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Single Molecule Force Spectroscopy of Modular Proteins in the Nervous System

Review

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Mechanosensitive Modular Proteins

Life is stressful, in a mechanical sense. Living tissues are constructed upon a scaffold of interconnected proteins, polysaccharides, and lipids that is in constant motion. When a biological polymer is fastened to this scaffold at more than one position, via covalent bonds or receptor–ligand interactions, relative movement of the anchor points causes stretching forces to be applied to the molecule. Such anchored proteins have important roles in the extracellular matrix (ECM), in the cytoskeleton, and in muscle. In the nervous system, cell adhesion molecules are intimately involved in synaptogenesis, as well as in plastic changes at mature synapses. Recent advances in single molecule spectroscopy with the atomic force microscope (AFM) have made it possible to study mechanical stress at the level of single proteins. This review will discuss the use of the AFM to investigate force-induced changes in protein conformation and how such changes might relate to protein function. We will begin by discussing the structure and function of proteins in the nervous system that are exposed to stretching forces.

Many proteins that experience stretching forces share a common structural feature, that of modularity. Such proteins contain multiple, individually folded domains that frequently belong to the immunoglobulin (Ig), fibronectin type III (FN-III), or cadherin domain superfamilies. Each of these superfamilies is characterized by β sandwich structures consisting of about 90–100 amino acids (Vaughn and Bjorkman, 1996) and are distinguished by the patterns of interaction between their β strands. The Ig (Halaby and Mornon, 1998) and FN-III domains are found in a wide range of proteins, with the latter group thought to occur in about 2% of all animal proteins (Bork and Doolittle, 1992). Although not all proteins containing these domains are exposed to stretching forces, proteins under stress frequently contain multiple copies of such domains (Figure 1). Neural cell adhesion molecules (NCAM) contain five Ig domains and 2 FN-III domains (Chothia and Jones, 1997), cadherins contain five or more cadherin domains (Chothia and Jones, 1997), and human cardiac titin, the giant molecule responsible for passive muscle elasticity, contains 112 Ig and 132 FN-III domains (Labeit and Kolmerer, 1995). Modular proteins involved in cell–cell adhesion also possess intracellular domains (see Figure 1) that act both to connect to the cytoskeleton and to activate signal transduction pathways (Hynes, 1999). Other proteins exposed to stress, such as the cytoskeletal protein spectrin, are composed of repeating α-helical domains (Pascual et al., 1997). Each of these types of domains, Ig (Rief et al., 1997a; Carrion-Vazquez et al., 1999b), FN-III (Oberhauser et al., 1998), cadherin (M. Carrion-Vazquez and J. M. F., unpublished data), and spectrin α-helical domains (Rief et al., 1999b), unfolds when exposed to axial stress. Moreover, since proteins containing these domains are exposed to stress in situ, it is possible that mechanically induced conformational changes may be relevant to their function (Erickson, 1994).

The modular structure of this group of proteins may allow individual domains to respond to force in different ways. Different Ig domains of human cardiac titin, for example, have different mechanical stabilities (Li et al., 2000). Domains may also undergo different degrees of conformational change depending on the applied force. Mechanical extension of modular proteins has shown that relatively low forces may cause small conformational changes (Marszalek et al., 1999a), by breaking noncovalent bonds between a few amino acids at the periphery of a fold, without altering the domain core. Greater forces, however, may cause sequential domain unfolding of modular proteins, such as with titin (Rief et al., 1997a), tenascin (Oberhauser et al., 1998), and spectrin (Rief et al., 1999b). Such unfolding events could enable a protein to undergo enormous increases in length in a highly controlled fashion. Minor structural variations may allow each of the constituent domains of a modular protein to undergo conformational changes at different applied forces. Disulfide bonds between cysteines within a folded domain, such as those found in many Ig domains (Chothia and Jones, 1997), might prevent the unfolding of the core of the domain while allowing force-induced extension of the remainder of the fold. Furthermore, the relief of mechanical tension induces domain refolding in many proteins (Rief et al., 1997a; Oberhauser et al., 1998; Carrion-Vazquez et al., 1999b), enabling the protein to undergo multiple unfolding–refolding cycles. These observations suggest that partial or complete protein unfolding and refolding might occur in situ and that such a process could regulate protein function.

Involvement of Mechanical Forces in Neuronal Function

The nervous system is replete with modular proteins that are exposed to stress (Figure 2). Such proteins are found in the ECM (such as fibronectin and tenascin), in the cytoskeleton (such as spectrin and dystrophin), and make *trans*-synaptic connections (such as cadherin, NCAM, and the cell adhesion molecule L1). The AFM experiments mentioned above have shown that protein domains similar to those found in these proteins may unfold when exposed to mechanical stress and can refold when the force is removed. Could force-induced conformational changes regulate protein function in the nervous system?

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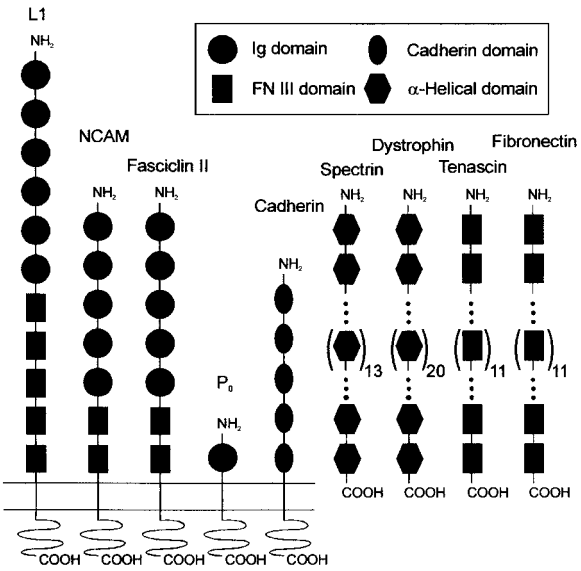

Figure 1. Mechanosensitive Domains in Modular Proteins Found in the Nervous System

Schematic representations of several proteins that are exposed to mechanical stress and that contain domains known to be mechanosensitive. Ig domains are indicated by circles, FN-III domains by rectangles, cadherin domains by ovals, and α-helical domains by hexagons. Large numbers of repeats are indicated by enclosing the repeating domain in parentheses and placing the number of repeats as a subscript. Other domain types are not shown. L1, NCAM, fasciclin II, P_0, and cadherin have transmembrane regions and intracellular domains involved in interactions with the cytoskeleton and signal transduction. Spectrin, dystrophin, tenascin, and fibronectin do not have transmembrane regions, but each are linked to the cell scaffold such that they are exposed to stretching forces.

Mechanical stress applied to a modular protein could regulate its function by either altering its interaction with a receptor molecule to which it is bound and thereby generating a signal, or by changing the conformation of one or more of its constituent protein domains. An example of the former mechanism may occur with the integrins, a family of cell surface proteins that bind to fibronectins in the ECM. When microscopic magnetic beads coated with anti-integrin antibodies were used to exert mechanical force on integrin receptors expressed in a cell line, an increase in internal Ca^{2+} was generated (Pommerenke et al., 1996). Stress on the integrin receptor at frog neuromuscular junction mediates immediate changes in neurotransmitter release in a fashion not dependent on Ca^{2+} release (Chen and Grinnell, 1995; Chen and Grinnell, 1997). Such changes might be triggered by mechanical deformation of the integrin molecule or by an alteration or breakage of the receptor–ligand interaction between integrin and components of the ECM. Force-induced partial or complete unfolding of domains within a modular protein could also prolong the lifetime of its interaction with a receptor molecule. If domains unfold prior to breakage of the receptor–ligand bond, the distance over which the bond may be maintained is increased, as is the total work required for breakage (Oberhauser et al., 1998; Evans and Ritchie, 1999; Smith et al., 1999). This may be a fundamental principle underlying biological adhesion (Smith et al., 1999). The unfolding of domains in a protein could also serve to regulate the elastic properties of the link between two anchors. This may occur with the giant muscle protein titin, which is linked to large multiprotein complexes at the muscle Z and M lines (Labeit and Kolmerer, 1995). Unfolding and refolding of titin Ig and FN-III modules may help to maintain the tension during the extension and relaxation of muscle and thereby contribute to the passive elasticity of muscle (Kellermayer et al., 1997; Tskhovrebova et al., 1997; A. Minajeva et al., submitted). Partial or total unfolding of a domain could also selectively alter the activity of that domain. Activation or inactivation of an enzymatic activity could, for example, generate a force-induced signaling mechanism. Domain unfolding could regulate the interaction between that domain and a receptor molecule, despite the lack of direct stress on the bond. Conformation changes due to stretching forces could expose or disassemble structures involved in binding and thereby favor or inhibit the interaction.

The clearest example of a role for force-induced unfolding in the nervous system involves the activation of the modular protein fibronectin during ECM formation. It is known that mechanical stress generated through anchored proteins is required for the development of fibronectin fibrils (Halliday and Tomasek, 1995) and that cell migration in tissue culture can generate forces consistent with protein unfolding (Galbraith and Sheetz, 1997). Fibronectin fibrils in the matrix between cultured cells are under tension since when they are severed they can contract to less than one-fourth of their extended length (Ohashi et al., 1999). This degree of stretching strongly suggests that fibronectin in the ECM may be in a partly unfolded state. Evidence suggests that the force-induced unfolding of a specific FN-III domain uncovers a "cryptic" site that is necessary for binding of other molecules of fibronectin and therefore for fibronectin polymerization (Hocking et al., 1994; Morla et al., 1994; Ingham et al., 1997; Zhong et al., 1998).

Although there is no direct evidence for mechanical unfolding of other neuronal proteins in vivo, it is likely that FN-III domains in other proteins could unfold under stress just as well. The other types of domains discussed in this review, Ig, cadherin, and spectrin domains, have mechanical stabilities roughly comparable to FN-III domains and therefore may also unfold under physiological conditions. Force-induced domain unfolding may be a common mechanism during the formation of cell–cell interactions and interactions between cells and the ECM and, thus, may be important during neuronal development. Similar changes may also occur in the adult nervous system. Recent experiments using two-photon imaging of neurons in cultured hippocampal slices suggest that neuronal dendrites are structurally dynamic, with filopodia constantly expanding and retracting (Maletic-Savatic et al., 1999). In particular, dendrites sprout new spine-like protrusions in response to stimuli similar to that evoking LTP (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999). These data suggest that cell adhesion proteins may experience changing mechanical stress in the adult nervous system.

Cell adhesion molecules may be involved in rapid changes in synaptic function. At the frog neuromuscular junction, physiological stretch enhances neurotransmitter release, and this effect is suppressed by peptides

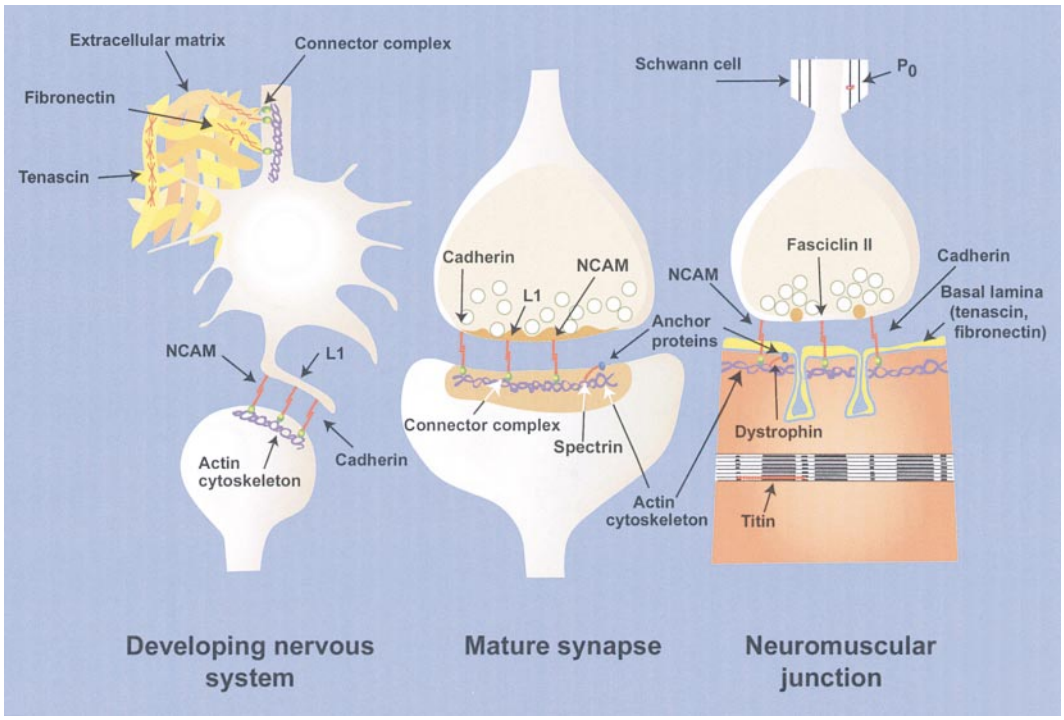

Figure 2. Mechanosensitive Modular Proteins in Excitable Cells

A schematic representation of the location of mechanosensitive, modular proteins (shown in red) in the developing nervous system, at a mature synapse, and at the neuromuscular junction. The "connector complex" links the mechanosensitive proteins to the cytoskeleton.

containing the arginine-glycine-aspartic acid (RGD) sequence, which mimics the portion of fibronectin that binds to integrin and thereby blocks their interaction, or by antibodies to integrins (Chen and Grinnell, 1995). This effect may be mediated through a direct mechanical interaction of integrins with some component of the exocytotic machinery (Chen and Grinnell, 1997). Mechanical force could be transmitted through an ECM protein to an integrin molecule through changes in the binding interaction. A molecular simulation of force-induced unfolding of a FN-III domain suggests that the RGD sequence deforms at an early stage of domain extension (Krammer et al., 1999). Force might thereby change or break the interaction between receptor and ligand and cause a change in the function of the integrin molecule.

Several neural recognition molecules have been proposed to have a role in synaptic plasticity. RGD peptides were also reported to block hippocampal LTP (Staubli et al., 1990), suggesting a role for integrins. A *Drosophila* mutant that shows a decrease in short-term olfactory memory has been shown to have a defect in genes for an α-integrin molecule and their behavioral defect may be rescued by transient expression of the wild-type gene in adulthood (Grotewiel et al., 1998). Antibodies directed toward L1 and NCAM, as well as peptide fragments of L1, inhibit the development of LTP (Luthl et al., 1994). Intracerebral injection of antibodies against L1 and/or NCAM can impair learning or retrieval tasks (Doyle et al., 1992a, 1992b; Scholey et al., 1993), as can inactivation of the NCAM gene in transgenic mice (Cremer et al., 1994). A role for a homolog of NCAM (apCAM) has also been identified in memory storage in *Aplysia* (Bailey et al., 1992). Hippocampal LTP, but not synaptic transmission at rest, was also blocked by antibodies toward cadherins (Tang et al., 1998). One explanation for this phenomenon is that the *trans*-synaptic bond between cadherin molecules was disrupted during intense activity and that this somehow leads to enhanced transmission. It is known that the interaction between cadherin molecules is dependent on Ca^{2+}. The block of LTP by cadherin antibodies was prevented by high concentrations of external Ca^{2+}, which was interpreted to mean that the formation of LTP depends upon the disruption of cadherin bonds resulting from depletion of Ca^{2+} in the synaptic cleft during intense activity (Tang et al., 1998). The breakage of the cadherin bond could alter cell function by propagating a mechanical signal to either the pre- or postsynaptic cell.

Force Measurement at the Molecular Level

Understanding the mechanical properties of proteins requires the measurement of force at the level of single molecules. This requires a means of anchoring the molecule of interest at its two ends, a means of moving one of the ends, and a means of measuring the force with which the molecule resists extension. Single molecule force spectroscopy was first accomplished by attaching one end of a DNA molecule to a glass surface and the other end to a magnetic bead (Smith et al., 1992). Extension of the molecule was measured optically by monitoring the position of the bead as it was exposed to combinations of hydrodynamic and magnetic forces. In later experiments, one end of a DNA molecule was attached via a glass bead to a piezoelectric actuator, while the other was attached to a force transducer, either a bead

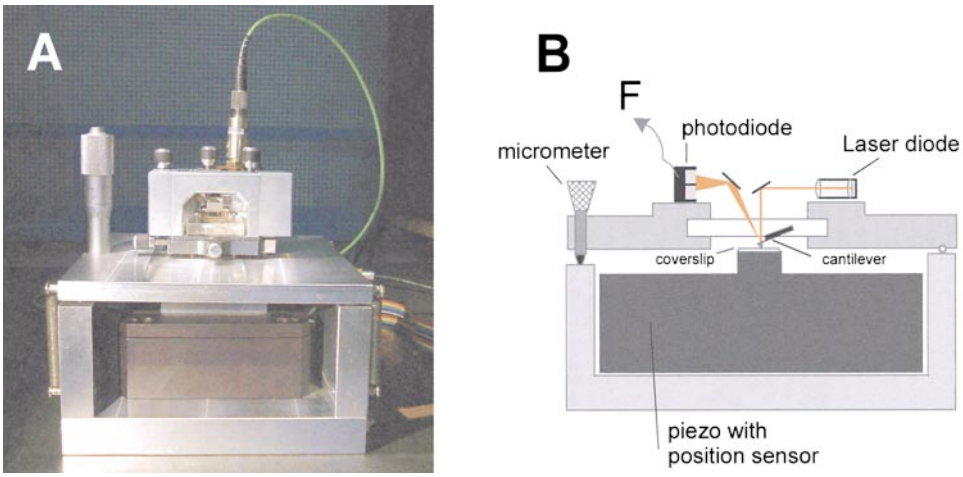

Figure 3. The Atomic Force Microscope

(A) A photograph of a custom-built AFM used in many of the studies described in this review. The main parts of the instrument are an AFM detector head (Digital Instruments; small box) mounted on a single axis piezoelectric positioner (Physik Instrumente; large box).

(B) A schematic diagram of an AFM. A laser beam is deflected off the cantilever onto a photodetector, to detect changes in the angle of the cantilever. Bending of the cantilever can be calibrated to determine the force with which the molecule resists extension.

in an optical trap (Smith et al., 1996) or a flexible optical fiber (Cluzel et al., 1996). Laser tweezers have since been used to explore the elastic properties of DNA molecules (Baumann et al., 1997, 2000), single protein molecules (Kellermayer et al., 1997; Tskhovrebova et al., 1997), DNA–protein complexes (Hegner et al., 1999), and the activity of RNA or DNA polymerases on single extended DNA molecules (Davenport et al., 2000; Wuite et al., 2000). The use of the AFM to stretch single molecules developed out of its original use in measuring the contours of microscopic samples (Binnig et al., 1986; Hansma et al., 1988; Wong et al., 1998; Binnig and Rohrer, 1999). In the force-measuring mode of the AFM (Fisher et al., 1999a, 1999b), proteins or other biological polymers are stretched between a microscopic tip at the end of a flexible cantilever and a flat substrate whose position is controlled by a highly precise piezoelectric positioner (see Figure 3). This arrangement may be achieved by pressing the cantilever tip against a substrate that has been coated with a fine layer of pure protein. By a mechanism as yet not well understood, a protein molecule may be adsorbed to the cantilever tip such that a portion of the protein (in the best of cases, essentially all of the protein) becomes suspended between the tip and the substrate. The molecule may then be stretched by retracting the substrate using the piezoelectric positioner. The force with which the molecule resists extension then causes the cantilever to bend. The angle of deflection is measured by reflecting a laser beam off the cantilever onto a photodetector, which detects changes in the vertical position of the incident laser light. By calibrating the response of the cantilever to applied force, changes in the angle of deflection of the cantilever can be converted to a measurement of the force on the molecule with a resolution at the level of piconewtons (pN).

The AFM and the Extension of Single Molecules

The AFM has been used to stretch individual molecules of polysaccharides (Rief et al., 1997b; Li et al., 1998; Marszalek et al., 1998, 1999b), DNA (Rief et al., 1999a; Clausen-Schaumann et al., 2000), and proteins (Rief et al., 1997a, 1998, 1999b; Oberhauser et al., 1998, 1999; Carrion-Vazquez et al., 1999a, 1999b; Marszalek et al., 1999a; Muller et al., 1999; Li et al., 2000; Yang et al., 2000; Oesterhelt et al., 2000). The data that are obtained may be expressed as a "force–extension curve", which shows the relationship between the force exerted on the cantilever and its distance from the substrate. Models of polymer elasticity predict that when an ideal polymer is stretched the amount of force needed to lengthen the molecule is determined by the loss of entropy of the molecule. The entropy of such a polymer is maximized when the polymer is randomly coiled and minimized when the polymer is fully extended and hence has no freedom of motion. The amount of force required to extend a polymer therefore increases steeply as it approaches its fully extended length. Deviations from entropic elasticity indicate force-induced changes in the structure of the components of the polymer that alter their end-to-end length. Deflections in the force extension curves for certain polysaccharide molecules, for example, have been shown to be due to conversion of the polysaccharide rings in the chain from the energetically favored "chair" conformation to the longer "boat" conformation (Marszalek et al., 1998, 1999b). When a folded protein is stretched, the entropic resistance to extension increases progressively until at some point the bonds holding one of the constituent folds together are broken and the fold unravels, thereby increasing the effective length of the protein and decreasing the force on the cantilever to near zero (Figure 4). The effective length of the protein is extended by a distance determined by the number of amino acids in the fold. For a multidomain protein, each domain will unfold consecutively, resulting in a force–extension curve with a series of peaks in a "saw-tooth" pattern (Rief et al., 1997a; Oberhauser et al., 1998). By fitting the upswing of each of the peaks to a model of polymer elasticity, such as the worm-like chain model, one can determine the total

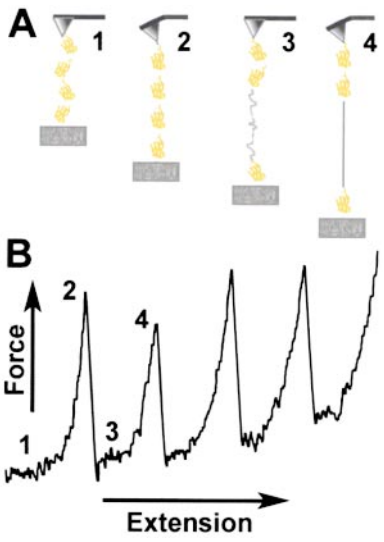

Figure 4. The Forced Extension of Modular Proteins Exhibits a Saw-Tooth Pattern

(A) A schematic representation of the stages of extension of a modular protein and the corresponding force–extension relationship (B). Initial stretching requires little force (1). As the molecule becomes fully stretched, resistance increases to a peak (2). At some point, the increased force causes unfolding of one of the domains in the protein (3), which releases the force on the cantilever. Further extension of the molecule stretches the unfolded sequence and the force on the cantilever again increases (4).

length of the polymer prior to the unfolding of each domain. The interval between events thus reveals the length increment caused by the unraveling of the peptide chain in each domain. The force at which domains unfold is variable because unfolding is a probabilistic event. Unfolding is therefore, like bond rupture (Bell, 1978; Alon et al., 1995; Merkel et al., 1999), dependent on the rate at which a molecule is extended—the faster the extension, the greater the force that is required. The distribution of forces at a given rate does, however, give an indication of the mechanical stability of the fold. These are key concepts in understanding the possible physiological relevance of mechanical protein unfolding, since they imply that it may occur at low or no force, but that the likelihood will increase as the applied force increases. The extension of a molecule in the ECM during synaptogenesis, for example, would be expected to unfold protein domains at lower forces than are required during an AFM experiment, if the extension occurs at a slower rate.

The Force-Induced Unfolding of an Immunoglobulin Domain

The best-understood mechanical domain is the I27 module of human cardiac titin (Figures 5A and 5B). This domain formed part of the first recombinant protein pulled on the AFM, which was the segment of titin containing Ig domains 27 through 34 (I27–I34) (Rief et al., 1997a). Extension of native titin, or portions of titin containing sequences of several titin Ig domains, resulted in force–extension patterns showing the saw-tooth patterns indicative of domain unfolding. Extension of the

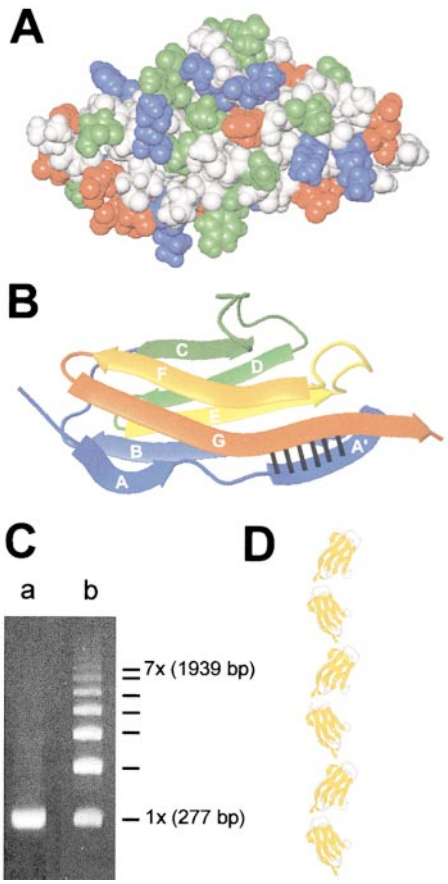

Figure 5. The Structure of the I27 Module of Human Cardiac Titin and the Construction of an I27 Polyprotein

(A) A space fill model of the I27 module of human cardiac titin. Positively charged residues are shown in blue, negatively charged residues in red, polar residues in green, and hydrophobic residues in gray.

(B) A schematic diagram of the mechanical topology of the β sandwich structure of I27 with each of the β strands shown in different colors. The critical hydrogen bonds between the A′ and G strands are shown in black.

(C and D) An agarose gel (C) stained with ethidium bromide showing cDNAs of the I27 monomer (lane a) and a ladder of concatamers consisting of various numbers of I27 monomers (lane b). Expression of these concatamers results in a polyprotein consisting of several repeats of the I27 domain (D).

titin segment I27–I34 yielded up to eight unfolding peaks ranging in force from 150 pN to 300 pN. (Since the adsorption of the protein to the cantilever tip occurs at random positions, the number of unfolding peaks observed is frequently smaller than the total number of modules in the protein.) The force of unfolding tended to increase with each unfolding event, creating a staircase pattern and suggesting that the less mechanically stable domains unfold prior to the more stable ones (Li et al., 2000). These experiments also demonstrated that force-induced unfolding is reversible. Upon relaxation of the protein, the domains readily refold and can undergo repeated unfolding–refolding cycles (Rief et al., 1997a; Carrion-Vazquez et al., 1999b).

It became clear, however, that the amount of information that may be derived from the extension of native

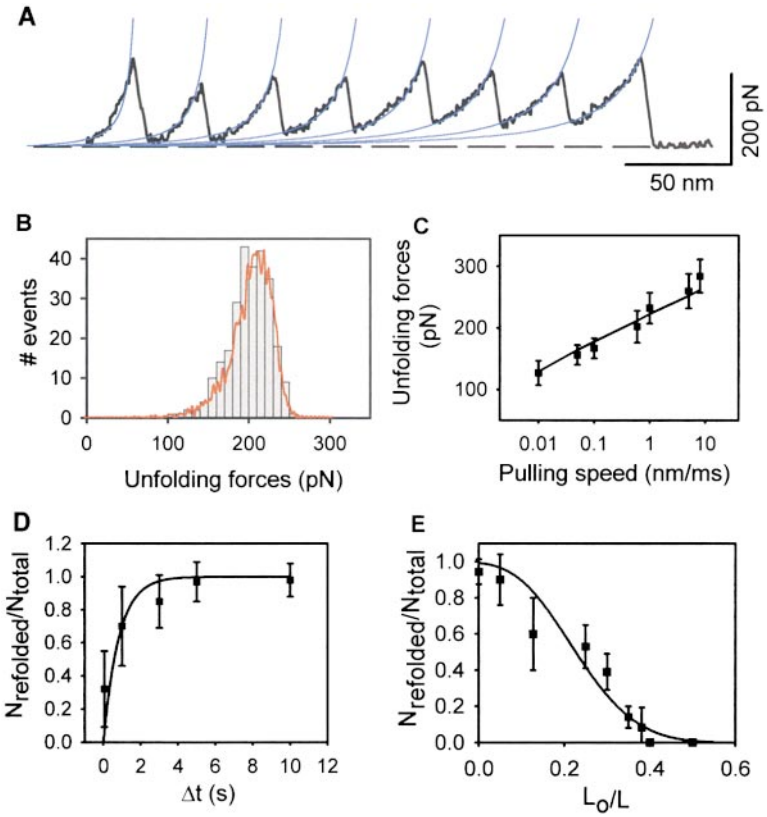

Figure 6. Force-Induced Unfolding and Refolding of the I27 Polyprotein

(A) A force–extension curve for recombinant $I27_8$. Consecutive unfolding events are fit using the worm-like chain model of polymer elasticity (blue lines).

(B) An unfolding force frequency histogram for $I27_8$. The red lines correspond to a Monte Carlo simulation of unfolding assuming a rate constant of $3.3 \times 10^{-4}\ s^{-1}$, an unfolding distance of 0.25 nm, and a pulling rate of 0.6 nm/ms.

(C) The force-induced unfolding of I27 domains at different pulling speeds.

(D) Unfolding–refolding cycles of $I27_8$ using a double pulse protocol. A plot of the refolded fraction ($N_{refolded}/N_{total}$) versus interval between the pulses (Δt). The solid line is a fit of the data assuming a refolding rate of $1.2\ s^{-1}$.

(E) A plot of the fraction of $I27_8$ modules refolded during a 5 s interval between two extensions, $N_{refolded}/N_{total}$, as a function of the degree of relaxation expressed as the relaxed length (L_0) divided by the extended length (L) (see Carrion-Vazquez et al., 1999b, for details).

proteins was limited because there were no means to relate the sequence or structure of a specific domain within the protein to its mechanical properties. Extension of segments containing a single Ig domain are impractical because unfolding peaks for such a protein would be difficult to distinguish from the nonspecific interactions that are seen as the cantilever tip is pulled away from an adsorbed protein layer (Carrion-Vazquez et al., 2000). Molecular biological techniques were therefore developed to allow production of protein constructs consisting of several repeats of a single domain (Carrion-Vazquez et al., 1999b). Such proteins, dubbed "polyproteins," do not occur in nature. Polyproteins made it possible to determine the mechanical stability of specific domains and to identify deviations from entropic behavior that could illuminate the conformational changes that constitute the force-induced unfolding pathway. Furthermore, the construction of engineered polyproteins has enabled mutagenesis experiments to alter the amino acid sequence within a folded domain to identify the determinants of mechanical stability.

The I27 domain of human cardiac titin was chosen for construction of the first polyprotein to be studied with AFM (Carrion-Vazquez et al., 1999b), because its tertiary structure had been defined by NMR (Improta et al., 1996), its thermodynamic stability was known (Politou et al., 1995), and its mechanical unfolding pathway had been modeled (Lu et al., 1998). Polyproteins with 8 or 12 tandem repeats of I27 (Figures 5C and 5D) were expressed, purified, and stretched using AFM techniques (Carrion-Vazquez et al., 1999b). The force extension curve of the I27 polyprotein (Figure 6A) differed from that of native titin in important ways. First, rather than displaying a staircase pattern of unfolding peaks, which indicate a heterogeneous distribution of unfolding forces, the peaks for I27 polyproteins were closely and randomly distributed around a mean of about 200 pN (Figure 6B). This allowed the first estimates of the mechanical stability of a protein domain. Second, the length increment between unfolding events was also homogeneous, allowing an accurate measurement of the length of the amino acid chain that is contained within the folded domain (28.1 ± 0.17 nm; Carrion-Vazquez et al., 1999a). This distance gives an indication of the mechanical structure of the domain, since it reveals the number of amino acids between the bonds forming the major mechanical resistance to domain unfolding (see below). Measurement of domain size is facilitated by the repetitive nature of polyproteins, since it allows the fitting and averaging of many unfolding events simultaneously. Third, polyproteins have enabled detailed measurements of the kinetics of I27 unfolding and refolding. Measurements of the dependence of unfolding on the rate of extension (Figure 6C) were used to estimate of a rate of unfolding at zero force of $3.3 \times 10^{-4}\ s^{-1}$ (Carrion-Vazquez et al., 1999b). The rate dependence of unfolding also allowed an estimate of the unfolding distance (the distance over which the force must be applied to reach the transition state between the folded and unfolded states) of 0.25 nm (Carrion-Vazquez et al., 1999b). This suggests that extension of the domain by only the length of a single water molecule causes unfolding. The refolding kinetics of a modular protein may be studied using a two-pulse protocol (Figure 6D). The molecule is

stretched twice, with a variable delay between the two extensions, and the degree of refolding is determined by the proportion of domains that have been able to refold (i.e., the number of peaks in the second force–extension curve divided by the number in the first). Analysis of refolding in a fragment of human tenascin-C resulted in a pattern that suggested two exponential rates of refolding (Oberhauser et al., 1998). This complexity is likely derived from the heterogeneity of the FN-III domains in tenascin. Refolding in the I27 polyprotein, however, showed a single exponential rate of refolding of 1.2 s^{-1} (Carrion-Vazquez et al., 1999b). This was the first direct mechanical measurement of the kinetics of domain refolding. A similar protocol has been used to determine the dependence of refolding on the extent to which the protein chain is relaxed before reextension (Figure 6E). These data suggest that refolding is also highly dependent on the force. Fourth, the simultaneous extension of several identical domains has enabled the identification of subtle conformational changes that occur prior to the all-or-none unfolding event (see below; Marszalek et al., 1999a).

The size of the measured length increment caused by I27 unfolding (28.1 nm; Carrion-Vazquez et al., 1999a) gives important structural information about the domain. Since each amino acid contributes about 0.40 nm to a stretched peptide chain (Carrion-Vazquez et al., 1999a), this increment corresponds to a length of about 70 amino acids. Since this is less than the total number of amino acids in an I27 fold (89), some of the amino acids must be extended along the axis of stretch before domain unfolding occurs. Amino acids are therefore either "force bearing", those that are exposed to mechanical force before unfolding, or "hidden", those that are exposed to force only after unfolding has occurred. The AFM experiments predict that the I27 domain resists mechanical disruption at a linkage between two or more amino acids that differ by about 70–75 positions in the I27 sequence. The structure of I27 therefore suggests that the mechanical breakpoint occurs between amino acids in two β strands called the A′ and G strands. This is consistent with molecular simulations of force-induced extensions of I27 that have suggested that bonds between these two strands pose the major resistance to forced unfolding (Lu et al., 1998). Studies of the mechanical properties of polyproteins constructed using mutants of I27 have supported this hypothesis (Figure 7A). When a set of five glycines was inserted into a hairpin loop within the I27 fold (i.e., among the hidden amino acids), the interval between unfolding peaks on the force extension curve was, as expected, increased by about 2.0 nm per domain (Carrion-Vazquez et al., 1999a). Insertion of glycines in the force-bearing portion of the sequence, however, had no effect on the unfolding interval because these amino acids were fully stretched before the unfolding of domains occurred (Carrion-Vazquez et al., 1999a).

Extension of the neighboring Ig domain of human cardiac titin, I28, shows that different domains may have different mechanical properties. Polyproteins of I28, like those of I27, display a prominent hump in their force–extension curve prior to domain unfolding (Marszalek et al., 1999a). The actual force of unfolding, however, is higher for I28 (257 ± 27 pN versus 204 ± 26 pN; Li et al., 2000). This is contrary to what would have been expected based on thermodynamic measurements, since these suggest that I27 is more stable than I28. Thermodynamic stability is therefore not a good indicator of mechanical stability. While thermodynamic stability is determined by the difference in the free energy of the folded and unfolded state, mechanical stability seems to be determined by the height and shape of the energy barrier posed by unfolding. Extension of polyproteins comprised of repeating units of I27–I28 showed that the domains with lower mechanical stability (the I27 domains) unfolded prior to the more stable domains (I28) regardless of their relative position in the polypeptide chain (Figure 7B) (Li et al., 2000).

The I27 polyprotein also made possible comparisons

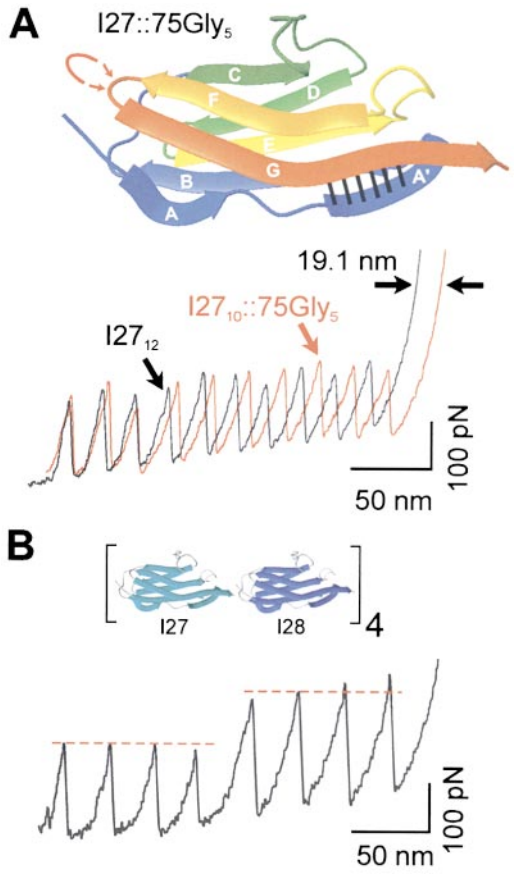

Figure 7. Force-Induced Extension of a Mutant I27 Polyprotein with Amino Acids Added to the Hidden Core and of a Polyprotein Consisting of Alternating I27 and I28 Domains

(A) Structure of the I27 module, showing the location at which five glycine residues were inserted to make the I27::75Gly$_5$ mutant. When the force–extension curves for I27 and I27::75Gly$_5$ are superimposed, there is a clear increase in the length increment for unfolding of the mutant protein. In the example shown, the unfolded length of the mutant is 19.1 nm longer for ten modules, suggesting that each unfolded I27::75Gly$_5$ module is 1.9 nm longer than the I27 module.

(B) The schematic diagram of the structure of a chimeric polyprotein consisting of alternating I27 and I28 domains. The force–extension relationship for this protein shows that the less mechanically stable I27 domains unfold at lower force than the more stable I28 domains, regardless of their relative position in the polyprotein.

between the rates of unfolding and refolding measured with AFM to those measured using chemical denaturation. The extrapolation of rates of unfolding at various pulling speeds to zero force resulting in a value (3.3×10^{-4} s^{-1}) very close to the value determined from experiments with different concentrations of guanidinium chloride extrapolated to zero denaturant (4.9×10^{-4} s^{-1}; Carrion-Vazquez et al., 1999b). This suggests that the bond breakage that forms the rate-limiting step in domain unfolding is the same during chemically or force-induced denaturation. It remains to be seen whether this tight correlation will hold for other proteins and domains, particularly proteins not exposed to mechanical stress in vivo. The refolding rates for the polyproteins, however, were slower than those measured following chemical denaturation of I27 monomers (1.2 s^{-1} compared to 32 s^{-1}, respectively). This may be due to the reduced rotational freedom of domains tethered in a modular protein and may better reflect the kinetics of refolding of tethered proteins in situ.

One of the great advantages of measuring force-induced unfolding with AFM is that deviation from the expected entropic behavior can indicate intermediates in the unfolding pathway. These observations may reveal the pathway through which proteins unfold and refold and how domains respond to forces lower than those required for full unfolding. Such changes were identified in the pathway of unfolding for the I27 domain in polyprotein experiments (Marszalek et al., 1999a). Prior to the unfolding of any domains a slow increase in force (a "hump") with an amplitude of 108 ± 19 pN is observed in the force–extension curve of poly I27 (Figure 8). The hump corresponds to an elongation of each I27 domain, which are 4.4 nm in length (Improta et al., 1998), by 0.66 nm, a 15% increase. Furthermore, subsequent unfolding peaks also display humps, but of progressively smaller size. Molecular simulations show the hump is likely to be caused by the straightening of the final few "force-bearing" amino acids on each I27 domain (Marszalek et al., 1999a), following the breakage of two hydrogen bonds bridging the A and B strands (Figure 8B). This conformational change occurs prior to, and at lower force than, the breakage of the hydrogen bonds of the A′G patches in any of the domains. The hump does not appear in a mutant polyprotein in which one of the amino acids thought to be involved in the interaction (at position 6) was mutated from a lysine to a proline (I27-K6P; Figure 8A). Thus, each of the I27 molecules undergoes a small forced extension before any of them unfold. The presence of humps prior to the second and subsequent unfolding peaks suggests that the AB bonds reform very quickly when unfolding of a domain relieves the tension on the polyprotein. Thus, the A and B strands form a hinge-like mechanism that regulates the length of the I27 module.

Mechanical unfolding has also allowed the identification of misfolding events in polyproteins. These events were observed as "missing peaks" during AFM refolding experiments (Oberhauser et al., 1999; Figures 9A and 9B). The amplitude of the force peaks before and after the gap were normal (i.e., close to 200 pN). When the length increment caused by the unfolding event following the gap was measured, it was found to correspond

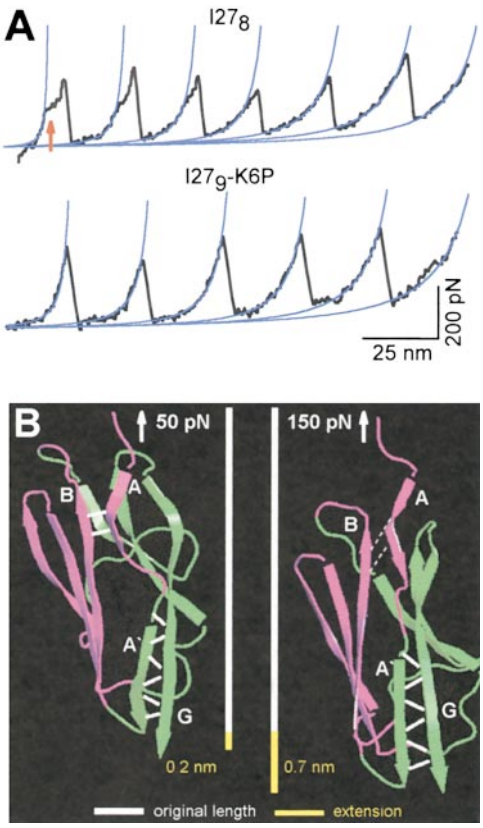

Figure 8. Identification of an Unfolding Intermediate in the Extension of I27 and the Prevention of This Intermediate by a Point Mutation

(A) Force extension curves for $I27_8$ (upper trace) and for $I27_9$–K6P (lower trace) showing fits of domain unfolding according to the worm-like chain model (blue lines). Note that while $I27_8$ shows a prominent hump in the rising phase of the initial force peaks, no such humps are seen for $I27_9$–K6P.

(B) Steered molecular dynamics simulations of the structure of I27 following 1 ns extension with either 50 pN (left) or 150 pN force (right). With 50 pN of force the domain is extended by 0.2 nm, but the hydrogen bonds between strands A and B are maintained. At 150 pN force, however, these bonds are broken and the domain is extended by 0.7 nm. (Adapted from Marszalek et al., 1999a.)

to two domains plus the length of force-bearing amino acids between two domains. This strongly suggests that the unfolding peak preceding the gap is due to breaking the interaction between the A′ strand of one domain and the G strand of the neighboring domain (Figure 9C). Such misfolding events were also observed in refolding experiments using tenascin (Oberhauser et al., 1999), showing that such events can also occur in native proteins and might occur in situ. These events were never observed on the first extension of a protein, however, which suggests that they only occur when multiple consecutive domains are simultaneously unfolded. The rates at which they occur is also low (about 2% in the I27 polyprotein and 4% in tenascin). The remarkable fidelity of domain refolding suggests that rapid reformation of secondary structures favors the interaction between A′ and G strands within the same domain.

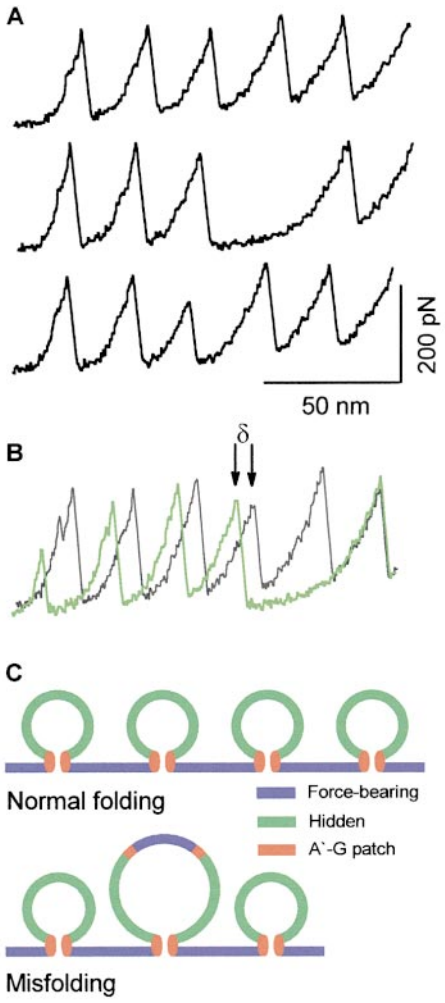

Figure 9. Misfolding of I27 Domains

(A) Consecutive force–extension curves for $I27_8$ at 10 s intervals. Note that the middle force–extension curve appears to have a "missing" force peak.

(B) Superimposition of traces 2 (green) and 3 (black) from (A) (aligned at full extension of both proteins) shows that the interval between unfolding peaks before and after the missing peak is equal to the length of two domains *plus* a distance δ. This distance, ~6 nm, is close to that expected if the misfolded domain were to encompass two adjacent domains plus the stretch of force-bearing amino acids between them.

(C) A schematic model of how such misfolding events are likely to occur. Interaction between the A′ and G patches of adjacent domains would create a misfold that included the hidden amino acids from the 2-folds as well as the stretch of force-bearing amino acids between the 2-folds.

Unfolding of Other Domains

The cytoskeletal protein spectrin is also a modular protein, but in contrast to the proteins described above, contains repeating α-helical domains. Extension of spectrin results in a force extension curve with unfolding peaks of about 25–35 pN (Rief et al., 1999b). The mechanical stability of spectrin α-helices is thus much lower than that of the titin I28 domain (which unfolds at about 260 pN), despite the similarity in their thermodynamic stability (Politou et al., 1995; DeSilva et al., 1997).

Other protein domains that have been used to construct polyproteins to be extended with the AFM include the C2A domain of synaptotagmin, which has a β sandwich structure, and calmodulin, which has an α-helical structure. The C2 domain has been proposed to be the calcium sensor that initiates membrane fusion during neurotransmitter release (Sudhof and Rizo, 1996), while calmodulin is a primary mediator of Ca^{2+}-dependent processes in the cell. Neither of these molecules is exposed to force in situ, and it was of interest to see if such domains had different mechanical properties than domains that are exposed to stress. Extension of the C2A domain was found to yield unfolding peaks of only about 60 pN (Carrion-Vazquez et al., 2000). Simulations of force-induced extension of the C2A domain suggest that these low forces reflect a mechanical topology that is different than Ig and FN-III domains (Lu and Schulten, 1999). The hydrogen bonds in the A′G patch of the I27 domain are perpendicular to the axis of extension, a "shear" configuration, and therefore must be broken simultaneously for the two strands to be moved relative to one another. The hydrogen bonds holding the C2 domain together, in contrast, are parallel to the axis of extension (a "zipper" configuration). This means that the bonds may break sequentially, and that the strands may tend to separate at lower force (Carrion-Vazquez et al., 2000). The shear configuration may reflect a common structural feature of β sandwich domains that resist mechanical unfolding. Unfolding of calmodulin domains appear to occur at forces beneath the sensitivity of the AFM used (Carrion-Vazquez et al., 2000). The force–extension curve therefore did not appear to have force peaks and had a shape similar to that predicted by entropic elasticity. Polymers consisting of multiple repeats of T4 lysozyme, fashioned by engineering cysteines at both ends of the molecule and linking them with disulfide bonds, have also been stretched with the AFM (Yang et al., 2000). Unfolding of these domains, which are a mixture of α-helices and β strands, also occurred at close to 60 pN.

In addition to stretching single protein molecules from purified samples, the AFM has been used to stretch integral membrane proteins from their locations in situ (Muller et al., 1999; Oesterhelt et al., 2000). In elegant experiments combining AFM surface imaging and single molecule force spectroscopy, individual molecules of a bacterial protein (Muller et al., 1999) or of bacteriorhodopsin (Oesterhelt et al., 2000) were identified and extracted from tightly packed arrays on bacterial membranes. These data suggest that AFM might provide a useful method to probe the structures of integral membrane proteins, such as ion channels.

AFM studies with tenascin have shown that proteins found in the ECM of the nervous system can undergo force-induced unfolding and refolding (Oberhauser et al., 1998). Extension of a large splice variant of human tenascin-C, which has 15 FN-III domains, resulted in force–extension curves with a saw-tooth pattern containing up to 12 unfolding peaks occurring at a mean force of 137 pN. These data were used to construct a model of how the presence of multiple domains in a protein might influence its binding to a receptor molecule (Figure 10). If two cells were linked via an interaction

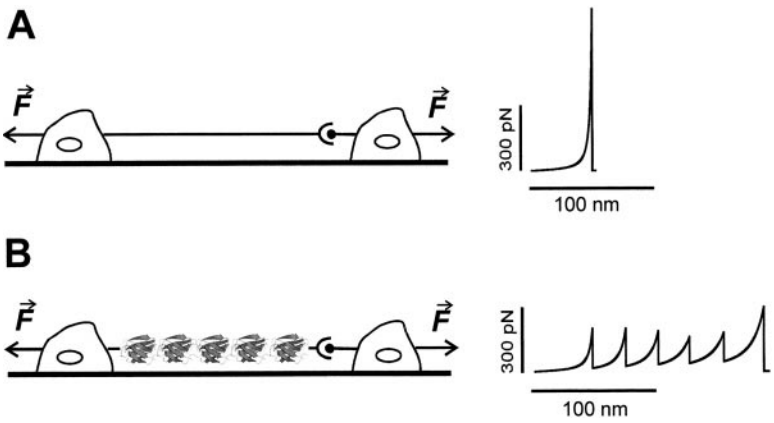

Figure 10. The Mechanical Unfolding of Protein Domains Helps to Maintain Mechanical Bonds between Cells

A cell surface receptor mediating a cell–cell interaction is modeled either as a short protein lacking extensible domains (A) or as a modular protein composed of 5 FN-III domains (B). The traces on the right are Monte Carlo simulations of the mechanical stretching of the bond with the final peaks in both cases representing the rupture of the bond (Oberhauser et al., 1998). In (A), the bond ruptured after a short extension of the protein at a high force (about 800 pN). In (B), the bond was maintained over a much greater extension due to the unfolding of the FN-III domains. The final rupture force was smaller (about 300 pN), but the total work, given by the areas under the two curves, was greater.

between a cell surface receptor on one cell and a short protein lacking extensible domains on the other, force on the bond would cause it to rupture at high force following a short extension. If, however, the protein contained domains that unfold in response to stress, the interaction between the cells would be maintained over a much greater extension. The final rupture of the bond would tend to occur at a lower force, but the total work that was required to unfold the domains and break the bond (given by the area under the curve) would be greater. Although the rate of extension of molecules in vivo may vary widely, this general principle will apply whatever the rates of extension and forces involved, assuming that unfolding occurs at a force lower than does dissociation. This model therefore suggests that mechanosensitive domains may be important for regulating the lifetime of cell–cell and cell–ECM interactions in the nervous system.

Future Perspectives: Addressing Neurobiological Questions with the AFM

Understanding the role of mechanical force in regulating protein function in the nervous system requires an understanding of the conformational changes that proteins undergo in response to axial force. In a modular protein, structural differences in the constituent domains may allow each of them to undergo a unique series of conformational changes as the applied force is increased. The complex summation of these changes may correspond to a pattern of steps in the regulation of protein function. The AFM offers an unprecedented opportunity to characterize force-induced conformational changes in neuronal proteins. How, for example, do the domains in a fibronectin molecule unfold in response to stress? What are the conformational changes responsible for the activation of fibrillogenesis? Force-induced extension of modular proteins with the AFM will therefore contribute to our understanding of how these proteins function during development and morphological plasticity.

The combination of AFM techniques with molecular biology has enabled experiments to test the involvement of single amino acids in determining mechanical stability (Marszalek et al., 1999a; H. Li et al., submitted). The presence in Ig domains of small groups of interacting amino acids that act as barriers to force induced unfolding (Lu et al., 1998; Carrion-Vazquez et al., 1999a, 1999b) suggests that point mutations of these domains might greatly alter their response to force. A mutation of the I27 domain can, for example, prevent the interaction that underlies the formation of an intermediate conformation during force-induced unfolding (Marszalek et al., 1999a). Recent data also shows that replacement of single amino acids involved in the A′–G interaction can influence positively or negatively the force at which the I27 domain unfolds (H. Li et al., submitted).

One might think that a single mutation in a large modular protein is unlikely to greatly influence its overall elasticity. But because inappropriate force-induced unfolding of a single domain would radically alter the length of the entire domain, the mechanical properties could be drastically changed by a single amino acid replacement. Furthermore, where the unfolding of a single domain in a modular protein acts as a signal, such as thought to occur with fibronectin, a point mutation that alters the mechanical stability of a specific domain could have a drastic effect on function. The ability of point mutations to alter mechanical behavior suggests that mutations of proteins whose function is regulated by mechanical force might lead to human disease. One candidate for a protein targeted by "mechanical" mutations is the cell adhesion molecule L1. A large number of mutations in L1 are associated with developmental abnormalities, some of which are thought to cause structural changes in the protein (De Angelis et al., 1999). Single molecule force spectroscopy with the AFM may help to elucidate whether mutations are capable of altering the mechanical properties of proteins such as L1, and whether such changes could be responsible for developmental or other defects in humans.

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