM (338-572AA) plasmid using the following primers: ACTCA GATCTCGAGCGCCACCATGGAGAACCAAAACCAG (sense) and GGCG AATTCGAAGCTTCGCGTCTGGGCTCTAGC (anti-sense) for the tandem dimer LIM sequence, and ATCCGCTAGCGCTCGCCACCATGGAG AACCAAAACCAG (sense) and TGAGCTCGAGATCTGGCGTCTGG GCTCTAGC (anti-sense) for the tandem trimer LIM sequence. The PCR products were digested with XhoI/EcoRI (tandem dimer LIM) or NheI/BglII (tandem trimer LIM), then ligated into the GFP-tagged single LIM plasmid (a gift from Dr. Hiroaki Hirata, Nagoya University). The LIM inserts were sequence verified. Using Lipofectamine 2000 and G418 (Invitrogen, Carlsbad, CA), MDCK cells were stably transfected with the GFP-tagged LIM plasmids.

2.3. VASP co-localization analysis

The accumulation of VASP at focal adhesions was quantified using ImageJ. First, focal adhesions were identified by a local maximum search algorithm using the GFP channel, then assigned individual ROIs. Second, the intensities of zyxin, LIM variants and VASP in focal adhesions were quantified. The numbers of focal adhesions analyzed were 614, 440, 490, and 326 for GFP-tagged zyxin, single LIM, tandem dimer LIM, and tandem trimer LIM expressing cells, respectively. All images were taken at the same exposure using identical optics.

2.4. Live-cell confocal microscopy

All samples were imaged on a Zeiss Axio Observer equipped with a Yokogawa spinning disk confocal system, a 40× objective, 488 and 561 nm solid-state lasers, and a CoolSNAP HQ camera. The microscope system was controlled and automated by Slidebook software (Intelligent Imaging Innovations, Denver, CO, USA). Live cells were imaged on glass bottom dishes (MatTek, Ashland, MA, USA) in a temperature-controlled chamber at 37 °C.

2.5. Micro-fabrication of miniature force sensor

Fabrication of micro-pillar arrays was described previously [4]. Briefly, the micro-pillar master was etched with deep reactive ion etcher and consisted of dimensions of 2 μm in pillar diameter, 6 μm in height, and 4 μm in pitch (AdvancedMEMS, Berkeley, CA, USA). Using Polydimethylsiloxane (PDMS), the negative mold was casted, then PDMS pillars were fabricated. A droplet of 100 μg/mL fibronectin (BD Biosciences, San Jose, CA, USA) solution spiked with rhodamine fibronectin (Cytoskeleton, Denver, CO, USA) in 1:5 ratio was deposited on the pillar tips for 10 min, then the pillars were wetted in PBS with 1% bovine serum albumin and 0.05% Triton X-100. The pillars were then washed with PBS and the growth media. This approach created a fibronectin mesh on the pillar array, making it easier for cells to adhere and spread. Due to re-organization of fibronectin by migrating cells, some cells were able to deflect distant pillars using fibronectin bundles. We avoided this phantom deflection in our analysis. The traction force was calculated based on the displacement of pillar tips from the original positions and pillar stiffness of 2.5 MPa. To quantitate the GFP intensity, we defined a ROI for each pillar and thresholded to identify the GFP positive puncta, which was normalized to corresponding background intensity using ImageJ. The numbers of cells analyzed were 20, 12, and 3 for single, tandem dimer, and trimer LIM expressing cells. See detailed protocol in Ref. [4].

3. Results and discussion

3.1. Expression and localization of tandem LIM proteins

Since the LIM domain is solely responsible for force-sensitive accumulation of zyxin [4], we hypothesized that the force-sensitive accumulation of LIM domain proteins would be enhanced by the presence of multiple LIM sequences. Based on the GFP-tagged human zyxin LIM domain (338-572AA), we generated tandem dimer and trimer LIM constructs (Fig. 1A), then transfected these plasmids, and isolated MDCK cell lines stably expressing these genes (Fig. 1B). Using a GFP antibody, we compared the levels of zyxin LIM variants in stable cell lines and selected clones expressing similar levels of LIM proteins (Fig. 1B). This approach allowed us to analyze how multiple LIM domains at a given quantity of LIM proteins affect force-sensitive localization.

Exogenous GFP-tagged zyxin proteins localized to focal adhesions and along stress fibers of single MDCK cells (Fig. 1C). This localization is similar to endogenous zyxin proteins observed through zyxin antibody staining [7], suggesting that the GFP tag does not compromise its localization. When the N-terminus of zyxin was truncated and only the LIM domain was expressed, the single LIM domain localized to both focal adhesions and along actin stress fibers (Fig. 1C). Both tandem dimer and trimer LIM proteins localized at focal adhesions and along stress fibers similarly to GFP-tagged zyxin and single LIM proteins (Fig. 1C). These results suggest that the presence of multiple LIM domains does not prevent focal adhesion and actin stress fiber localization.

3.2. The LIM proteins displace VASP from focal adhesions

The exogenous expression of zyxin LIM domain was shown to displace endogenous zyxin from focal adhesions. Due to the lack of a VASP binding site in the LIM domain, the zyxin LIM domain is unable to recruit VASP to focal adhesions [7–10]. VASP is an actin anti-capping protein that directly binds to the proline-rich domain in the N-terminus of zyxin. Since VASP is a critical regulator of actin dynamics, we analyzed VASP displacement in the tandem LIM protein expressing cells.

In the full-length zyxin expressing cells, VASP localized at focal adhesions (Fig. 2A), whereas significantly less VASP was localized at focal adhesions in the single LIM domain expressing cells (Fig. 2A). Quantitative analyses of VASP and zyxin intensities show that, when the GFP–zyxin signal is used to define the area of focal adhesion, VASP increasingly accumulated with increased zyxin (Fig. 2B, top). This observation is consistent with the notion that zyxin recruits VASP to focal adhesions. In contrast, only a few VASP at focal adhesions were observed in the single LIM domain expressing cells (Fig. 2A and B).

In the tandem dimer or trimer LIM expressing cells, VASP was not localized to focal adhesions (Fig. 2A). The VASP intensity in both tandem LIM expressing cells was lower than the single LIM expressing cells (Fig. 2B, bottom). The extra LIM domain is most likely preventing endogenous zyxin accumulation at focal adhesions and thereby, also preventing VASP from accumulating at focal adhesions. The similar levels of VASP in the tandem dimer and trimer LIM expressing cells suggest that one extra LIM domain is sufficient to completely remove VASP from focal adhesions in these cells.