Head/tail interaction of vinculin influences cell mechanical behavior

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This study evaluates the influence of vinculin in closed conformation on the mechanical properties of cells. We demonstrate that MEF_vin−/− cells transfected with the eGFP-vinculin mutant A50I (talin-binding-deficient-vinculin in a constitutively closed conformation) show 2-fold lower stiffness and focal adhesion density compared to MEF_vin+/+ and MEF_Rescue cells. MEF_A50I cells are as stiff as MEF_vin+/+ cells with similar focal adhesion density. Further, 2D traction microscopy indicates that MEF_A50I and MEF_vin−/− cells generate 3- to 4-fold less strain energy than MEF_vin+/+ and MEF_Rescue cells. These results demonstrate that vinculin’s mechano-coupling function is dependent on its conformational state.

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1. Introduction

Adhesion is important for ensuring cell survival and proliferation. This attachment is driven by extracellular matrix contacts (EMC), which trigger biochemical/biomechanical signals inside the cell. The ~116 kDa vinculin molecule with its larger head and smaller tail domain is one of the pivotal proteins for focal adhesion formation and for its maintenance and regulation [1,2]. Vinculin is known to act as a mechano-coupler that connects the actin cytoskeleton via talin to the integrin-receptor [3–7]. Vinculin-deficient cells are still able to form focal contacts, however, they are more motile and less adhesive than wildtype cells [8–10]. Furthermore, vinculin-deficient cells are less stiff and generate fewer tractions in 2D and 3D than wildtype cells [11,12].

The vinculin molecule itself is a highly conserved intracellular protein which exists in two different conformations [13,14]. In the closed or autoinhibited form the vinculin head keeps the tail region pincer-like in place [13–15]. In that state, binding of the vinculin tail to its binding partners is not possible. Talin or α-actinin (hydrophobic) binding to the head region (residues 1–258) activates and transfers vinculin to the open conformation [14,16,17]. This decreases the affinity between the vinculin head and tail, which allows for binding of paxillin, actin, and phospholipids [1]. FRET experiments demonstrated that vinculin must be in the open conformation for focal adhesion recruitment [16]. Mutating alanine at position 50 on vinculin to isoleucine (A50I) prevents talin binding [13,18]. This mutation inhibits head/tail dissociation of vinculin and increases the vinculin turnover rate in focal adhesions (FAs) [18]. Weakening the head/tail interaction by mutating certain residues at the contact surface between head and tail (e.g. D974, K975, R976, R978A) is shifting vinculin to a constitutively open state. Cells transfected with these vinculin mutants showed more focal adhesions in comparison to wildtype cells [18,19].

To further analyze vinculin’s role as mechano-coupler, we used the A50I vinculin variant in magnetic tweezer and 2D-traction microscopic experiments. In additional experiments, we counted the number of focal adhesions for each cell type of similar size. The results suggest that force transmission is dependent on the number of focal adhesions, and that vinculin must be in an open conformation (i.e. incorporated in the focal adhesion complex) to transmit forces via the actin network for cell adhesion.

2. Materials and methods

2.1. Cell culture

MEF cell lines were maintained in a low glucose (1 g/L) Dulbecco's modified Eagle's complete medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (low endotoxin), 2 mM l-glutamine, and 100 U/mL penicillin/streptomycin and kept at 37°C with 5% CO2.

2.2. Cloning and expression of vinculin

Mouse vinculin-encoding cDNA was kindly provided by Dr. E.D. Adamson. To distinguish transfected from non-transfected cells, an eGFP cassette was cloned into pcDNA3.1 eukaryotic expression
vector (Invitrogen, Karlsruhe, Germany), using NheI and XhoI restriction sites. The polymerase-chain-reaction amplified vinculin wild-type (1–1066), and constructs were N-terminally fused to the eGFP cassette using AflII and XbaI restriction sites. The fused eGFP constructs are driven by a CMV promoter (Clontech, Mountain View, CA). The A50I variant of vinculin was generated by the exchange of alanine (A) on position 50 to isoleucine (point mutation) using site-directed mutagenesis. To transfect cells, $2 \times 10^5$ cells were seeded in a 30 mm Ø tissue culture dish for 24 h. The cells were then transfected with 4 µg DNA using Lipofectamine (Invitrogen, Karlsruhe, Germany). Before fluorescence microscopic and tweezer measurements, approximately $2 \times 10^5$ transfected cells were reseeded in a 30 mm Ø dish or on fibronectin-coated coverslips, respectively.

### 2.3. Magnetic tweezer experiments

We used a magnetic tweezer device as described in [20]. For measurements, $2 \times 10^5$ cells were seeded overnight into a 30 mm Ø tissue culture dish. Thirty minutes before the experiments, the cells were incubated with fibronectin-coated paramagnetic beads of 4.5 µm Ø (Invitrogen, Karlsruhe, Germany). A magnetic field was generated using a solenoid with a needle-shaped core (HyMu80 alloy, Carpenter, Reading, PA). The needle tip was placed at a distance of 20–30 µm from a bead bound to the cell using a motorized micromanipulator (Injectman NI-2, Eppendorf, Hamburg, Germany). During measurements, bright-field images were taken by a CCD camera (ORCA ER, Hamamatsu) at a rate of 40 frames/s. The bead position was tracked on-line using an intensity-weighted center-of-mass algorithm. Measurements on multiple beads per well were performed at 37 °C for 1 h, using a heated microscope stage on an inverted microscope at 40× magnification (NA 0.6) under bright-field illumination. Transfected MEFvin−/− cells were identified in fluorescence mode.

### 2.4. 2D-traction microscopy

Different MEFs were plated overnight onto collagen-coated polyacrylamide hydrogels (Young’s modulus of ~13,000 Pa) at 37 °C and 5% CO2 in DMEM medium. Cells were prepared according to a modified protocol by Pelham and Wang [21]. Adherent cells were detached by treating them with cytochalasin D and trypsin. The gel relaxed into an undeformed state after cell detachment. Comparing the position of fluorescent microspheres in the deformed and undeformed state, the traction field can be obtained using a difference-with-interpolation algorithm with a spatial resolution of 2.5 µm and an accuracy of 8 nm. Traction were computed according to a Fourier-based-algorithm [22] and expressed as elastic strain energy of the matrix.

### 3. Results and discussion

The focal adhesion protein vinculin (1066 residues) with its head (residues 1–821) and tail (residues 858–1066) domains exhibits a bistable behavior, operating in either an open or a closed conformation. It is believed that (i) in the closed state, intramolecular interactions between the head and tail obstruct the exposure of cryptic binding sites for proteins and surface binding sites for phospholipids and (ii) in the open state, when the head and tail are dissociated, the cryptic binding sites in the helical bundles are exposed [13,18,19,23]. The dissociation is assumed to be cooperative, and the activation is most likely to be allosteric. According to Chen et al. [16] several conformational states are likely, exposing different combinations of cryptic binding sites for other focal adhesion proteins during the turnover of focal adhesion complexes, which changes cellular dynamic behavior.
In this study, we mutated alanine on position 50 of the vinculin molecule to an isoleucine (A50I) to prevent the protein talin from binding and to maintain the vinculin molecule in a closed state [13,18]. To test cell mechanical changes induced by an open vs. closed conformation, we measured the cell stiffness of MEF vinculin variants using the magnetic tweezer device (Fig. 1). We observed that the tentatively open wildtype/rescue (MEFvin+/+ MEFRescue) cells vs. the closed MEFvinA50I cells were about twofold stiffer at 2 nN force. The stiffness of vinculin-deficient (MEFvin−/−) cells was similar to that of MEFvinA50I mutant cells. These results support the hypothesis that in the closed state (A50I) of vinculin, the binding of other focal adhesion proteins is compromised, and that the binding sites for other focal adhesion proteins on vinculin are only accessible in the open state of vinculin [18].

Two-dimensional traction microscopic measurements further support this view. We could demonstrate that the contractile forces generated via the intracellular integrin-FA-actomyosin link are 3–4-fold higher in MEFvin+/+ and MEFRescue cells compared to MEFvinA50I and MEFvin−/− cells (Fig. 2). Previously, it was reported that focal adhesion formation depends on internal tension [2,11,24,25]. We determined the number of focal adhesions in MEF cell lines of similar spreading area (Fig. 3). MEFvin+/+ and MEFRescue cells showed similar density of focal adhesions, whereas MEFvinA50I and MEFvin−/− cells showed between 40%–50% fewer focal adhesions. The size of the focal adhesions in these different cell lines was similar (Fig. 4). These results support the view that the reduced focal adhesion density in MEFvinA50I and MEFvin−/− compared to MEFvin+/+ and MEFRescue cells might be a secondary effect due to the reduced force generation [11].

The interaction of vinculin with other focal adhesion proteins like α-actinin, α- and/or β-catenin, and/or talin as well as vinculin phosphorylation have been reported to influence vinculin’s linkage with the actin cytoskeleton to allow for cellular force generation [7,11,26–29]. It has previously been reported that the A50I mutation in vinculin head ablated talin binding due to steric hindrance and that the loss of talin binding correlated with significant enhancement of vinculin dynamics in focal adhesions [18,30]. These reports concluded that the high vinculin dynamics is characteristic of the head domain that requires the talin binding site for vinculin activation and proper FA incorporation. All these findings point into the direction of a distinct state (open or closed) of vinculin which has mechanical implications on the cell behavior. In summary, only the open
conformation of vinculin facilitates contractile force generation and proper focal adhesion formation in cells.

Further detailed experimental studies are still needed to answer the following questions: (i) what is the influence of vinculin’s phosphorylation on the binding of talin and actin to vinculin and (ii) how does vinculin’s activation influence the mechanical load (i.e. strengthening and reinforcement) of focal adhesions.

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