Reversible Unfolding of Individual Titin Immunoglobulin Domains by AFM

Matthias Rief, Mathias Gautel, Filipp Oesterhelt, Julio M. Fernandez, Hermann E. Gaub*

Single-molecule atomic force microscopy (AFM) was used to investigate the mechanical properties of titin, the giant sarcomeric protein of striated muscle. Individual titin molecules were repeatedly stretched, and the applied force was recorded as a function of the elongation. At large extensions, the restoring force exhibited a sawtoothlike pattern, with a periodicity that varied between 25 and 28 nanometers. Measurements of recombinant titin immunoglobulin segments of two different lengths exhibited the same pattern and allowed attribution of the discontinuities to the unfolding of individual immunoglobulin domains. The forces required to unfold individual domains ranged from 150 to 300 piconewtons and depended on the pulling speed. Upon relaxation, refolding of immunoglobulin domains was observed.

Proteins acquire their unique functions through specific foldings of their polypeptide chains. Their stability or resistance to unfolding are typically investigated by chemical or thermal denaturation (*1*). Many proteins, however, are designed to withstand forces rather than heat or a harsh change in their chemical environment; their role is to maintain a certain structure against an external load (examples are cytoskeletal or muscle constituents). Because the energy landscape of protein folding (*2*) is yet unknown, proteins' mechanical properties or unfolding forces cannot be derived by thermal or structural analysis; they must be measured. Here we report direct measurements of the mechanical properties of individual proteins by means of single-molecule force spectroscopy (*3*). Titin is a prominent example of a protein whose mechanical properties are essential for its biological function. The passive tension developed by muscle sarcomeres when stretched is largely due to the rubberlike properties of this giant protein, which is also known as connectin (*4*, *5*). The mechanically active region of titin in the sarcomeric I band is assembled from immunoglobulin (Ig)-like domains arranged in tandem (tandem Ig) and a small fraction of nonmodular sequences rich in proline, glutamate, valine, and lysine (the PEVK region) (*6*). Antibody labeling experiments have elucidated the mechanical role of both regions in intact sarcomeres (*7*, *8*). They suggest that the tandem Ig chain is an extensible chain that resists stretching at longer sarcomere lengths, whereas the PEVK region is increasingly extended under stronger forces.

In our experiments, native titin molecules were allowed to adsorb from solution [10 to 100 μg/ml in phosphate-buffered saline (PBS) at pH 7.4] onto a freshly evaporated gold surface for 10 min. After being rinsed with PBS, the sample was probed in the fluid cell of a custom-built force microscope. The AFM tip (Si_3N_4 tips; Digital Instruments, Santa Barbara, California) was brought to the surface and kept in contact for several seconds in order to allow a fraction of the large protein to adsorb onto the tip (*9*). When the tip was retracted, extension curves like the ones shown in Fig. 1 were recorded. The majority of the traces exhibited marked forces at tip-gold distances of more than 1 μm. This indicates that long molecular structures bridged the tip and the gold surface. In all extension traces from native titin, the start region is the least well defined. We attribute the high force peaks in the beginning to multiple molecular interactions between the tip and the gold surface, which rupture upon further separation. For larger extensions, the force curves typically exhibited a sawtoothlike discontinuity. The periodicity was between 25 and 28 nm. At pulling velocities of 1 μm/s, the maximum force of the sawtooth peaks varied from 150 to 300 pN. The maximum length increase upon unraveling of an Ig domain is expected to be 31 nm (*10*), which lead us to the working hypothesis that the sawtooth pattern might reflect the successive unraveling of individual domains of a single titin molecule. In some recordings, the sawtooth pattern was preceded by a monotonical increase of the force upon stretching (Fig. 1, second trace). This range may reflect the extension of denatured stretches of the protein or of naturally less well ordered segments such as the PEVK region.

To test this hypothesis in a well-defined experimental system, two model recombinant titin fragments were constructed, consisting of a four- or eight-Ig segment stretch in the I-band region of titin. We called

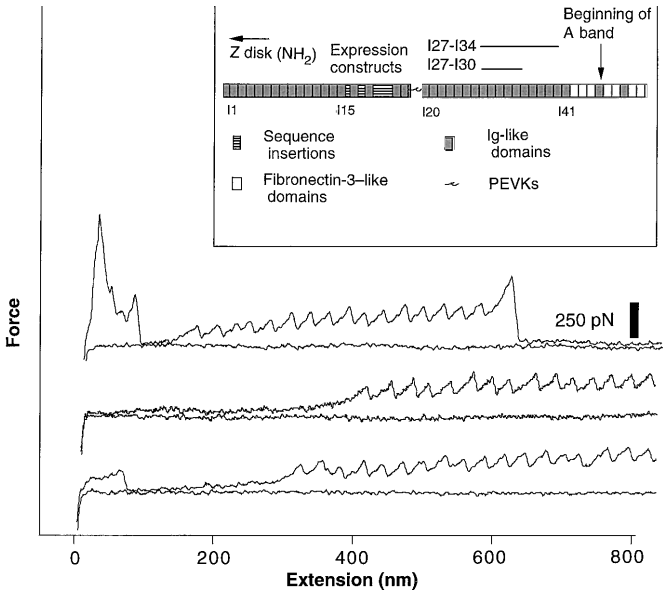

Fig. 1. Force extension curves obtained by stretching titin proteins show periodic features that are consistent with their modular construction. Native titin proteins (10 μg/ml in PBS) were allowed to adsorb onto a gold surface. Three typical approach and retract cycles are shown. The AFM tip approaches the surface covered with the protein (lower trace), and segments of the adsorbed titin are picked up at random by an AFM tip and then stretched (upper trace). We frequently observed a sawtooth pattern in the retraction curves, with as many as 20 force peaks that varied between 150 and 300 pN and were spaced between 25 and 28 nm. The sawtooth pattern was in most cases preceded by a spacer region of variable length, where the force extension curve was not well defined and varied widely. All experiments were done at room temperature. Titin is a large modular protein composed of 244 repeats of Ig-like and fibronectin-like domains (inset). These domains are 89 to 100 amino acids long. Each domain folds into a seven-stranded beta-barrel. The sawtooth pattern observed while stretching titin segments is consistent with the sequential unfolding of individual titin domains.

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them Ig4 and Ig8, respectively (*11*). Covalent binding of the fragments to the gold surface was achieved by two cysteines at the COOH-terminal end. The extension experiment is depicted schematically in Fig. 2F. Several representative extension curves are shown in Fig. 2. All traces exhibit the sawtooth pattern, with a strict 25-nm periodicity measured at 100 pN (*12*). Because the tip could pick up any domain in the chain, the number of peaks within each force curve was randomly distributed. However, we never saw more than four equally spaced peaks in the case of Ig4 or more than eight peaks in the case of Ig8. The superposition of the traces reveals that this pattern was very persistent from experiment to experiment (Fig. 2, B and D) and also within the different constructs (Fig. 2E). The distance between the peaks is the same as that measured on native titin and agrees very well with the length difference between the folded and unfolded Ig domains. From this finding we conclude that this pattern reflects the forced unfolding of the Ig domains (*13*).

The superpositions also reveal that the left-hand slope of the peaks, which reflects the stiffness of the protein, decreases from peak to peak. This means that the stiffness of the stretched protein, which may be derived from the slope, is dominated by a spring that becomes softer (that is, longer) with the unfolding of each Ig domain. The results of a quantitative analysis applying a wormlike chain model to an extension curve of Ig8 are shown (Fig. 3). The experiment can be modeled by assuming a persistence length of 0.4 nm for the unraveled polypeptide and an increase in contour length of 28 to 29 nm per domain. This supports our picture of the stretching process (Fig. 2F). The analysis also shows that the measured increase in length of 25 nm per unfolded domain is shorter than the actual gain in contour length. This is due to the fact that the chain is not fully extended at this force.

A more subtle but persistent feature of the extension traces is that the peak maximum increases with increasing extension (*14*). Because every downstroke (peak to trough) reflects the unfolding of a domain and because the domains are different, this finding means that the weakest domains unfold first and the strongest last (*15*). Thus, the domains are sorted in the extension curves according to their unfolding force and not to their position in the chain. This rules out the idea that desorption of the domains from the gold surface makes a significant contribution to the measured unfolding force (*16*).

Titin Ig domains are believed to fold

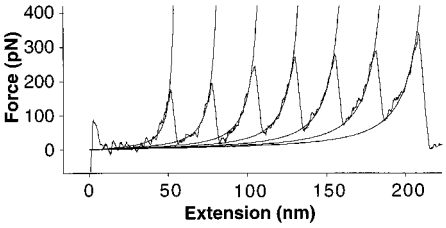

Fig. 3. The characteristic sawtooth pattern of unfolding can be explained as stepwise increases in the contour length of a polymer whose elastic properties are described by the wormlike chain model (WLC) (*27, 28*). The figure shows a force extension curve obtained by stretching of a single Ig8 titin fragment. The force extension curve shows a characteristic sawtooth pattern with seven peaks. The force extension curve [$F(x)$ versus x] leading up to each peak is well described by the WLC equation $F(x) = (kT/b)\,[0.25(1 - x/L)^{-2} - 0.25 + x/L]$ with a persistence length $b = 0.4$ nm and a contour length L that began at 58 nm for the first peak and then increased by 28 to 29 nm to fit consecutive peaks, reaching a maximum of 227 nm for the last peak. k is Boltzmann's constant and T is temperature. Thus, the WLC model predicts that the contour length of the polypeptide chain increases by 28 to 29 nm each time an Ig domain unfolds. This value is close to the 30 nm predicted by fully extending a polypeptide chain comprising 89 amino acids (minus a folded length of 4 nm). At a force of 150 to 300 pN, the polypeptide chain is not fully extended, hence the peaks are spaced by only ~25 nm. Unfolding of the first domain reduces the force to zero, whereas unfolding of consecutive domains reduces the force to a lesser extent. This effect is also well explained by this simple model: Upon reaching a certain force (peaks), the abrupt unfolding of a domain lengthens the polypeptide by 28 to 29 nm and reduces the force (troughs) to that of the value predicted by the force extension curve of the enlarged polypeptide.

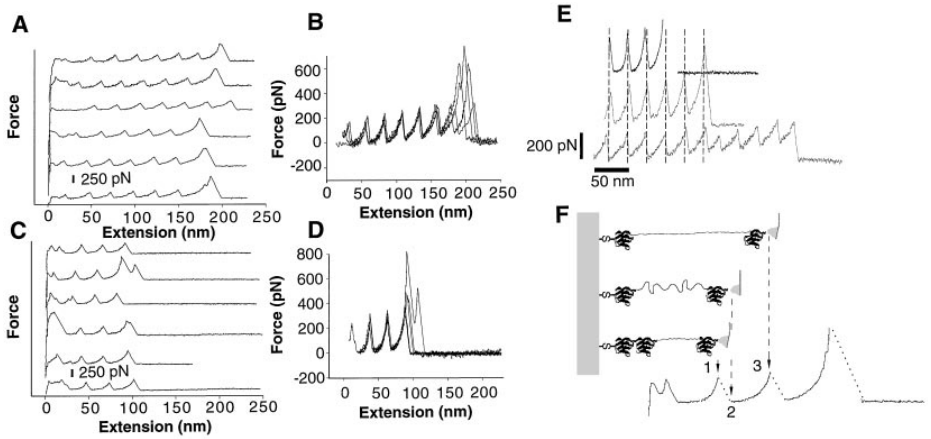

Fig. 2. Stretching well-defined recombinant titin segments produces force extension curves that are consistent with the unfolding of individual Ig domains. We constructed two recombinant titin fragments spanning either eight Ig domains (Ig8, spanning I27–I34; see inset in Fig. 1) or four Ig domains (Ig4, spanning I27–I30). The recombinant titin fragments were constructed with two additional cysteine amino acids added to the COOH-terminal end, providing a covalent anchor to the gold surface. The AFM tip picked up the other end by adsorption. (**A**) Stretching Ig8 domains produced force extension curves that contained up to eight equally spaced peaks of ascending force ranging from 150 pN to a maximum of 300 pN. Only curves showing a maximum number of peaks are presented here. (**B**) Force extension curves obtained from Ig8 proteins were superimposable and revealed a spacing between force peaks of 25 nm measured at 100 pN. The last peak in each recording is usually much higher than the preceding peaks of the sequence and does not reflect the unraveling of an Ig domain but is due to the detachment of the adsorbed construct from the tip. (**C**) Stretching of the shorter titin segment Ig4 produced force extension curves with a sawtooth pattern of up to four force peaks. (**D**) The force extension curves obtained with Ig4 were superimposable and revealed force peaks that were similar to those obtained with Ig8 and were also spaced by 25 nm. (**E**) Force extension curves obtained from all three titin forms (from top to bottom: Ig4, Ig8, and native). (**F**) We interpret our observations as evidence for sequential unfolding of individual titin Ig domains. This figure shows a possible sequence of events. (1) An Ig4, covalently attached to the gold surface, is picked up by adsorption by an AFM tip. As the AFM tip is retracted, the domains unfold. The sawtooth pattern results from the sequential unfolding of Ig domains, which are mechanically in series. Before a domain unfolds, the extended polypeptide will be stretched until a holding force of 150 to 300 pN is reached and unfolding becomes highly probable. (2) Unfolding of an Ig domain abruptly reduces the holding force because of an increase in the length of the extended polypeptide by 25 nm. (3) Continued retraction of the AFM tip again stretches the extended polypeptide until a force is reached where the next Ig domain unfolds. When a domain unfolds, the AFM tip snaps back 2 to 4 nm into its resting position. This leaves a blind window in the force curve within which no structure of the unfolding process can be observed.

spontaneously (*1, 17*). It is even speculated that reversible folding may play a physiological role (*10, 18*). We recorded subsequent extension traces of the same titin molecule (Fig. 4). After each extension, the molecule was allowed to relax completely. A complete cycle took approximately 1 s. As can be seen (Fig. 4), only a fraction of the domains refolded under these conditions. If a titin segment was relaxed to only about one-half of its length, the characteristic sawtooth pattern of the force extension curve disappeared, which suggests that refolding did not occur (Fig. 4, lower panel). However, relaxation to its full length reestablished the pattern of refolding seen before. These results suggest that refolding of titin Ig domains may occur in less than a second and requires the molecule to be relaxed. Our observation that titin Ig domains can be unfolded in a reversible manner raises the possibility that this mechanism is of importance under overstretch conditions. Rather than allowing irreversible damage to the sarcomere, this reversible mechanism would allow for the massive length gains observed in overstretched sarcomeres.

Unfolding caused by an external force can be viewed as a process in which the increasing external force lowers the activation barrier between the folded and unfolded state so that within the time span of the experiment, thermal fluctuations succeed in overcoming the unfolding barrier (*19–21*). The measured unfolding force is thus expected to depend on the extension speed. Figure 5 shows the average unfolding force measured from segments of native titin that were repeatedly extended and relaxed. The extension speed was varied over three orders of magnitude. As predicted by Evans and Ritchie (*19*), the unfolding force depends logarithmically on the extension speed. The solid line represents the results obtained from a simple Monte Carlo simulation. This simulation describes the data well and gives an estimate for the natural off rate of unfolding ($\alpha_0 = 3 \times 10^{-5}\ s^{-1}$). Our value is comparable with the one reported for a twitchin Ig domain ($\alpha_0 = 4 \times 10^{-4}\ s^{-1}$) (*17*). Our simulation also predicts a width for the unfolding potential of Δx = 0.3 nm, which is 100 times smaller than the full extension of the unfolding domain (ΔL = 31 nm). This result implies that the unfolding of a domain occurs as an all-or-none event and is consistent with the finding that we did not observe intermediate states during the peak-to-trough transitions that mark unfolding (see Fig. 3). An alternative estimate for the width of the unfolding potential is the ratio of the free energy of unfolding [10 kcal mol^{-1} (*10*)] to the unfolding force (150 to 300 pN), yielding Δx = 0.3 to 0.5 nm, which is close to the value predicted by the Monte Carlo simulation of Fig. 5.

Our experiments demonstrate that single-molecule force spectroscopy by AFM provides detailed insights into the mechanical properties of individual proteins at the level of single tertiary-structure elements. Compared with other techniques, the AFM approach offers a wider dynamic range and the ability to investigate shorter motifs with a high extension precision (*22, 23*). The measurable restoring forces start in the range of purely entropy-driven forces and are limited in the upper range only by the stability of the attachment. Future instrumental improvements should push both sides of the time window and allow the investigation of the dynamics of a broad range of protein-folding mechanisms.

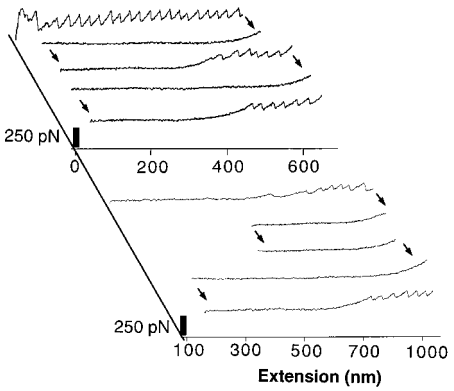

Fig. 4. Repeated stretch-relaxation cycles of single titin fragments demonstrate refolding. We stretched a segment of native titin until the characteristic sawtooth pattern of the force extension curve became apparent, and then we arrested the extension (upper panel). A force retraction curve was then obtained, relaxing the segment to a smaller length. Restretching of the same titin segment gave the same characteristic sawtooth pattern in the force extension curve, albeit with fewer peaks. This extension-retraction cycle could be repeated many times (>50 times) with similar results, which suggests that titin modules unfold reversibly. If a titin segment was relaxed to only about one-half of its length (lower panel), the characteristic sawtooth pattern of the force extension curve disappeared. However, relaxation to its full length restored the sawtooth pattern seen before. These observations suggest that refolding occurs only when the titin molecule is relaxed.

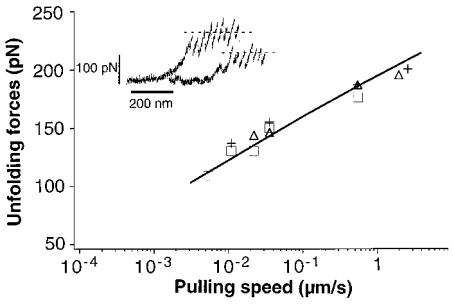

Fig. 5. The force required to unfold a domain is dependent on the pulling speed. The figure shows the average unfolding force measured while pulling segments of native titin at different speeds. The inset at upper left shows a typical experiment. A titin segment 500 nm long was pulled at a speed of 0.5 μm/s. The resulting force extension curve shows nine peaks averaging 190 pN. When the speed was reduced to 0.01 μm/s, the average force dropped to 130 pN. The graph shows the result of three different experiments (□, △, and +). A thousandfold reduction in the pulling rate reduced the average force required for unfolding by ~100 pN. These data are well described by a Monte Carlo simulation of the unfolding of consecutive domains stretched at a constant speed (solid line). The Monte Carlo simulation was done as follows: The force generated by stretching the polypeptide chain is calculated with the WLC model shown in Fig. 3, using the parameters from these data (a persistence length of 0.4 nm; unfolding of a domain always lengthens the polypeptide by 28 nm). The probability of unfolding was calculated as $P = \alpha\Delta t$, where Δt is the polling interval and α is the rate constant for unfolding given by $\alpha = \alpha_0 \exp(F\Delta x/kT)$ (*19*), where α_0 is the unfolding rate in the absence of an external force, F is the applied force, and Δx is the width of the unfolding potential. The simulation was done by stretching the polypeptide chain by a small amount, computing the resulting force with the WLC model, calculating the domain unfolding probability for that force, and then polling the domains with a random number generator in order to define their status. The data were well described using Δx = 0.3 nm and $\alpha_0 = 3 \times 10^{-5}\ s^{-1}$. The simulations gave force extension curves similar to the data. From repeated trials, we computed the average unfolding force at different pulling speeds.

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11. Titin fragments of interest were amplified by polymerase chain reaction from primary lambda cDNA clones and cloned into pET 9d. NH_2-terminal domain boundaries were as in (*24*). The clones were fused with an NH_2-terminal His^6 tag and a COOH-terminal Cys^2 tag for immobilization on solid surfaces. The identity of the cloned fragments was verified by DNA sequencing with the use of a standard automated sequencer. Expression of the fragments was induced in BL21(DE)3 by 0.1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3 hours. The proteins were expressed solubly and were purified from bacterial lysates as described (*25*). Protein was stored frozen in aliquots in 20 mM sodium phosphate (pH 7) and 5 mM dithiothreitol (DTT). Circular dichroism spectroscopy in the far ultraviolet was recorded in storage buffer on a Jasco J-710 spectropolarimeter fitted with a thermostatted cell holder. For further details, see (*1*). The spectra were typical for the beta-barrel structure of titin Ig domains and confirmed the fold of the constructs. Native cardiac titin was purified from bovine heart tissue following the protocol of (*26*), except that the final ammonium sulfate precipitation was omitted. Electrophoresis of the preparation on 3% SDS–polyacrylamide gel electrophoresis showed essentially undegraded titin. The protein was stored at 0.5 mg/ml in 200 mM sodium phosphate (pH 7), 50% glycerol, 5 mM EGTA, 5 mM DTT, and leupeptin (2 μg/ml) at −20°C.
12. The characteristic pattern of the force extension curves observed upon stretching of titin fragments is sensitive to denaturing and cross-linking agents. Incubation of Ig8 in a solution containing 6.6 M urea produced force extension curves that were either featureless or peaked at long extensions with a variable spacing. Treating Ig4 titin fragments with glutaraldehyde (0.1 to 5%) also eliminated the sawtooth pattern in the force extension curves. Instead, we could only observe a featureless and short-ranged force extension curve. These observations suggest that we could either destroy the tertiary structure of the protein by denaturation or that we could render it rigid by cross-linking.
13. It cannot be ruled out that fibronectin III domains, which have a structure similar to that of Ig domains, also contribute to the sawtooth pattern of native titin.
14. The first unfolding peak is typically camouflaged due to multiple adsorptions. The absolute position of the first peak varies because of a random pickup of the protein with respect to the anchoring cysteines, we believe, but also because of rearrangements of the adsorbed segments of the protein on the tip.
15. Note that the unfolding force is the maximum force of the peaks above the baseline of the free cantilever on the approach part of the cycle and not the difference between the peaks and the troughs.
16. Geometric effects can also diminish the observed rupture force of the first peaks. When the line defined by the cysteine tag to the gold surface and the adhesion point on the AFM tip is not in parallel with the direction of pulling, the measured rupture force of the first peak can be up to 15% below the actual force. However, this effect will be unmeasurable from the third peak on.
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10 March 1997; accepted 9 April 1997

Folding-Unfolding Transitions in Single Titin Molecules Characterized with Laser Tweezers

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Titin, a giant filamentous polypeptide, is believed to play a fundamental role in maintaining sarcomeric structural integrity and developing what is known as passive force in muscle. Measurements of the force required to stretch a single molecule revealed that titin behaves as a highly nonlinear entropic spring. The molecule unfolds in a high-force transition beginning at 20 to 30 piconewtons and refolds in a low-force transition at ~2.5 piconewtons. A fraction of the molecule (5 to 40 percent) remains permanently unfolded, behaving as a wormlike chain with a persistence length (a measure of the chain's bending rigidity) of 20 angstroms. Force hysteresis arises from a difference between the unfolding and refolding kinetics of the molecule relative to the stretch and release rates in the experiments, respectively. Scaling the molecular data up to sarcomeric dimensions reproduced many features of the passive force versus extension curve of muscle fibers.

Passive force develops when a relaxed muscle is stretched; this force is responsible for restoring muscle length after release, and it is required for maintaining the structural integrity of the sarcomere in actively contracting muscle (*1*). Titin (*2*), a giant 3.5-MD protein, spans the half-sarcomere, from the Z line to the M line (*3*) (Fig. 1A). Because titin is anchored both to the Z line and to the thick filaments of the A band, passive force while the sarcomere is stretched is probably generated by extension of the I-band segment of the molecule (*4, 5*). It has been suggested (*6, 7*) that the elasticity of titin derives from the reversible unfolding of the linear array of ~300 immunoglobulin C2 (Ig) and fibronectin type III (FNIII) domains (*8*) that make up the molecule. In addition, a unique proline (P)-, glutamate (E)-, valine (V)-, and lysine (K)-rich (PEVK) domain recently identified in titin (*9*) has been hypothesized to form a semistable region that operates as a low-stiffness spring (*9*).

Here, we stretched titin by attaching each of its ends to a different latex bead (*10*), one of which was held by a movable micropipette and the other was trapped in force-measuring laser tweezers (*11*) (Fig. 1B). The micropipette was then moved at a constant rate while the force generated in the molecule was continuously monitored. When a maximum predetermined force (f_{max}) was reached, the process was reversed to obtain the release half-cycle. The force versus extension (f versus z) curves from these experiments (Fig. 2) illustrate several characteristics of the data. (i) The end-to-end distance (z) at which the common f_{max} is reached varies considerably, probably as a result of a variation in the location of the bead attachments in titin. (ii) Many molecules were extended far beyond the ~1-μm contour length of native titin (*12*). (iii) Normalizing the curves to the same length scale reveals that the force generated for a given fractional extension (the ratio of z to the contour length, L) also varies from experiment to experiment, probably reflecting different numbers of titin molecules within the tethers. (iv) All curves show hysteresis.

To identify single-molecule tethers and determine their length, we segregated the data into classes by comparing them with the predictions of two entropic elasticity models: the freely jointed chain [FJC (*13*)] and the wormlike chain [WLC (*14*)] models. The FJC model did not describe the data, but the WLC model fit the stretch data at low to moderate forces and the release data at moderate to high forces (*15*). The WLC model describes the chain as a deformable continuum (rod) of persistence length A, which is a measure of the chain's bending rigidity. For a WLC, z is related to the external force (f) by $fA/k_BT = z/L +$

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