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A mechanical unfolding intermediate in an actin-crosslinking protein

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Many F-actin crosslinking proteins consist of two actin-binding domains separated by a rod domain that can vary considerably in length and structure. In this study, we used single-molecule force spectroscopy to investigate the mechanics of the immunoglobulin (Ig) rod domains of filamin from *Dictyostelium discoideum* (ddFLN). We find that one of the six Ig domains unfolds at lower forces than do those of all other domains and exhibits a stable unfolding intermediate on its mechanical unfolding pathway. Amino acid inserts into various loops of this domain lead to contour length changes in the single-molecule unfolding pattern. These changes allowed us to map the stable core of ~60 amino acids that constitutes the unfolding intermediate. Fast refolding in combination with low unfolding forces suggest a potential *in vivo* role for this domain as a mechanically extensible element within the ddFLN rod.

In a moving cell, the cytoskeleton undergoes constant reorganization. The protein components of the cytoskeletal meshwork are therefore subject to mechanical deformation. Despite its obvious importance, we know little about the mechanical response of the individual cytoskeletal components to a force. Many actin crosslinking proteins share a common building scheme: two actin-binding domains are connected by a rod domain. The length and structure of the connecting rod varies considerably among actin crosslinkers. The family of filamins has rod domains of the Ig fold. Depending on the specific filamin isoform, the number of rod domains can vary from 6 to 24 (ref. 1). Here we have used single-molecule mechanical experiments in combination with protein engineering to characterize the mechanical response to a stretching force of the rod domains of the *Dictyostelium discoideum* filamin ddFLN.

ddFLN consists of an N-terminal actin-binding domain and a rod domain of six Ig domains (1–6, N terminus to C terminus; **Fig. 1a**). Fucini *et al.*[2] and McCoy *et al.*[3] have shown that ddFLN dimerizes into an antiparallel homodimer via Ig domain 6. Here we explore the response of the ddFLN rod domains to a stretching force using single-molecule force spectroscopy.

RESULTS

Mechanical stability of the ddFLN rod

We investigated the stability of the dimer bond between each domain 6 of the two ddFLN monomers using a construct containing the entire dimerized rod lacking the N-terminal actin-binding domain (ddFLN1–6). The dimers were incubated on a freshly evaporated gold surface and picked up by an atomic force microscope (AFM) tip (**Fig. 1b**). In force-extension curves obtained for this construct (**Fig. 1c**), similar to those of many other modular proteins investigated so far, a sawtooth pattern reflects the unfolding of individual Ig domains[4,5]. In the experiment, the molecules can be picked up anywhere along their contour, and so the number of unfolding peaks per force curve can vary from zero to the total number of domains in the rod. Many of the traces (see **Fig. 1c**) exhibit clearly more than six unfolding peaks, which is the number of domains present in a ddFLN monomer. This result shows that under deformation forces the individual domains in the rod unfold before the dimer bond breaks. A lower estimate for the average strength of the dimer bond of ~200 pN at pulling velocities between 100 nm s^{-1} and 1 μm s^{-1} can be obtained from the average force of the last rupture in our traces. This number is a lower estimate because we cannot distinguish rupture of the dimer bond from desorption of the molecule from either the tip or the substrate. Both events disrupt the mechanical connection between tip and substrate.

Domain 4 unfolds via an intermediate state

Most unfolding peaks show clear two-state unfolding of the Ig domains. The distance between two adjacent peaks is $\Delta L = 31.5 \pm 0.1$ nm ($n = 636$) and thus is consistent with the gain in length expected for the unfolding of a 100-residue domain. However, in the initial part of the force-extension curves, unfolding events with intermediate peaks are observed in the sawtooth pattern (green arrows, **Fig. 1c**). This indicates that at least one of the six Ig domains does not unfold in a two-state manner but via an intermediate state. To identify the Ig domain with these special mechanical properties, we studied the domains individually. In force spectroscopy experiments, single-molecule unfolding events must be distinguished from multiple

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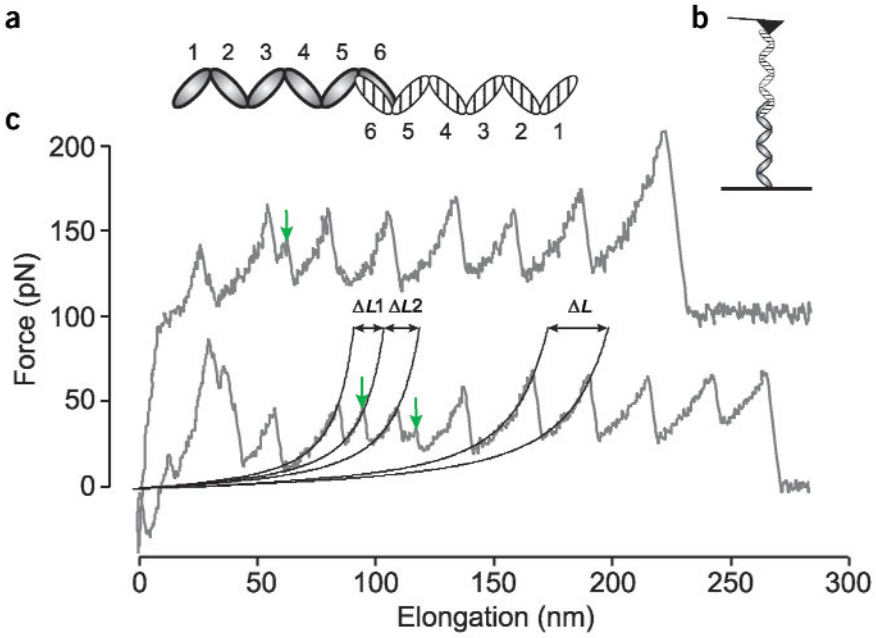

Figure 1 Single-molecule mechanics of ddFLN. (**a**) Schematic illustration of the rod construct ddFLN1-6. The rod consists of six Ig domains and forms a tail-to-tail dimer. (**b**) Experimental geometry. (**c**) Sample traces of force-extension curves of ddFLN1-6. The sawtooth pattern reflects unfolding of seven (upper trace) and eight Ig domains, respectively. At low forces and extensions, intermediate peaks (green arrows) indicate the presence of unfolding intermediates. The fits to the left-hand slopes of the lower unfolding trace were calculated using the worm-like chain model (see Methods). The length change $\Delta L1$ corresponds to unfolding from the native to the intermediate state, $\Delta L2$ to unfolding from the intermediate to the fully unfolded state and ΔL to unfolding from the native to the fully unfolded state.

interactions and nonspecific background. Protein domains are often studied by constructing polyproteins composed of identical subunits[6]. The resulting sawtooth pattern serves as a selection criterion for single-molecule events. However, the interpretation of data from polyproteins may become complicated if the individual domains do not unfold in a two-state manner. We therefore chose a different strategy combining the advantage of a clear single-molecule fingerprint with the ability to carry out experiments with only a single protein domain. We sandwiched each of the ddFLN Ig domains between four C-terminal and four N-terminal Ig domains from the muscle protein titin (**Fig. 2**). Titin Ig domains are mechanically well characterized and provide a clear sawtooth pattern allowing the detection of single unfolding events[7]. Sample traces of two such constructs (Ti/ddFLN2 and Ti/ddFLN4) with domains 2 and 4, respectively, from ddFLN are shown in **Figure 2**. At large extensions the sawtooth pattern of titin domains unfolding at forces between 150 and 250 pN is clearly visible. Because ddFLN domains unfold at forces <100 pN (**Fig. 1c**), the sandwiched ddFLN domain unfolds in the initial part of the force curve with the titin domains still folded (arrows, **Fig. 2**). Domain 4 shows a clear double peak that indicates an unfolding intermediate. In contrast, domain 2 unfolds cooperatively without any intermediate visible.

The number of amino acids forming the structured part of the intermediate can be estimated by measuring the distance between the respective unfolding peaks. This was done for the dimeric construct (ddFLN1–6), for a monomeric construct lacking the dimerization domain 6 (ddFLN1–5) and for Ti/ddFLN4. The fits to the rising slope of the peaks (**Fig. 1c**) yield a distance of $\Delta L1 = 15.1 \pm 0.1$ nm and $\Delta L2 = 16.9 \pm 0.1$ nm ($n = 296$) for ddFLN1–6, $\Delta L1 = 14.2 \pm 0.1$ nm and $\Delta L2 = 16.0 \pm 0.2$ nm ($n = 53$) for ddFLN1–5 and $\Delta L1 = 14 \pm 0.2$ nm and $\Delta L2 = 16.6 \pm 0.2$ nm ($n = 17$) for Ti/ddFLN4. Assuming a length of 0.36 Å per residue, a length change of 14–15 nm for $\Delta L1$ indicates that ~40 residues unfold during the first unfolding step and the remaining 60 residues constitute the structured portion of the intermediate. Also, the forces required for unfolding from the ground state to the

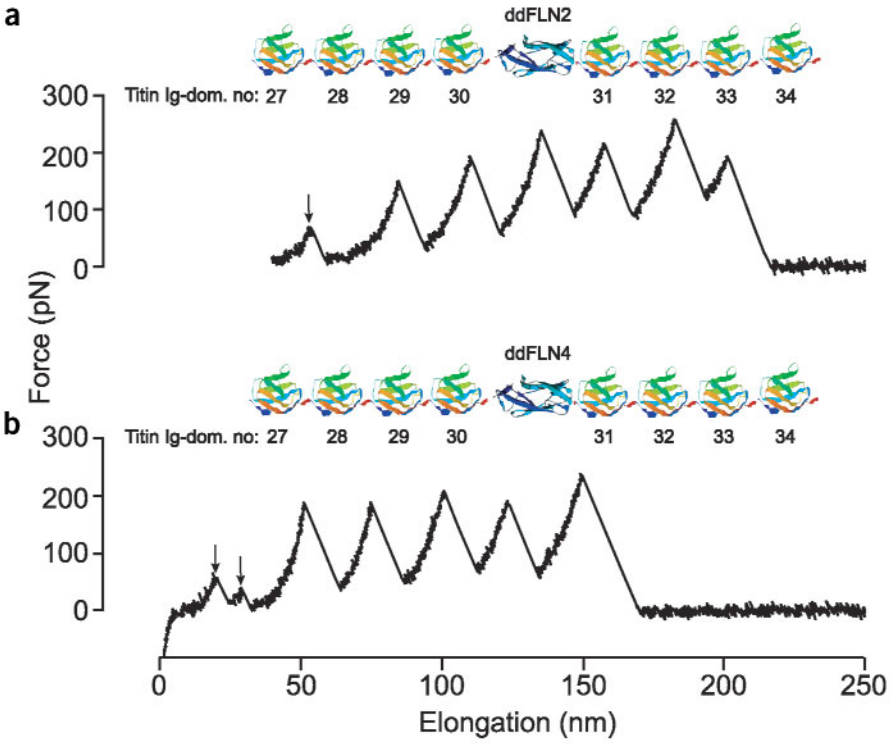

Figure 2 Single-domain force spectroscopy. (**a**) Schematics of the construct Ti/ddFLN2, with rod domain 2 sandwiched between Ig domains I27–30 and domains I31–34 from titin. A force-extension curve of this construct is shown below. The peak marked with an arrow is due to the unfolding of domain 2 showing no intermediate states. (**b**) Schematics of the construct Ti/ddFLN4. The force-extension curve of this construct shows two peaks (marked with arrows) for the unfolding of Ig domain 4 via a stable unfolding intermediate.

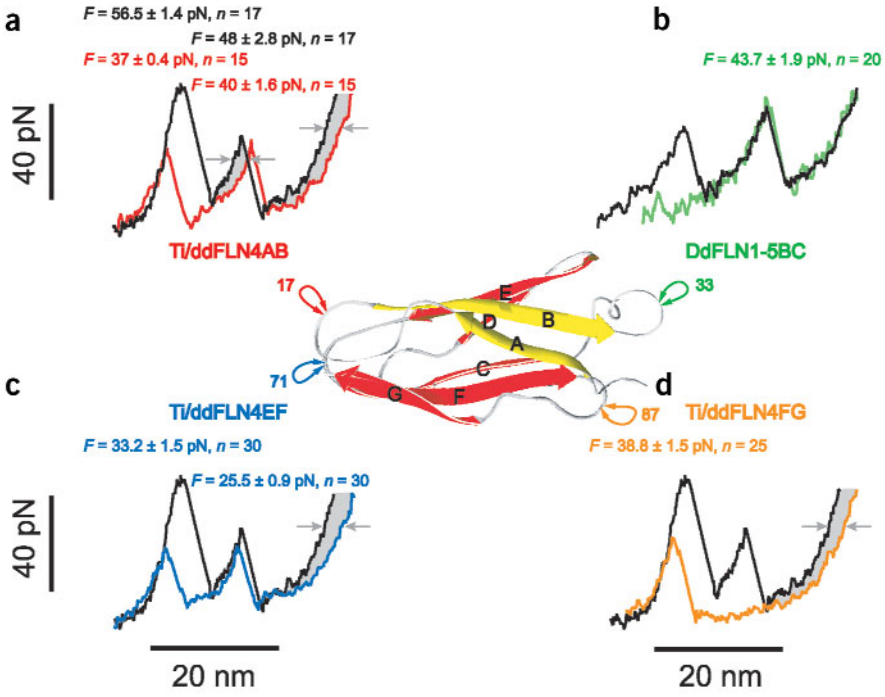

Figure 3 Characterization of the unfolding intermediate structure in single-molecule experiments using loop mutations. The NMR structure of ddFLN domain 4 is shown in the center[8]. Five glycine residues were inserted in various loops of the domain. The numbers above each peak are the average unfolding forces measured for the respective peaks and constructs. (**a**) Superimposed are the average curves of Ti/ddFLN4 (black) and Ti/ddFLN4AB (red). The gray area reflects the additional change in contour length after the first step of unfolding of Ig domain 4 owing to the five glycines integrated at position 17. (**b**) Superimposed are the average curves of ddFLN1–5 (black) and ddFLN1–5BC (green). (**c**) Superimposed are the average curves of Ti/ddFLN4 (black) and Ti/ddFLN4EF (blue). The gray area reflects the additional change in contour length after unfolding of the structured portion of the intermediate owing to the five glycines integrated at position 71. (**d**) Superimposed are the average curves of Ti/ddFLN4 (black) and Ti/ddFLN4FG (orange). The gray area reflects the additional change in contour length after unfolding of Ig domain 4 owing to the five glycines integrated at position 87.

intermediate (peak 1) are consistently higher (62.9 ± 3 pN, n = 296, ddFLN1-6) than those for unfolding from the intermediate to a completely unfolded structure (peak 2) (52.5 ± 3 pN, n = 296, ddFLN1-6).

Structured portion of the intermediate

To unequivocally identify the amino acids that form the structured portion of the intermediate, a more elaborate approach is necessary. Insertion of residues into the various loops of the domain[6] should result in length changes of $\Delta L1$ or $\Delta L2$ depending on the position of the inserts. If the inserted amino acids are part of the structured portion of the intermediate, $\Delta L2$ will increase; if not, $\Delta L1$ will increase. We chose four different positions in the loops of domain 4 (**Fig. 3**) and inserted five glycine residues into each of these positions. **Figure 3** shows averaged force-extension traces for the unfolding of domain 4 in the mutated constructs Ti/ddFLN4AB, ddFLN1–5BC, Ti/ddFLN4EF and Ti/ddFLN4FG (see Methods). We chose the positions for inserts considering that the unfolding of middle strands during the first step would be highly unlikely. For any scenario involving strands other than terminal ones, the domain would have to break into two folded portions plus an unfolded middle portion in the first step. We did not observe such additional intermediate peaks. Insertion in the loop connecting strands A and B (AB loop) increases $\Delta L1$ by 1.7 ± 0.4 nm (n = 17) and leave $\Delta L2$ unaffected (**Fig. 3a**). At the same time, the average unfolding force for peak 1 is reduced but the unfolding force of peak 2 is unchanged (**Fig. 3a**). This indicates that the amino acid insert occured in the part of the protein that unfolds during the first unfolding event but that the insert is not part of the structured portion of the intermediate. We obtained a similar result for a five-glycine insert into the BC loop of domain 4 (**Fig. 3b**). The effects are even more pronounced and the first unfolding peak vanishes completely. However, the unfolding force of the second peak is unchanged, as is $\Delta L2$. This insertion apparently affects the stability of the domain so much that it does not fold completely and unfolding starts from the intermediate as the stable ground state. The BC loop is therefore not part of the structured portion of the intermediate either. In contrast, glycine inserts into the EF loop result in a length increase of $\Delta L2$ by 1.6 ± 0.4 nm (n = 30); this suggests that the EF loop is part of the folded intermediate structure (**Fig. 3c**). This insertion reduces the average unfolding force of both peak 1 and peak 2. The insert in the FG loop destabilizes the intermediate state so substantially that in the averaged trace (**Fig. 3d**) peak 2 can no longer be detected.

Combining all the information obtained from the loop variants, we suggest the following sequence of events for the mechanical unfolding of domain 4: during the first unfolding event strands A and B (yellow parts of the structure in **Fig. 3**) detach from the domain and unfold. Strands C–G (red parts in **Fig. 3**) form a stable intermediate structure, which then unfolds in the second unfolding event. This result agrees well with our above estimate of ~40 amino acids unfolding in the first unfolding event, judged from the length gain of 14–15 nm. According to the NMR structure[8] strand C starts at Phe43.

DISCUSSION

Intermediates in Ig domains

A force-induced intermediate structure for the Ig domain I27 from the muscle protein titin has been reported[9,10]. However, this intermediate is very similar to the full native structure and lacks only seven amino acids at the N terminus. In contrast, the unfolding intermediate we find for domain 4 of ddFLN lacks the complete strands A and B and is only 60% of the size of the native folded domain. This result expands the current picture of the interactions stabilizing an Ig domain against mechanical forces. A patch of six hydrogen bonds between the A′ and G strands includes the key residues controlling mechanical stability of the Titin I27 Ig domain[9]. Mutations in this localized region had marked effects on the unfolding force whereas mutations outside this region did not affect mechanical stability[11]. In contrast, all of our mutations in various regions of the domain had a pronounced effect on mechanical stability. Thus, the stability of domain 4 cannot be attributed to a localized patch of hydrogen bonds. In fact, even the core of only 60 residues that forms the structured portion of the inter-

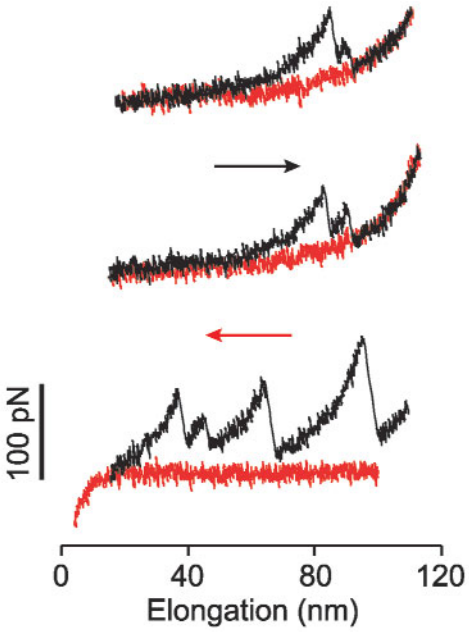

Figure 4 Series of three unfolding (black) and refolding (red) cycles of a single ddFLN1-5 rod. The curves were recorded at an pulling velocity of 200 nm s^{-1}. The lowest black curve reflects the unfolding of domain 4 (intermediate peak) at low forces and two more stable domains at higher forces of a fully folded molecule. Relaxation (middle and upper red curves) and subsequent restretching of the molecule (middle and upper black curves) shows, that only domain 4 folds fast enough at the time scale of the experiment (~0.5 s).

mediate seems to fold independently into a stable structure, as our results on the ddFLN1–5BC construct show. The different interactions that stabilize this domain in comparison to I27 of titin may also explain the lower unfolding forces of the Ig domains of ddFLN (60–90 pN) as compared with unfolding forces of titin Ig domains (100–300 pN) at similar pulling velocities. Sequence details can strongly influence stability and unfolding pathways even for domains similar structures. Earlier findings that β-structures are mechanically more stable than domains with an all α-fold seem therefore not generally applicable[12–14]. For fibronectin type 3 domains, molecular dynamics simulations have predicted the existence of an unfolding intermediate similar to the one observed here[15]. A clear experimental indication of this intermediate, however, is missing. Oberhauser *et al.*[16] have observed possible intermediate states for the domain FN1 from fibronectin. However, folding of this domain seems to be compromised by the absence of its neighboring domains. Notably, in an equilibrium NMR study, McParland *et al.*[17] report an unfolding intermediate in an Ig domain from microglobulin. This intermediate is different from that described by our results in that it lacks strands A and G, with the other strands forming a native-like β-sheet. Although unfolding pathways in these experiments using denaturants cannot be directly related to our forced-unfolding data, these studies show that different domains with the same fold can adopt various stable substructures during unfolding.

Physiological relevance of the intermediate

What are the physiological implications of the mechanical behavior of domain 4, which is distinct from that of all the other Ig domains in this protein? Notably, domain 4 not only has the lowest unfolding force of all rod domains in ddFLN but also refolds fastest. Repeated unfolding and refolding experiments with ddFLN1–5 (**Fig. 4**) illustrate this behavior. The lowest trace shows the unfolding of the folded rod, with domain 4 unfolding first and two other domains following at higher extensions. If the unfolded polypeptide is relaxed and subsequently extended again, only domain 4 refolds within the experimental timescale of 0.5 s (middle and top trace, **Fig. 4**). Low unfolding forces in combination with fast refolding make domain 4 unique among the rod domains of ddFLN. The presence of the intermediate could serve two purposes: reducing the overall unfolding forces and, at the same time, ensuring fast refolding. An intermediate can reduce unfolding forces if it breaks up the free energy barrier to unfolding of a two-state folder (native→unfolded) into two barriers (native→intermediate→unfolded) and if each of them is lower than the two-state unfolding barrier. If the intermediate that we observed during forced unfolding is also transiently populated during the folding process, it may guide the protein through a productive folding pathway and hence speed up folding. With its low unfolding forces and fast refolding kinetics, domain 4 may act as an extensible element within the ddFLN rod; this would allow the rod to double in length compared with its folded length. Notably, in the much longer rod of human filamin A containing 24 Ig domains, such intermediates have not been observed[18].

We have shown here that mechanical single-molecule unfolding experiments in combination with site-directed mutagenesis can yield structural information about unfolding intermediates in single protein domains. We cannot yet determine whether the β-strands in the structured portion of the intermediate will retain their native fold or adopt a non-native structure. Future experiments combining mechanical and fluorescence experiments may be able to address this question, even on the single-molecule level.

METHODS

Cloning and protein expression. The full-length rod construct ddFLN1-6 containing six Ig rod domains and the shortened construct ddFLN1-5 are identical to those described elsewhere[2]. The titin construct I27-I34 was as described[7]. To insert single ddFLN domains into the titin construct, two restriction sites, *Nco*I and *Bste*II, were introduced between I30 and I31 using the QuikChange mutagenesis kit (Stratagene). The vector was digested and the ddFLN domain of choice was introduced and religated. Protein purification was done using an N-terminal His_6-tag. Glycine inserts were introduced using the QuikChange protocol. The four constructs Ti/ddFLN4AB, ddFLN1–5BC, Ti/ddFLN4EF and Ti/ddFLNFG were expressed with five glycines inserted at positions 17, 33, 71 and 87. All mutations were introduced into the Ti/ddFLN4 construct except for ddFLN1–5BC, which was derived from ddFLN1–5. The presence of the inserts was verified by DNA sequencing.

Force spectroscopy of single proteins. All single-molecule force measurements were made on a custom-built AFM[19]. Calibration was done in solution by using the equipartition theorem. Si_3N_4 tips with spring constants between 8 and 15 pN nm^{-1} were used. A drop of protein solution was placed on an amino-reactive aldehyde surface or, alternatively, on a freshly evaporated gold surface and incubated for 5 min at room temperature. Subsequently the sample was thoroughly rinsed with PBS buffer to remove excess protein. The force curves of all constructs except ddFLN1–6 (v = 25 nm s^{-1}, 1.5 μm s^{-1}) were collected at a pulling speed ranging from 250 to 350 nm s^{-1}. Experiments were conducted at room temperature.

Data analysis. For quantitative analysis the force-extension curves were fit to an analytical approximation of the worm-like chain model[20], $F(x) = (kT / p)[0.25 (1 - x / L)^{-2} - 0.25 + x / L]$. p denotes the persistence length, L the contour length, k is Boltzmann's constant, T is the temperature in Kelvin and x is the distance between the attachment points of the protein. For the high-force regime (forces >30 pN), a value of p = 0.5 nm provided the best fit; this value was p = 0.9 nm for the low-force regime (forces <30 pN). To improve the signal-to-noise ratio for the curves presented in **Figure 3** an average of ten

curves for each construct was computed. In addition to the averaged traces in **Figure 3** we also fitted the individual data traces for each construct and computed average values. All data were analyzed using Igor pro (Wavemetrics).

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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