CELL ADHESION

Direct single-molecule quantification reveals unexpectedly high mechanical stability of vinculin—talin/α-catenin linkages

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The vinculin-mediated mechanosensing requires establishment of stable mechanical linkages between vinculin to integrin at focal adhesions and to cadherins at adherens junctions through associations with the respective adaptor proteins talin and α-catenin. However, the mechanical stability of these critical vinculin linkages has yet to be determined. Here, we developed a single-molecule detector assay to provide direct quantification of the mechanical lifetime of vinculin association with the vinculin binding sites in both talin and α-catenin, which reveals a surprisingly high mechanical stability of the vinculin—talin and vinculin—α-catenin interfaces that have a lifetime of >1000 s at forces up to 10 pN and can last for seconds to tens of seconds at 15 to 25 pN. Our results suggest that these force-bearing intermolecular interfaces provide sufficient mechanical stability to support the vinculin-mediated mechanotransduction at cell-matrix and cell-cell adhesions.

INTRODUCTION

Cells contact and sense the extracellular environment through the formation of focal adhesions (FAs) with the extracellular matrix (ECM). They can also communicate with neighboring cells through the formation of cell-cell adhesions, such as adherens junctions (AJs). These physicochemical cell-matrix or cell-cell communications are mainly carried out through physical linkages of mechanosensitive proteins (Fig. 1, A and B) (1–3). One key mechanosensor is vinculin, which acts as a bridge between the actin cytoskeleton and the respective adhesion sites (FAs or AJs), through association with either talin or α-catenin and actin filaments (Fig. 1, A and B) (1–3).

Vinculin is composed of eight α-helical bundles organized into five structural domains, VD1-4 and a tail domain (Vt) (4). It interacts with talin and α-catenin mainly through the N-terminal VD1 domain and with actin filaments through the C-terminal Vt domain (Fig. 1, A and B). Cells lacking or overexpressing vinculin exhibit altered migration, morphology, motility, and alterations in many other mechanical properties (1). Mice lacking vinculin suffer severe developmental abnormalities and die at embryonic day 10.5 (5). On the other hand, fruit flies genetically programmed to overexpress cardiac vinculin exhibit remodeled cytoskeleton networks and prolonged contractility in aging fly hearts and a substantially longer life span compared to normal flies (6).

Vinculin is recruited to cell-matrix adhesion through binding to multiple vinculin binding sites (VBSs) in talin and to cell-cell adhesion through binding to the VBS in α-catenin (7, 8). For both talin and α-catenin, the VBSs are buried in the α-helical bundles (9, 10). In living cells, talin and α-catenin are subject to mechanical stretching caused by actomyosin contractility (1–3, 11, 12). Mounting evidence have suggested that these buried VBSs are mechanically exposed by a few piconewton forces (13–15), activating binding by vinculin through a high-affinity VBS—VD1 interaction (16), which, in turn, releases the Vt domain from the vinculin head domain, making the Vt domain available for binding to actin filaments. Hence, a vinculin-mediated force transmission pathway from talin or α-catenin to cytoskeleton is established, which is believed to be crucial for the vinculin-dependent mechanotransduction at cell-matrix and cell-cell adhesions (1–3, 11).

The crucial mechanotransduction roles of vinculin at these adhesion sites imply that the vinculin-mediated force transmission pathway should have a sufficient mechanical stability to support its mechanotransduction function. As this force transmission pathway is assembled by noncovalent interaction between talin/α-catenin and vinculin and that between vinculin and actin, the mechanical stability of the pathway is determined by the stability of two intermolecular interfaces, VBS—VD1 and Vt—actin. A recent single-molecule study reported that vinculin forms a directionally asymmetric catch bond with F-actin and can withstand tensile forces of 4 to 15 pN for 2 to 12 s when force is applied toward the pointed end of the actin filament (17). Hence, to understand the mechanical stability of these vinculin-mediated force transmission pathways, it is critical to quantify the mechanical stability of the VBS—VD1 interfaces.

In this work, we developed a single-molecule detector assay, with which we directly quantified the mechanical lifetime of the VBS—VD1 interfaces associated with the vinculin—talin interaction and the vinculin—α-catenin interaction (Fig. 1, C and D) (7, 8). Our results showed that, for both cases, the VBS—VD1 interfaces could survive for >1000 s at forces up to 10 pN and seconds to tens of seconds over a force range of 15 to 25 pN. Together with the previous quantification of the vinculin—actin interface that has a lifetime of 2 to 12 s within 10 pN (17), the findings suggest that both of the two critical interfaces involved in the vinculin-dependent mechanotransduction pathways at cell-matrix and cell-cell adhesions are able to provide sufficient mechanical stability required for the mechanotransduction functions of vinculin. In general, the single-molecule detector assay developed in this work can be further implemented to quantify the mechanical stability of other force-bearing intermolecular interfaces in various crucial mechanotransduction pathways..

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RESULTS

Single-molecule detector for VBS—VD1 interface

The single-molecule detector designed for direct quantification of the mechanical lifetime of two interacting proteins comprises three essential components—two interacting protein partners A and B and a long flexible unstructured peptide linker that connects A and B. When the single-molecule detector is specifically tethered and stretched by forces, it can exist in two states, a paired state where A and B form a linked complex A—B that is associated with a looped long flexible linker and an unpaired state where A and B are separated and remained linked by the flexible linker. The paired and unpaired states of the detector have a significant extension difference over physiological forces that can be detected by the magnetic tweezers setup (18–20). Therefore, when force is increased from lower forces where A and B form a linked complex to higher forces until the complex is disrupted, one would observe stepwise extension increases due to the release of the flexible linker as a result of the disruption of the linked complex and potentially unfolding of the structural domains of A or B in the detector. When force returns to the lower forces, the detector re-pairs when A and B refold and subsequently re-form the linked complex, allowing the detector to be un-paired in the next force-increase scan. Therefore, the detector can undergo multiple un-pair and re-pair cycles during the force-increase and force-decrease scan cycles, respectively. By directly measuring the lifetime of the detector until the linked A—B complex ruptures at various forces, we can quantify the mechanical lifetimes of the linked A—B complex using the single-molecule detector assay.

In this single-molecule detector design, the long flexible linker provides several advantages while it does not affect the quantification of the mechanical stability of the interface of the A—B complex: (i) It serves as an amplifier of the rupturing signal through a large extension increase upon rupture of the linked complex. (ii) It allows rapid re-formation of the ruptured complex at lower forces, which largely increases the experimental throughput. (iii) It provides a molecular signature to eliminate any contamination from non-specific interactions. (iv) Being a highly flexible unstructured peptide chain that has a bending persistence of ~0.8 nm and a contour length of ~0.38 nm per residue (21), when the complex forms, the long looped linker does not exert significant force to the interface. According to the structure of the complex, the end-to-end distance

Fig. 1. Schematics of experimental design. (A and B) Vinculin-mediated mechanotransduction pathways at FAs (A) and AJs (B). The key proteins involved in the mechanical linkages are illustrated. The example VBS—VD1 structures [Protein Data Bank: 1xwj (24) and Sy04 (23)] are highlighted in the zoomed-in box on each panel. (C) Design of the single-molecule VBS—VD1 detector (N to C terminus): avi-tag, two repeats of titin I27 domain, vinculin D1 domain, formin FH1 domain, talin/a-catenin VBS, two repeats of titin I27 domain, and spy-tag. Between two adjacent domains, there is a short flexible linker (GGGSG). (D) Single-molecule detection of the VBS—VD1 interaction: VBS—VD1 complex forms at low forces (left) and ruptures at higher forces (right) with higher extension (i.e., the end-to-end distance of the detector along force direction). The illustration in the dashed box in the middle describes two additional possible rupture transitions during force increase: (i) VD1 and VBS disengaged while the VD1 remain folded and (ii) the VD1 partially unfolds and then leads to the rupture of the linked VBS—VD1 complex.
of the looped linker can be measured (~2 nm for the VBS—VD1 complex). On the basis of the force-extension curve of the long linker (196 amino acids in this study) using a worm-like chain model (22), the looped linker exerts a contractile force of <0.2 pN to the interface, which is at least 10-fold smaller than the forces applied to the complex in our experiments. Thus, since the linker does not participate in the molecular interaction involved in the formation of the interface, and since it does not introduce significant mechanical perturbation to the complex, it does not affect the quantification of the force-dependent lifetime of the molecular interface of interest.

On the basis of the principles of the single-molecule detector design described above, the single-molecule detector of VBS—VD1 is composed of (from N to C terminus) a VBS from talin or α-catenin (7, 23, 24), an unstructured long flexible peptide linker [Diaph1 FH1 domain (21), 182 residues, the 583rd to 764th amino acids], and a VD1 domain (4). The detector is spanned between two repeats of the titin I27 domain at each side, which serve as molecular spacers and specificity controls due to its well-characterized high mechanical stability (25–27). Figure 1C shows a sketch of the single-molecule detector design, and more details of the detector sequences could be found in text S1. The N and C termini of the single-molecule detector contains a biotinylated avitag and spy-tag, respectively, which enables specific tethering of the detector between a streptavidin-DNA-coated superparamagnetic bead and a spycatcher-covered coverslip surface, respectively (Fig. 1D) (28, 29). The DNA handle (572 bp) coated on the bead serves as a spacer between the bead and the coverslip surface. Force is applied to the detector using magnetic tweezers (18–20).

The single-molecule detector of VBS—VD1 is designed to mimic the in vivo pulling geometry of vinculin on talin or α-catenin where the N terminus of talin or α-catenin is bound to the adaptor proteins at cell-matrix or cell-cell adhesions, respectively, while the C terminus of vinculin is bound and stretched by actin filament (Fig. 1. A to D). Two sequences from talin, VBS1<sup>talin</sup> and VBS2<sup>talin</sup>, used in the study are from the first rod domain of talin 1 (VBS1, the fourth helix, residues 607 to 635) and the third rod domain of talin 1 (VBS2, the 12th helix, residues 852 to 876), respectively. One sequence from α-catenin, VBD<sup>α-ca</sup>tenin<sub>3</sub>, is from the first modulation domain of α-catenin (residues 325 to 360). The VD1 is the residues 2 to 258 of vinculin (text S1). Here, we note that two α helices (residues 304 to 316 and 328 to 353) within the α-catenin first M domain were reported to interact with vinculin D1 domain, where the α helix of residues 328 to 353 inserts into the first vinculin D1 α-helix bundle (8), while the α helix of residues 304 to 316 interacts with the second vinculin D1 α-helix bundle (30). We used the α helix of residues 325 to 360 of α-catenin for VD1 binding in our study. The VBS—VD1 detector consists of minimal domains (i.e., VBS and VD1) that involve the physical interface of vinculin to talin or α-catenin, thereby minimizing the possible signals that could occur during mechanical rupture of the complex and hence leading to a clear and precise quantification. On the other hand, the kinetics of the minimized interface of the complex might differ from that of a full-length protein complex with the presence of other domains.

**Force-loading rate-dependent rupture of the VBS—VD1 complex**

At low forces (~1 pN), in the detector, the VD1 binds to VBS and forms a stable linked VBS—VD1 complex (paired state). When force is linearly increased with time at a loading rate of 1 pN s<sup>−1</sup>, the linked VBS—VD1 complex ruptures at forces of >15 pN, indicated by a large (~100 nm; red dashed boxes in Fig. 2, A and B) stepwise extension jump. This step size is consistent with concurrent un-pairing between the linked VBS—VD1 complex that releases the FH1 loop and unfolding of the VD1 domain (text S2). When force is subsequently decreased at a loading rate of ~0.1 pN s<sup>−1</sup>, the unfolded VD1 refolds with two steps, at ~5 and ~2 pN (magenta arrows in Fig. 2, A to D, and fig. S1). Following refolding of VD1, the linked VBS—VD1 complex re-forms at ~1 pN (blue arrow in Fig. 2, A to D). The linked VBS—VD1 complex in the detector could undergo tens to hundreds of cycles of mechanical rupture and re-formation, enabling direct quantification of the force-dependent lifetime of the linked VBS—VD1 complex.

Figure 2 (E and F) shows the normalized histograms of rupture forces recorded with three force-loading rates of 0.2, 1, and 5 pN s<sup>−1</sup>. The histograms show that the peak forces of the VBS—VD1 rupture occurs at ~19, ~22, and ~25 pN for talin VBS1 and ~17, ~19, and ~22 pN for α-catenin VBS, at corresponding force-loading rates. The talin VBS2—VD1 shows slightly higher mechanical stability indicated by peak rupturing forces of ~28 and ~30 pN at force-loading rates of 1 and 5 pN s<sup>−1</sup>, respectively (fig. S2). To quantify the mechanical lifetime of the VBS—VD1 interface, we fitted the histogram of the force-dependent rupture rate using the classic Bell’s model (31): $k_u(F) = k_u^0 e^\beta (F) - k_u^0$ , where $\beta = \frac{1}{k_u^0}$, $\Delta$ is the transition distance and $k_u^0$ is the extrapolated zero-force rupture rate (text S3). The best fitting of the histograms determines the $k_u^0$ and $\Delta$ to be 6.5 ± 6.5 × 10<sup>−3</sup> s<sup>−1</sup> and 2.4 ± 0.2 nm (mean ± SD) for VBS<sup>talin</sup>—VD1 and 5.7 ± 8.4 × 10<sup>−7</sup> s<sup>−1</sup> and 2.8 ± 1.3 nm for VBD<sup>α-ca</sup>tenin—VD1, respectively.

When we stretched the VD1 domain without the VBS in the detector, two unfolding events were observed at ~4.5 and ~12 pN at a loading rate of 1 pN s<sup>−1</sup> (Fig. 2, G and H, and fig. S1). This construct contains only the VDI domain, the unstructured flexible long linker, and the four I27 domains spacer (sketch in Fig. 2G and see text S1). As the unstructured flexible long linker does not contribute to large unfolding steps (21) and the four titin I27 domains unfold with very slow kinetics (10<sup>−3</sup> s<sup>−1</sup> per domain) at forces below 20 pN (25–27), therefore, the two unfolding steps of this construct obtained during a force-increase scan can only be from the two four-helix bundle domains (VD1a and VD1b) of VDI in the construct. As the unfolding force of either of the VDI α-helix bundles is much smaller than the rupture forces of the VBS—VD1 complex for both talin and α-catenin, these results suggest that the VBS—VD1 is an integral complex that is associated with higher mechanical stability than the original VDI domain.

**The mechanical lifetime of VBS—VD1 complex**

To directly quantify the mechanical lifetime of the linked VBS—VD1 complex, we implemented a force-jump cycle procedure. In each cycle, force was sequentially changed through the following values (Fig. 3A): (i) a force of ~1 pN for 10 s to maintain the VBS and VD1 in a complex paired state, (ii) a force of ~3 pN for 1 s to confirm that the detector is in the paired state, (iii) a rupture force in the range of 3 to 30 pN for a duration Δt until the complex rupture is observed, (iv) a force of ~3 pN for 30 s to allow refolding of VD1, and (v) a force of ~1 pN for 100 s to ensure re-formation of the complex. At each rupture force, the force-jump procedure was repeated for
Fig. 2. Force-dependent rupture of the VBS—VD1 complex. (A and B) Force bead height curves of VBS—VD1 detectors during force cycles with force increase at a loading rate of 1 pN s\(^{-1}\) and force decrease at a loading rate of –0.1 pN s\(^{-1}\) for the interfaces formed between the VD1 and the talin VBS1 (A) or \(\alpha\)-catenin VBS (B), respectively. The red dashed boxes indicate the region of VBS—VD1 rupture, magenta arrows indicate the two-step refolding of the VD1 domain, and the blue arrows indicate repairing of VD1 and VBS. Each colored line indicates one independent force-increase and force-decrease cycle, smoothed (10-point fast Fourier transform) from raw data (gray). (C and D) The zoom-in of refolding and repairing events during force-decrease scans of the VBS—VD1 detectors. (E and F) The normalized histograms of VBS—VD1 rupture forces at force loading rates of 0.2, 1, and 5 pN s\(^{-1}\). The gray lines are corresponding to a Gaussian fitting of the histograms (the goodness of fit, \(R^2 > 0.9\)). (G and H) Force bead height curves of VD1 during force-increase scans at a loading rate of 1 pN s\(^{-1}\) (G) and the resulting normalized histograms of VD1 unfolding forces (H). The yellow-green arrows indicate the unfolding events of VD1. The gray line is the double-Gaussian fitting of the histogram (the goodness of fit, \(R^2 > 0.9\)). The corresponding numbers of data points (\(N\)) for the histograms are labeled on the figure panels. The sketch in the top panel shows the domains involved in the construct for Fig. 2 (G and H).
Fig. 3. Force-dependent lifetime of the VBS—VD1 complex. (A) Experimental procedures of force-jump assay. Bottom: Typical example of 29 cycles of the force-jump experiments. Top: Zoomed-in view of two force-jump cycles. The red arrows indicate the rupture of the complex, and the blue arrows indicate the re-formation of the complex. The light gray horizontal arrows indicate the bead height change mainly caused by the rotation of the bead due to torque rebalance during force jump. (B to F) Examples of the extension change time traces of the VBS—VD1 complex rupture at various forces. Each large extension jump step indicates an event of VBS—VD1 complex rupture. In (F), the ~10-nm extension increase/decrease steps are the force-induced unfolding/refolding of the I27 domains in the detectors (fig. S3). The colored arrows indicate the VBS—VD1 rupture events at these forces. (G) The force-dependent lifetime of the VBS—VD1 complex. The blue/magenta hollow cycles represent the individual lifetimes measured at the corresponding forces. The blue/magenta solid squares represent the characteristic lifetime at the corresponding forces. The light blue/magenta line is the fitting curve based on Bell's model, and the blue/magenta line is the fitting curve based on Arrhenius law. The horizontal error bars represent the 10 to 15% uncertainty in force calibration due to the heterogeneity of the beads. The vertical error bars represent the SE.
Figure 3G shows the resulting force-dependent lifetime of the linked VBS—VD1 complex. The results show that VBS\textsubscript{talin}1—VD1 and VBS\textsubscript{\textalpha}—catenin—VD1 complexes can last >1000 s at forces up to 10 pN and seconds to tens of seconds over a force range of 15 to 25 pN. The force-dependent lifetime of the linked VBS\textsubscript{talin}1—VD1 and linked VBS\textsubscript{\textalpha}—catenin—VD1 complexes exhibits deviation from the prediction by Bell’s model at forces of <10 pN (Fig. 3G, light blue and light magenta lines). Therefore, we fitted the data based on the more generous Arrhenius law (blue and magenta lines in Fig. 3G and see text S3). A possible cause of the deviation from Bell’s model prediction is discussed in Discussion. In addition, it is reasonable to hypothesize that at even lower forces, the lifetime of the linked VBS—VD1 complex might be further deviated from Bell’s model. To test it, we kept the VBS\textsubscript{talin}1—VD1 detector at a lower force of ~1 pN for over several hours to measure the lifetime of the complex at this force (fig. S4). Consistent with the hypothesis, the lifetime of the complex at ~1 pN is $5 \times 10^3$ s (Fig. 3G), which is more than three orders of magnitude shorter than that predicted by Bell’s model.

**DISCUSSION**

The force-dependent lifetime of VBS—VD1 interfaces provides important insights into the molecular mechanism underlying the vinculin-mediated mechanotransduction at cell-matrix and cell-cell adhesions. Previous studies reported that tension across vinculin in stable FAs lies in a scale of a few piconewtons, with a lifetime of tens to hundreds of seconds (12, 32–34). Our findings show that the VBS—VD1 interface could survive for >1000 s at forces up to 10 pN. In addition, the vinculin—actin interface at the other end of the linkage was reported to have a lifetime of 2 to 12 s over the same force range (17). Together, these results suggest that both of the critical force-bearing interfaces involved in the vinculin-mediated pathways are able to support the mechanical stability required for the mechanotransduction functions of vinculin at cell-matrix and cell-cell adhesions.

Our single-molecule quantification revealed an interesting trend of the force-dependent lifetime of the VBS—VD1 interfaces. At above 10 pN, force exponentially accelerates the dissociation of VBS—VD1 interfaces, which is consistent with the well-accepted Bell’s model that predicts that force applied to a molecular complex should exponentially reduce the lifetime of the complex (31). However, below 10 pN, the lifetime of the VBS—VD1 interfaces becomes much less sensitive to the applied force. As a result, at forces below 5 pN, the measured mechanical lifetime of the VBS—VD1 interfaces is more than two orders of magnitude shorter than that extrapolated on the basis of Bell’s model fitting. As this force range overlaps with that measured in vinculin-mediated force transmission pathways (12, 32–34), this interesting force dependence could play an important functional role to stabilize the VBS—VD1 interface within the physiologic force range. Moreover, as the VBS peptides from various vinculin binding proteins share the highly consensus vinculin binding sequence motif LXXAAXXVAXXXLXXA (7), it is likely that the high mechanical stability of the VBS—VD1 interfaces are also shared among these VBS peptides from various proteins. In support of this view, the interfaces formed between the three tested VBS peptides (two from talin and one from \textalpha-catelin) and VD1 all show high mechanical stability.

It is worthwhile to discuss the mechanism underlying the deviation from the prediction by Bell’s model. While Bell’s model has been well supported with numerous experiments conducted using atomic force microscopy typically at a high force range (>100 pN) (26, 35, 36), deviations from the model prediction have also been recently reported for a number of molecules at a lower force range (27, 37). Several recent experimental and theoretical analyses suggest that the structural-elastic properties of molecular complexes and the low-force entropic extension fluctuation may cause these deviations (27, 38, 39). We showed that, by taking into account the structural-elastic properties of the VBS—VD1 and the low-force entropic extension fluctuation, the force-lifetime data for the VBS—VD1 interfaces can be explained on the basis of the force-dependent change of the barrier height, and the fitting suggests that the transition state might involve production of a short peptide of seven to nine residues under force. This adds another example supporting the structural-elastic properties and low-force entropic extension fluctuation as a critical determinant of the mechanical stability of molecular complexes (27, 38, 39). The model we used to fit the data is based on Arrhenius equation with a constant prefactor, $k^\text{Arrh}(F) = k^0_\text{Arrh} \exp[-\beta \Delta \phi^*_\text{Arrh}(F)]$, where $\Delta \phi^*_\text{Arrh}(F)$ is the force-dependent change of the height of the energy barrier calculated based on the structural-elastic property of the linked VBS—VD1 complex. This model only depends on the force-induced change in the barrier height, without assuming the pathway or the dimensionality of the underlying energy landscape. However, this does not exclude previous mechanisms based on parallel dissociation pathways that might play a role in the observed deviation from Bell’s model prediction at low forces (40).

Our single-molecule quantification revealed that the VBS—VD1 forms an integral complex of helix bundles that is associated with much higher mechanical stability than the VD1 domain alone. Briefly, there are three main potential possibilities regarding the transitions during mechanical rupture a complex formed by two components. The first possibility is an intuitive picture that the two components (VBS and VD1 in this study) disengaged first, and then, each folded component unfolds (i.e., disengagement followed by unfolding). In this first scenario, the disengagement would occur before unfolding; therefore, three smaller transition steps would be observed (one disengagement and two following steps from unfolding the two subdomains in VD1). The second possibility is that one of the components unfolds first, and it leads to disengagement of the two components (i.e., unfolding followed by disengagement). In this second scenario, the disengagement would occur concurrently with or slightly after the unfolding of the domain. The third possibility is that the two components form an integral complex associated with a different mechanical stability compared to the individual component and ruptures as an entity under mechanical forces (i.e., concurrent unfolding and disengagement at a force different with the unfolding force of the individual component). We observed that a single large step rupture of the complex at forces of ~20 pN with a loading rate of 1 pN s\(^{-1}\) (Fig. 2, A to F) and that the rupture force is much larger than the original unfolding force of VD1 (~5 and ~12 pN with a loading rate of 1 pN s\(^{-1}\); Fig. 2G). These observations support the third possibility that concurrent unfolding and disengagement of the integral complex formed with a higher mechanical stability that is differed from VD1.

A recent work reported that during *Shigella* invasion, the VBS from *Shigella* IpaA can interact with four \textalpha helices (H1–H4) of talin R1 rod domain and form a new stable five \textalpha-helix bundle structure.
(41). Since talin and vinculin and likely the IpaA in vivo are under mechanical force when the respective mechanical linkage is activated, the talin rod is expected to be unfolded by mechanical force, and the buried $\alpha$ helix would be exposed to interact with their partners and form new helix bundle complexes under mechanical stretching. It will be interesting and important to investigate the mechanical stability of these new formed complexes, which can be, in general, directly probed and quantified using the single-molecule detector assay developed in this study.

Mechanotransduction plays critical roles in various crucial cellular processes, and dysregulated mechanotransduction is implicated in various diseases such as cancer (2, 3). Mechanotransduction processes begin with mechansensing, which involves dynamic assembly of various supramolecular force transmission linkages each consisting of multiple physically noncovalent linked proteins. These linkages allow the cells to sense the level of mechanical force in the linkages and force-dependent interactions between the molecules in the linkages and signaling proteins. Previous studies have mainly been focused on understanding individual proteins that constitute the linkages and the proteins that interact with the linkages (13–15, 42, 43). However, the roles of the force transmission linkages as a functional entity have largely remained unexplored. On the other hand, targeting force transmission linkages has numerous advantages. It provides a more systematic view of mechanotransduction, and it may lead to development of new approaches to modulate mechanotransduction by targeting the physical connectivity of the linkages.

Our results define the mechanical stability of the interfaces of vinculin—talin and vinculin–$\alpha$-catenin. Combined with previous results obtained on the mechanical stability of the interfaces of vinculin—actin (17), we now have complete information of the mechanical stability of vinculin as an adapter between talin or $\alpha$-catenin and actin cytoskeleton. However, the complete mechanotransduction pathway from the extracellular environment to the nucleus includes a number of other important physical linkages. At cell–matrix adhesions, these are between ECM—integrin, integrin—$\alpha$-helix, talin, and talin—actin, while at cell-cell adhesions, they are between cadherin—cadherin, cadherin–$\beta$-catenin, $\beta$-catenin–$\alpha$-catenin, and $\alpha$-catenin–actin (2, 11). A comprehensive knowledge of the mechanical lifetime of all these connections will be crucial for a deeper understanding of the mechanotransduction pathways at cell-matrix and cell-cell adhesions. The single-molecule detector method developed in this study provides a powerful assay that can potentially be extended to quantify the mechanical lifetime of the physical interfaces of these force-bearing intermolecular interactions within the mechanotransduction pathways of the cell at a molecular level over physiological force range.

In addition to directly quantify the mechanical stability of the interfaces of the intermolecular or the intramolecular interactions, the single-molecule detector assay can also be further implemented for probing the effects of chemical compounds or other biomolecules on the interactions of the interfaces. For instance, a biomolecule that enhances the interaction would likely increase the mechanical stability of the interfaces. In contrast, a biomolecule that inhibits or suppress the interaction would likely decrease the mechanical stability of the interfaces or inhibit/suppress the formation of the interfaces. The shift of the mechanical stability of the interfaces in the presence of various biomolecules could be readily probed and quantified by the single-molecule detector assay.

MATERIALS AND METHODS
Single-protein manipulation and analysis
A vertical magnetic tweezers setup (18, 20) was combined with a disturbance-free, rapid solution-exchange flow channel for conducting in vitro protein stretching experiments (19). In magnetic tweezers experiments, the height of the molecule-tethered superparamagnetic bead from the coverslip surface was recorded. At a constant applied force, the bead height change was the same as the molecule extension change. When the applied force changed, the bead height change was a combination of the bead reorientation due to the force change–induced torque rebalance and the force-dependent elastic extension change of the molecule (44, 45). Hence, a force jump (which took $\leq 0.25$ s in our setup) was usually accompanied with a concurrent stepwise bead height change. On the other hand, during linear force-increase/force-decrease scans with a loading rate of 0.1 to 5 pN s$^{-1}$ used in our study, the stepwise bead height change was the same as the stepwise extension change of the molecule. It was because the force change over the time window ($\leq 0.01$ s, the temporal resolution of our setup) of the stepwise transition was negligible ($\leq 0.001$ to 0.05 pN). All experiments were performed in solution containing 1 x phosphate-buffered saline, 1% bovine serum albumin, 2 mM dithiothreitol, and 10 mM sodium l-ascorbate at 21° ± 1°C. Details of plasmid constructs, protein expression, and the kinetic determination based on Bell’s model and Arrhenius law model can be found in the Supplementary Materials.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/12/eava2720/DC1

Text S1. Plasmid constructs and protein expression.
Text S2. Transition step size to residue number conversion.
Text S3. Un-pairing kinetics and force distribution.
Fig. S1. Force-dependent VD1 domain unfolding and refolding.
Fig. S2. Force-dependent rupture of complex formed by talin-VBS2 and VD1.
Fig. S3. A representative bead height distribution of the VBS-VD1 detector before and after the complex rupture at a low force of ~4.7 pN.
Fig. S4. Long time measurement of the time extension fluctuations of the VBS$^{\text{link}}$-VD1 detector at ~1-pN forces.

View/request a protocol for this paper from Bio-protocol.

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