Title
Spider Silk Peptide Is a Compact, Linear Nanospring Ideal for Intracellular Tension Sensing

Permalink
https://escholarship.org/uc/item/3vb94049

Journal
Nano Letters, 16(3)

ISSN
1530-6984

Authors
Brenner, Michael D
Zhou, Ruobo
Conway, Daniel E
et al.

Publication Date
2016-03-09

DOI
10.1021/acs.nanolett.6b00305

Copyright Information
This work is made available under the terms of a Creative Commons Attribution License, available at https://creativecommons.org/licenses/by/4.0/

Peer reviewed
Spider Silk Peptide Is a Compact, Linear Nanospring Ideal for Intracellular Tension Sensing

Michael D. Brenner,†‡ Ruobo Zhou,‡ Daniel E. Conway,§ Luca Lanzano,∥ Enrico Gratton,∥ Martin A. Schwartz,§⊥ and Taekjip Ha*†‡§#∇○

†Department of Chemistry and ‡Department of Physics and Center for the Physics of Living Cells, University of Illinois at Urbana−Champaign, Urbana, Illinois 61801, United States
§Laboratory for Fluorescence Dynamics, Department of Biomedical Engineering, University of California, Irvine, California 92697, United States
∥Laboratory for Fluorescence Dynamics, Department of Biomedical Engineering, University of Virginia, Charlottesville, Virginia 22908, United States
⊥Yale Cardiovascular Research Center, Departments of Internal Medicine (Section of Cardiovascular Medicine) and Cell Biology, Yale University, New Haven, Connecticut 06511, United States
§Laboratory for Fluorescence Dynamics, Department of Biomedical Engineering, University of California, Irvine, California 92697, United States
#Institute for Genomic Biology, University of Illinois at Urbana−Champaign, Urbana, Illinois 61801, United States
∇Department of Biophysics & Biophysical Chemistry, Department of Biophysics, and Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland 21205, United States
○Howard Hughes Medical Institute, Baltimore, Maryland 21205, United States

*Supporting Information

ABSTRACT: Recent development and applications of calibrated, fluorescence resonance energy transfer (FRET)-based tension sensors have led to a new understanding of single molecule mechanotransduction in a number of biological systems. To expand the range of accessible forces, we systematically measured FRET versus force trajectories for 25, 40, and 50 amino acid peptide repeats derived from spider silk. Single molecule fluorescence-force spectroscopy showed that the peptides behaved as linear springs instead of the nonlinear behavior expected for a disordered polymer. Our data are consistent with a compact, rodlike structure that measures 0.26 nm per 5 amino acid repeat that can stretch by 500% while maintaining linearity, suggesting that the remarkable elasticity of spider silk proteins may in part derive from the properties of individual chains. We found the shortest peptide to have the widest range of force sensitivity: between 2 pN and 11 pN. Live cell imaging of the three tension sensor constructs inserted into vinculin showed similar force values around 2.4 pN. We also provide a lookup table for force versus intracellular FRET for all three constructs.

KEYWORDS: Force-sensor, spider flagelliform silk, optical tweezers, single-molecule FRET, force-fluorescence spectroscopy, FLIM

The ability to measure forces across proteins is critical for studies of mechanical regulation and mechanotransduction.1,2 Several candidates for intracellular force-sensing modules have been examined using computational modeling, biochemical3−5 or single-molecule force spectroscopic techniques.6,7 Although molecular scale force reporters based on DNA8,9 or polyethylene10,11 are available for examining extracellular structures, peptides with calibrated force-extension properties are better-suited as tension reporters in live cells due to their relative ease of incorporation using molecular biology methods. Optical reporters of intracellular tension have linked defined peptides to fluorescent proteins (FPs), which report strain through a fluorescence spectral shift12 or change in fluorescence resonance energy transfer (FRET) due to peptide extension.3,13 For tension sensors made of a defined peptide flanked by a donor FP and an acceptor FP, FRET efficiency decreases with increasing force. By embedding such modules into specific sites within protein networks in the cell, one can measure intracellular forces by taking fluorescence images, provided that a calibration exists that relates intracellular FRET values to force. These tools have contributed to our understanding of cytoskeletal forces in mechanotransduction14,15 in a number of biological systems.16,17

In earlier work, we used a spring with eight repeats of the peptide motif GPGGA,6 derived from the spider silk protein flagelliform,18 as the tension sensing element for FRET-based intracellular force measurements. A tension-sensing module (TSMod) consisting of the 40 amino acid (GPGGA)₈ peptide flanked by donor and acceptor FPs was inserted into the middle...
of the cytoskeletal linker protein vinculin to produce the vinculin tension sensor (VinTS). Vinculin has a head domain that associates with the membrane-bound integrin receptors via talin\(^{19}\) and a tail domain that associates with F-actin,\(^{20}\) thus transmitting forces between integrins and the actin cytoskeleton.\(^{11,21}\) Vinculin can also link \(\alpha\)-catenin to F-actin in a similar manner and thus is thought to be a major load-bearing component in cell−cell junctions as well.\(^{26,27}\) Vinculin was therefore the target of studies to monitor the tension in these structures\(^{5,6,13}\) and the calibrated VinTS construct has found widespread use in subsequent studies probing different aspects of integrin-mediated mechanotransduction.\(^{28−35}\) Because vinculin’s head and tail domains interact with binding partners independently,\(^{20,36}\) the FRET-based tension sensor could be inserted between the domains without affecting its cellular localization and functions.\(^{6}\) To convert the FRET values to forces, we utilized single-molecule fluorescence-force spectroscopy that combines single molecule FRET with optical tweezers,\(^{27,38}\) taking advantage of the subnanometer sensitivity in distance determination with FRET while applying piconewton (pN) forces using optical tweezers. Because of unfavorable photophysical properties of FPs, we used organic fluorophores for in vitro FRET measurements. By making certain approximations to relate in vitro and intracellular FRET values, these measurements allowed conversion of FRET efficiencies into force values.

The 40 amino acid (aa) peptide, termed F40, equilibrated its conformations rapidly upon stretching and relaxation, giving it favorable tension-sensing properties.\(^{6}\) Significant FRET changes occurred upon application of 1−6 pN of force, which is a useful range for many biological systems.\(^{39,40}\) In addition to VinTS, many studies have since utilized the F40-based TSMod in intracellular proteins including E-cadherin and \(\beta\)-spectrin,\(^{41−50}\) as well as in extracellular tension-sensing applications with an organic dye FRET pair.\(^{51}\) However, different tension sensing elements that report higher or lower forces would extend the reach of this approach. Here, we examined springs with 5, 8, and 10 repeats of GPGGA (referred to as F25, F40, and F50) using single molecule fluorescence-force spectroscopy and incorporation into vinculin in cells. In addition to identifying the force regimes for these constructs, these studies yielded the surprising result that all three peptides are better characterized as both linear springs and rigid rods rather than by a nonlinear entropic spring model expected for a disordered peptide.\(^{52}\) Overall, our new findings demonstrate that the flagelliform repeat peptide, though well ordered and rigidly folded, is a robust force sensor with a potentially tunable force sensitive range from 1 to 11 piconewtons.
**Force-Fluorescence Spectroscopy of Single Flagelliform Peptides.** We measured FRET efficiency of single, dual-labeled flagelliform repeat peptides as a function of force using a hybrid instrument combining optical tweezers and confocal microscopy as previously described. The peptides were expressed and purified as GST-fusions (Figure 1a). Upon thrombin cleavage of the GST tag, free peptides were purified by FPLC and HPLC. Mass spectrometry confirmed their correct sizes (Figure 1b). The peptides were engineered to contain cysteines at both ends of 5, 8, and 10 repeats of GPGGA, and amino-modified DNA oligonucleotides were attached to the terminal cysteines via SMCC bifunctional cross-linkers (Figure 1c,d,e). Purification of DNA–peptide conjugates from excess DNA was performed with FPLC or PAGE (Figure 1c,d). In some experiments, we used two different oligonucleotides so that among the three distinguishable protein–DNA conjugates only the construct containing one peptide and two distinct oligos was isolated. In other experiments, we used a single DNA oligonucleotide and isolated constructs containing one peptide and two identical oligonucleotides (see Supporting Information Figure 1 for more details). Both methods produced functionally equivalent constructs for optical tweezers experiments.

An oligonucleotide functionalized with 5′-biotin and 3′-Cy5 and another oligonucleotide with 3′-Cy3 were annealed to the oligonucleotides covalently linked to the peptide ends so that FRET between the donor (Cy3) and the acceptor (Cy5) depends on the extension of the peptide (Figure 2a, right). Constructs were immobilized on a polymer-passivated surface through a biotin moiety conjugated to one of the oligonucleotides and examined using total internal reflection fluorescence (TIRF). TIRF measurements can determine the intensities of the donor and acceptor from hundreds of molecules in parallel, allowing us to rapidly build histograms of FRET efficiency (Figure 2a, left). A single peak was observed for each construct, indicating that each peptide had a predominant conformation that was stable on the time scale of one second to minutes at zero force. As expected, FRET efficiency decreased with increasing peptide length.

For fluorescence-force spectroscopy, a 5′ overhang of Lambda phage DNA was annealed to the Cy3-labeled oligonucleotide with the other 5′ overhang of the Lambda DNA annealed to a digoxigenin-labeled oligonucleotide for attachment to a bead coated with antidigoxigenin (Figure 1f and bottom). The bead was trapped at a fixed position with a 1064 nm laser. The piezo-driven sample stage was moved laterally in the x- and y-directions until a preset force value was reached to determine the location of the surface tethered peptide. Then, the stage was used to exert gradual changes in force by moving the surface-bound peptide between 14 and 17 μm away from the trapped bead at the constant speed of 455 nm·s⁻¹ (Figure 2b). Forces of up to 25 pN were applied to the lambda DNA and the tethered peptide in this process. Single molecule fluorescence intensity time trajectories for Cy3 and Cy5 emissions were collected for several cycles of peptide stretching and relaxation (Figure 2c). Raising the force decreased FRET, as determined by a decrease in the acceptor signal and an accompanying increase in the donor signal.

**Flagelliform Peptides Display Linear Behavior upon Stretching and Scaling.** Averaged time trajectories for each construct (Figure 3a) yielded identical FRET vs force curves during stretching and relaxation, demonstrating that below 20 pN the peptide equilibrates rapidly to the new extension without any net energy dissipation. This lack of hysteresis was observed and quantified for individual molecule trajectories in Supporting Information Figure 2. As expected, F25 and F50 obtained from the TIRF assay depicted in the cartoon on the right. (b) Force-fluorescence spectroscopy assay with the bead held still while force is applied to each peptide as the stage moves laterally at constant speed. (c) Individual pulling traces show decreases in FRET as applied force increases. Multiple cycles can be obtained for a single molecule before fluorophore bleaching or tether breaking.

![Image](https://example.com/image.png)
The extension versus force curves were best described by linear fits within the observable FRET range and the compliance was taken as the slope of the linear fit. Fitting with the wormlike chain (WLC) model did not yield an improved fit across the entire distance vs force range (Supporting Information Figure 3). This linearity is surprising but does not rule out the possibility for the flagelliform peptide with no known structure to behave as a nonlinear, entropic spring within higher force regimes like many unfolded proteins.\textsuperscript{54–57} Compliance was proportional to the peptide length (Figure 3c), which is also consistent with a linear spring. The plot of the compliance versus the peptide length (including the terminal cysteines) was fit well by a straight line, yielding a normalized compliance of 0.012 nm/pN/a.a. The linear fit, when extrapolated to zero amino acids, gave a compliance of zero. This indicates that the SMCC cross-linkers maintain the same conformation throughout the stretching cycles and that the compliance values report exclusively on the flagelliform peptide and flanking cysteines. The contribution of the cross-linkers to the measured extension was estimated by linearly fitting zero-force extension versus the number of amino acids (Figure 3d). The extension extrapolated to zero amino acids is 3.9 nm. We attribute this extra extension to the SMCC cross-linkers because the expected length of two SMCC linkers is about 4 nm (Figure 1 middle and Supporting Information Figures 3 and 4) and because the SMCC linker with its aromatic structure\textsuperscript{60} is likely to be stiff.\textsuperscript{59}

We calculated the extension \( R \) of the peptide itself by subtracting the cross-linker length. At zero force, \( R \) is 1.4 nm for F25 and is twice as long, 2.8 nm, for F50. For an ideal polymer, \( R \sim N^{1/2} \), where \( N \) is the polymer length.\textsuperscript{60} If excluded volume effects are included,\textsuperscript{59} \( R \sim N^{5/2} \). Indeed, excluded volume effects are only present in far larger polymers than those between the FRET dyes in this study and are ignored as all flagelliform sizes are on the order of the persistence length.\textsuperscript{52,63} Defining \( N \) as the number of amino acids, fitting our data to \( R \sim N^\nu \) gave \( \nu = 1.01 \) (Figure 3d, inset, and Supporting Information Figure 5). The flagelliform peptide therefore does not behave as a disordered polymer but rather behaves as if it has a rodlike, folded structure with a defined zero-force equilibrium length. This rod is highly compact (\( R/L_0 = 0.14 \), with \( L_0 \) being the contour length) and the contribution of each amino acid to total length is \( \sim 0.5 \) Å (or 0.26 nm per 5 a.a. repeat, Figure 3d and Supporting Information Figure 5). Remarkably, F25 increased its length from 1.4 to 6.3 nm when no force is applied. FRET efficiencies were determined from fluorescence lifetime imaging microscopy (FLIM) as previously described.\textsuperscript{64} Representative lifetime images obtained from cell focal adhesions containing VinTL and VinTS constructs with the F25 peptide are shown in Figure 4b. When the VinTS-expressing cells were plated on fibronectin-coated surfaces, all three constructs localized to focal adhesions where they showed...
significant reductions in FRET efficiency compared to the cells expressing the corresponding VinTL fusions (Figure 4c). The VinTL constructs, presumably under no external tension, exhibited decreasing FRET with increasing flagelliform length. The FRET values differ between the intracellular FP and force-fluorescence constructs, the possible basis for which is depicted in Supporting Information Figure 4A. While the zero-force distance between the probes of the two constructs differ, it is assumed the peptide retains the same compliance and reversible unfolding within the TSMod cassette inside cells as in force-fluorescence measurements. FLIM measurements are probing heterogeneous environments (e.g., pH, ionic strength, viscosity) surrounding an unknown concentration of fluorophores, so extracting absolute lifetime components is not a standard practice.68 However, comparing the relative change in FRET values between TL- and TSMod constructs allows for straightforward, reproducible comparisons to data. To convert the intracellular FRET values to forces, we used a previously described procedure (see also Supporting Information).6 Using VinTL constructs as zero-force controls gave 2.12 ± 0.93, 3.23 ± 0.85, and 1.97 ± 0.89 pN of intracellular forces across a single vinculin at focal adhesion for F25, F40, and F50, respectively. These force values match within error the previously reported 2.5 pN for F40,6 therefore confirming the validity of VinTS as a robust force sensor with potential for tuning the force-sensitive range.

Here, we extended the sensitivity range of the flagelliform force sensor by constructing and analyzing two additional peptide lengths, F25 and F50. F50 had the highest compliance; that is, it changed its extension most with force, making it the most sensitive to small force changes. However, forces above ~5 pN caused the F50 extension to exceed the FRET sensitive range of about 9 nm. In contrast, F25 exhibited measurable FRET changes over a greater force range (2–11 pN). Because F25 has the lowest compliance, one would expect F25 to be less force-sensitive than F50. However, the actual readout is FRET efficiency. Because the initial, zero force FRET efficiency decreased with the length of the springs, the slopes of FRET versus force in the force range between 2 and 6 pN were nearly identical for all three constructs (Supporting Information Figure 6d). Therefore, for force values exceeding 2 pN, F25 provided the largest force range without sacrificing sensitivity. A recently developed genetically encodable tension sensor relying on a well-defined protein unfolding transition was shown to be sensitive to forces above 7 pN17 but the expanded FRET-reportable range of F25 to both low and high force regimes with simple fluorescence-to-force conversion makes it unique as a transducing element. However, small forces (<2 pN) would still be more sensitively measured with longer peptide linkers due to the low-force plateau seen with F25 (discussed below).

Although there have been two reports of linear spring behavior in proteins with well-defined structures involving ankyrin repeats69 and the yeast wall stress component sensor,70 the linear behavior of the flagelliform repeat is surprising because there is little evidence in the literature that it should fold into a well-defined structure. In our FRET analysis, we assumed that the orientation factor known as κ2 is 2/3 (i.e., isotropic averaging) mainly for lack of better estimates. Nevertheless, the apparent linearity does not appear to be due to any intrinsic limit in the dynamic range of our method because we found that single stranded DNA, which has nonlinear spring-like properties, indeed shows a nonlinear extension versus force according to the FRET vs force measurements and similar conversion.71

The nature of the flagelliform repeats’ structure can be inferred from our analysis. Zero-force extension of the peptide is approximately 14% of the contour length with each amino acid contributing about 0.5 Å, which suggests a highly compact structure. We suggest that these peptides form an ordered, rodlike coil structure (Supporting Information Figure 5) as indicated by the linear relationship between number of amino acids and extension (Figure 3d, inset). We showed that F25 can

Figure 4. FLIM analysis of VinTL and VinTS constructs. (a) The full-length vinculin, VinTS, and tailless mutant, VinTL, constructs were expressed in live cells with either the F25, F40, or F50 linker. Below, cartoon derived from crystal structures65–67 depicting a potential VinTL conformation with the closed form of vinculin. The flagelliform peptide is in magenta on the right. (b) Lifetime images of VinTL (left) and VinTS (right) with the F25 peptide. TL25 displays only high FRET within the analyzed focal adhesion regions. (c) VinTL FRET values decreased with increasing peptide length. VinTS FRET values are lower. The difference between VinTL and VinTS FRET values for a given construct report on force (Figure 4d). FRET versus force curves (d) were calculated from fluorescence-force spectroscopy curves in Figure 3b using conversion factors described in the Supporting Information Methods section for obtaining force values from intracellular FRET data in Figure 4c. Error bars are propagated from Figure 3b.

DOI: 10.1021/acs.nanolett.6b00305
Nano Lett. 2016, 16, 2096–2102

undergo a linear expansion of nearly 50% without hysteresis, and it is likely that the same is true for F40 and F50 although the limited distance range of FRET did not allow us to show this directly. The flagelliform repeat sequence is derived from spider capture silk, which can stretch as much as 500% if hydrated.6,7,24 Our data support an interesting possibility that while networks of flagelliform fibers can undergo net energy dissipation upon stretching and relaxation through breaking and reforming of cross-links,8 the linear springlike, reversible-folding of the individual peptides may account for some of the remarkable elastic-like elasticity of hydrated spider capture silk.7

We observed a plateau in the FRET versus force curve below 2 pN for F25. This observation is perhaps unsurprising considering the very good agreement between zero-force TIRF FRET efficiencies and lowest force (∼1 pN) confocal FRET efficiencies obtained from optical tweezers measurements (Figure 3a). This plateau is consistent with F25 having a defined rodlike shape, not unlike a mechanical, coiled spring. When an external force is applied to the spring, its orientation changes first, aligning in the direction of the applied force (Supporting Information Figure 6), an effect that requires force in the low pN range. FRET would not change during this alignment because the distance within the construct would not change. Alternatively, small increases in distance at low forces could be compensated for by higher FRET efficiency due to improved fluorophore alignment, thus giving rise to the plateau. Regardless of exact origin, this plateau is invisible or less pronounced in the longer constructs potentially because less intermolecular interaction. Using this method, the average force is needed to align a longer rod, that is, the plateau will be less sensitive to changes in forces below 2 pN for F40 or F50.

FLIM images of (tailless) VinTL constructs reveal predominantly high FRET and thus low applied forces within vinculin in focal adhesions. VinTS yielded lower FRET values with some regional heterogeneity within focal adhesions (Figure 4b and Supporting Information Figure 7f). The average FRET efficiencies from Figure 4c for VinTL (zero force) and VinTS (under tension) were used as input values for calculating force across vinculin according to the equation in Supporting Information Figure 4e. Reference curves relating intracellular FRET values for F25, F40, and F50 to intracellular forces are provided in Figure 4d. The approximations used to derive the curves should be generally applicable to other proteins as long as the N- and C-terminal domains of the protein of interest can bind cellular partners independently and there is negligible intermolecular interaction. Using this method, the average force value obtained here was ∼2.4 pN, which is in good agreement with 2.5 pN as we reported earlier.6 This study supplies clear evidence for the tunability of a single peptide repeat motif as an intracellular force sensor.

■ ASSOCIATED CONTENT

1 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.6b00305.

Materials and methods and supplementary figures. (PDF)