Development of a model to explain the effect of variable membrane compliance on single molecule adhesive bond force

by

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ABSTRACT

The intermolecular bond force existing between adhesive membrane receptors and extracellular matrix (ECM) molecules is believed to regulate key cell functions, such as growth, apoptosis, motility, and mechanotransduction. From a clinical perspective, understanding the mechanics of cell-matrix bonds may be key to unraveling the factors, which promote or inhibit wound healing as well understanding the mechanisms by which cancer cells grow and metastasize. Models describing molecular bond behavior have been studied for close to a century, but accumulation of knowledge in this area has accelerated in recent years due to the advent of methods, such as atomic force microscopy, to study biological forces in the piconewton range. Based on the work of Evans and others, the concept has emerged that molecular pairs do not possess characteristic bond strength, but rather that bond strength varies as a function of the rate at which a disrupting force is applied. On a theoretical basis, this effect may be explained by the complexity of the energy landscape typical of most biological bonds. Thus, bonds subjected to a lower rate of force loading exhibit weaker bond force, owing to the added contribution of thermal activation energy, while bonds subjected to a higher rate of force loading exhibit higher bond force. What is not generally considered is the way in which membrane compliance in cells may contribute to perceived force loading, and in turn, bond force. Our laboratory has previously determined a relationship between membrane compliance and bond force employing high-resolution force spectroscopy, whereby the more deformable domains of the cell membrane are associated with lower bond force and the less deformable domains are associated with higher bond force. The purpose of this thesis is to analyze this distinction in light of Evans' theory of bond energetics, and to develop a model accounting for the contribution of membrane mechanics to single bond force.

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1. Introduction

1.1 Goal

The ability of cells to adhere to and communicate with other cells and matrices is a critical biological function in multi-cellular organisms. Groups of membrane proteins, called cell-adhesion molecules, facilitate adherence and communication between adjacent cells (cell-cell adhesion) or between cells and the extracellular matrix (cell-ECM adhesion). The presence of these adhesions allow cells to self-aggregate into geometrically specific functional groups, which are essential for maintaining the structure and viability of tissues. The intermolecular bond force existing between adhesive membrane receptors and the ECM is believed to be the key factor for regulating multiple cell function, such as growth, apoptosis, motility, and mechanotransduction.

One important clinical application of understanding adhesion is the development of drugs affecting the migration or spread of cancer cells. It is well recognized that cancer cells exhibit alterations in regard to the presence and activity of growth factors and surface receptors. The spread of cancer cells is closely associated with how they are attached. For successful metastasis, cancer cells must be nearby the circulatory system, and exhibit a modified interaction with the extracellular matrix. It is thus reasonable to conclude that the strength of the cell-matrix interaction is an important determinant of cancer dissemination, and that drugs, which modify this interaction may turn out to be important new therapies.

A vast array of intracellular and extracellular chemical signals has been shown to effect adhesive events in living cells. Recent work from Dr. Gilbert's laboratory has demonstrated that in addition to these biochemical determinants of adhesion, the

mechanical properties of the membranes in which the adhesion receptors anchor themselves may also be key regulators of adhesive force. The presence of such regulatory influences may provide a physical mechanism by which a cell's biological function is affected, based on the magnitude and distribution of forces connecting these cells to the surrounding environment.

It is the purpose of this thesis to develop a theory explaining the way in which cell compliance, or deformability, impacts upon the strength of biological bonds. As suggested by Evans, the non-covalent bonds between macromolecules, such as the receptor-matrix bonds in question, are not finite and will ultimately submit if pulled with sufficient time and force. This is based on the fact that most biological bonds possess a complex array of energy barriers, due to the existence of multiple atomic relationships in the binding domain. Since low rates of force loading take better advantage of accumulating thermal energy, it is easier to disrupt bonds under low rates of force loading delivered over a longer period of time as compared to high rates of force loading delivered over a shorter period of time. My purpose will be to develop a modification of this model, which accounts for the effect of membrane compliance on the rate of force loading applied to the bond, and thus, bond force.

1.2 Overview of adhesive biology

Cell adhesion is the process by which a cell forms a crucial connection with another cell or surface. Adhesion thus constitutes the physical basis for cell-cell and cell-matrix interactions. Formation of architecturally sound tissues, cell migration and motility, and tumor metastasis are among the many processes that are mediated by the

highly dynamic process of adhesive bond formation and disruption. Cell-cell adhesion interconnects cells, allowing local communication in neighboring cells. This interaction makes the cells more resilient by connecting the cytoskeletons. Cell-ECM adhesion anchors the cells to a large network of proteins throughout the tissue. The extracellular matrix serves to enhance the mechanical stiffness of cells and tissues, as well as creating a web, or matrix, upon cells may migrate (Figure 1).

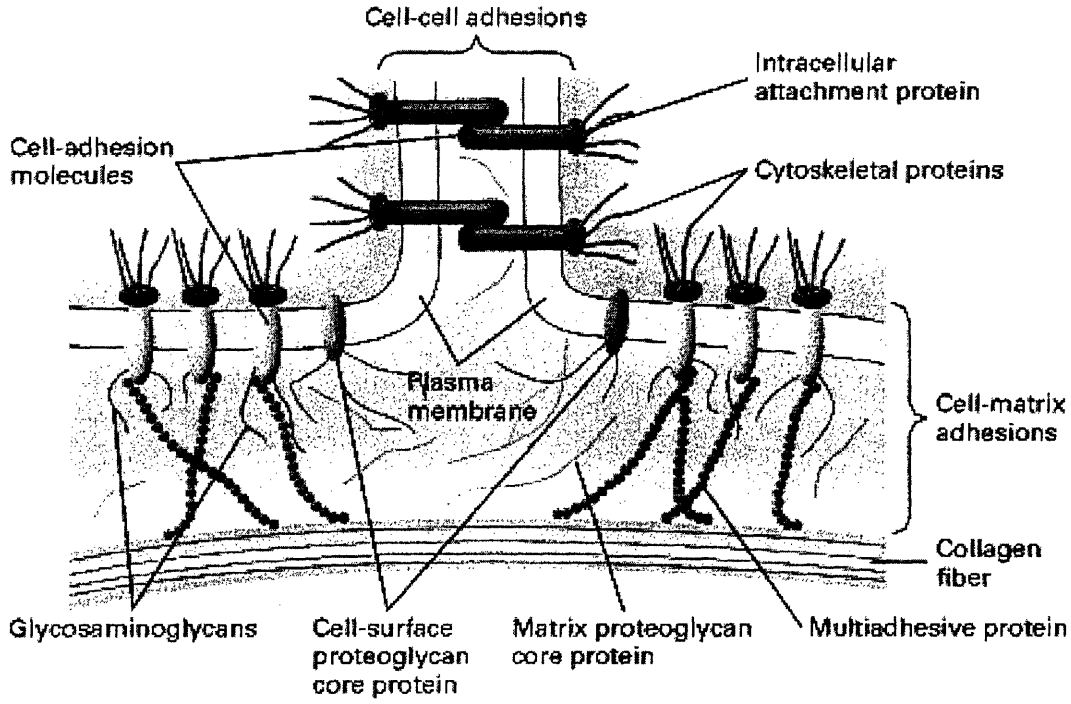


Figure 1 Overview of cells binding to each other and a matrix, including the participating proteins. Taken from Molecular Cell Biology. (Lodish *et al.* 2000)

Cell adhesion molecules (CAMs) describe a family of proteins expressed on the cell surface, which are capable of binding to another cell's receptors, or the ECM. There are five subclasses of CAMs: immunoglobulin (Ig) superfamily, selectins, mucins (including dystroglycan), and integrins (Lodish et al. 2000).

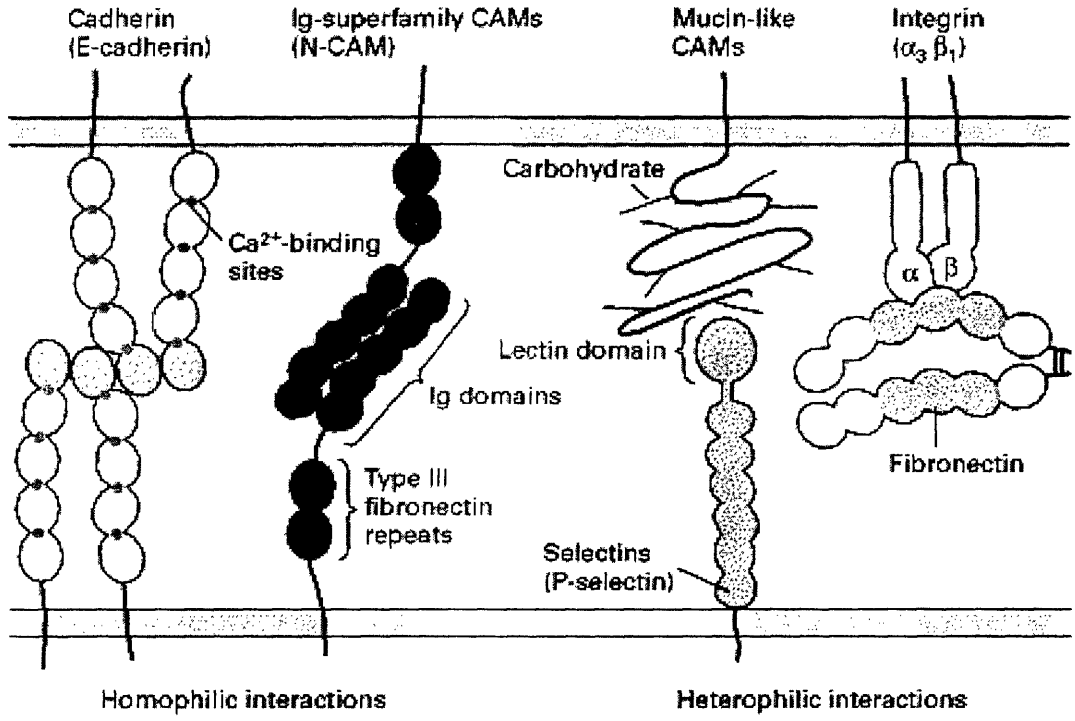


Figure 2 The five major families of cell adhesion molecules. Integrins mediate cell-matrix adhesion, while the other families regulate cell-cell adhesion. (Lodish *et al.* 2000)

It is generally regarded that the affinity of integrin for ECM proteins is relatively small (dissociation constant K_D of between 10^{-6} and 10^{-8} mol/liter) when compared with other cell-surface hormone receptors (K_D of 10^{-9} to 10^{-11} mol/liter). It has thus been postulated that the aggregation of numerous weak binding interactions at focal adhesions is necessary to anchor cells tightly to associated matrices. From a physiological perspective, this interaction is not static, but involves a dynamic process of bond formation and breakage, as well as the construction through polymerization of complex matrix assemblies.

1.3 Adhesion receptors

While numerous adhesion molecules have been described, the two most important receptors are believed to be integrin and dystroglycan. Similarly, the extracellular matrix is composed of numerous molecules, including collagen, fibronectin, and laminin. We will focus on laminin in this discussion since it constitutes a common ECM contact for both integrin and dystroglycan.

1.3.1 Integrins

Characteristically, integrins are heterodimeric proteins, containing linked α and β chains. These molecules exhibit large biological diversity, attributable to the large number of possible combinations of α and β subunits. Due to complexity added from the molecule's transmembrane domains, it has only recently been successfully studied by x-ray crystallography, thus allowing novel hypotheses regarding integrin structure and biological function (Garcia-Alverez *et al.* 2003, Adair *et al.* 2002). Crystallography has shown that both the alpha and beta subunits have an extra-cellular as well as an intercellular component, although the intercellular domains are much shorter than the extracellular domains. The extracellular beta subunit has been shown to consist of approximately 640 residues, including four cysteine-rich repeats that are EGF-like and have been identified as binding sites for many antibodies (Zang *et al.* 2001). The α subunit has been shown to contain approximately 920 residues, containing a β propeller domain, which has been proposed to be essential for bond formation (Zang *et al.* 2001). There are at least 22 different combinations of alpha and beta chains observed in

mammals (Lodish *et al.* 2000), most of which bind to specific proteins in the ECM or in other cells of pathogens.

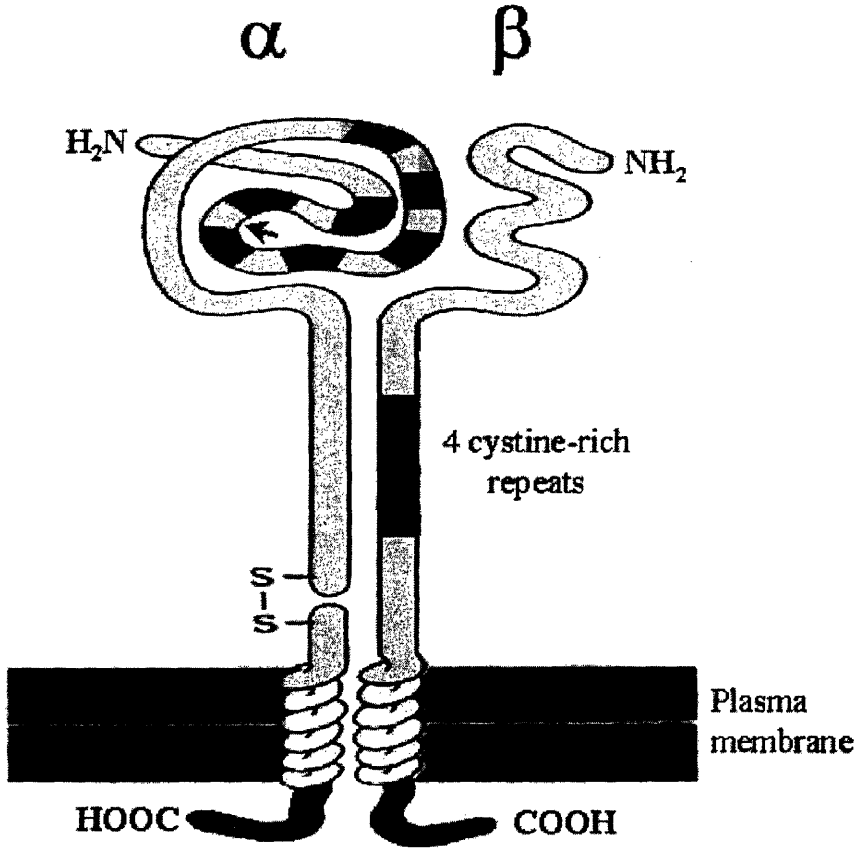


Figure 3 Integrin Structure: shows α and β subunits. Integrins are transmembrane receptors (taken from the web. URL:www.bendermedsystems.com/108.html).

The attachment of the cytoskeleton to the ECM occurs through two integrin dependent junctions: focal adhesions and hemidesmosomes (Lodish *et al.* 2000). The latter works by interconnecting lamina filaments from the ECM with fibronectin in the cytoskeleton. Focal adhesions form an actin rich "anchor" inside the cell between the substrate and the intracellular portion of integrin receptors. The integrin molecules cluster together to assemble and to bind to a focal adhesion (Lodish *et al.* 2000, Li *et al.*

1999). Integrins' greatest value is inherent in their capability for social behavior. Individually, these receptor-ligand bonds are weak, but in clusters they are collectively strong. The advantage to this system is that the individual bonds can be broken quickly as the cell's needs change such as in the motility of a platelet. The cell can also regulate the strength of the bond by controlling the number of receptors exhibited on locations of the membrane.

1.3.2 Dystroglycan

Dystroglycan (DG) belongs to the mucin-like family of CAMs because of a common genomic section. It takes its name from being a dystrophin-associated glycoprotein. Truly a complex, this glycoprotein is formed from a single polypeptide chain that is cleaved once, creating α-dystrogylcan (α-DG) and β-dystrogylcan (β-DG) of lengths 43-kd and 156-kD respectively (Figure 4(a)) (Ibraghimov-Beskrovnaya 1992, Winder 2001). The α-dystroglycan binds the extracellular matrix, specifically laminin, which will be discussed in the next section. The transmembrane β-dystroglycan binds its heterodimer α-dystroglycan extracellularly and the cytoskeleton through dystrophin or utrophin (Winder 2001), as shown in Figure 4(b). In this manner, α- and β-DG together span the sarcolemma and physically link the extracellular matrix and the cytoskeleton. Such a connection involving DG may be important for stabilizing the sarcolemma during contraction-induced stress, and thus play an important role in the generation of adhesive force associated with DG-ECM bonds. Reduction of dystroglycan complex expression in the sarcolemma thus interferes with the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix, and results in reduced contractile strength.

Dystrogylcan was first sequenced and cloned in 1992 (Ibraghimov-Beskrovnaya 1992). Two years prior, the protein had been shown to be either disrupted or missing in cases of Duchene muscular dystrophy (Ervasti et al. 1990). While at first thought only to be found in skeletal muscle tissue, it has since been found in neuronal and other type muscle tissues (Winder 2001). Dystroglycan is now recognized as an important anchorage producing cell-matrix receptor in diverse cells.

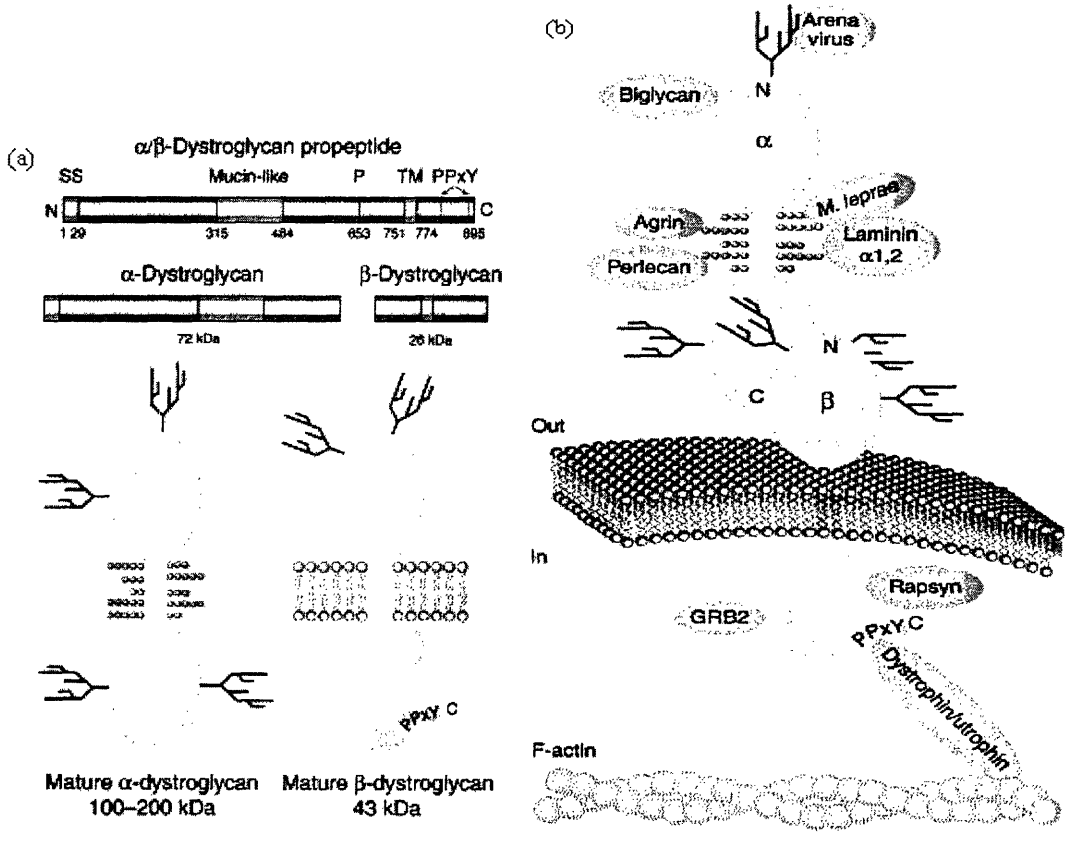


Figure 4 (a) The heterodimers of dystroglycan and the polypeptide chain portions. (b) The binding of α and β dystrogylan, shown here in blue. Note the extracellular a heterodimer binds laminin (taken from Winder 2001).

1.3.3 Extracellular matrix (laminin)

Laminin is present in almost every animal tissue, and constitutes a key molecular component of the extracellular matrix. The laminins can self-assemble, bind to other matrix macromolecules, and have unique and shared cell interactions mediated by integrins, dystroglycan, and other receptors on the cell membrane. Basement membranes are specialized cell-associated extracellular matrices, whose molecular architectures are created through binding interactions of unique monomers. These matrices have support and cell regulatory functions. The monomeric units of the basement membrane are, in themselves, large multi-domain glycoproteins and proteoglycans, each with several functions. Laminin and type IV collagen, for example, form polymeric networks as well as selectively bind and activate a number of different cellular receptors. α-Dystroglycan has been demonstrated to be a major laminin binding protein, in association with β-dystroglycan (Matsumura et al. 1997, Ferletta et al. 2003). Laminin is a protein found amidst collagen in the extracellular matrix. A huge molecule, laminin is shaped to stretch out arm-like sections which have high affinity binding for a wide variety of cell surface receptors and matrix components. Laminin is composed of three heterotrimers, dubbed α, β, and γ, which make up its unique structure shown in Figure 5 (Colognato *et al.* 2000).

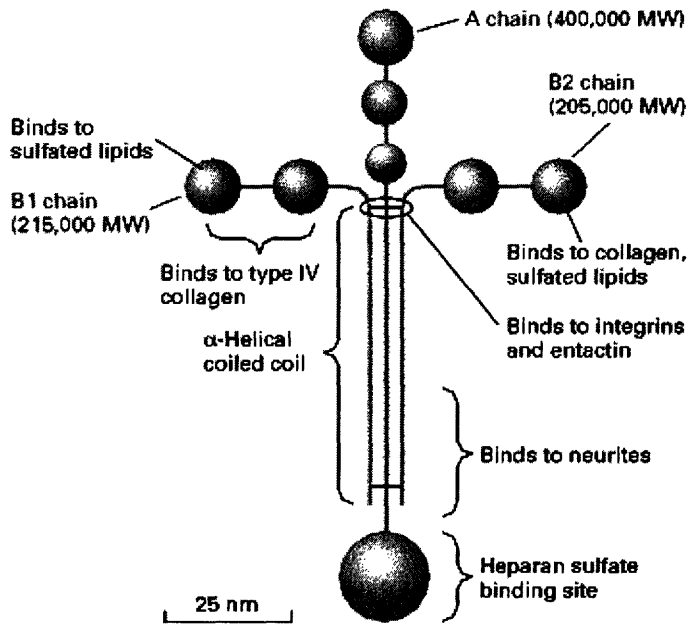


Figure 5 A laminin molecule, showing its structure (taken from Lodish *et al.* 2000)

1.4 Determination of single molecule adhesive force

Over the past decade since the advent of AFM it had become possible to measure single bond forces. High-resolution force spectroscopy (as a variant of atomic force microscopy) provides the ability to interrogate the pico-newton range of forces, which are characteristic of single biological bonds. Based on the fact that the application of a force to an intermolecular bond will reduce the activation energy of such a bond, and therefore accelerate bond dissociation, the force associated with ligand-receptor interactions can be determined.

2. Model and Notation

Physical theories of molecular bonding date back to Einstein's classic theory of Brownian dynamics (1905). Kramers theorized bound state escapement in liquids (1940) and Bell proposed the first specific models for cell adhesion in 1978. Recent work by Evans and others have really looked into the energetics of bond formation, establishing a fundamental relationship between the mechanics and thermochemistry of bonds.

A molecular bond derives much of its complexity from its array of non-covalent interactions. Two large molecules which bind present to each other binding sites in which numerous interactions take place: hydrogen bonds, ionic bonds, hydrophobic and van der Waals interactions (Figure 6). Although individually non-covalent bonds are weak interactions (between 1-5kcal/mol) together such bonds can be quite strong. They determine the shape of molecules and of complexes of molecules, the specificity of which sites will be complimentary, and most importantly, control how strongly, if at all, binding will be.

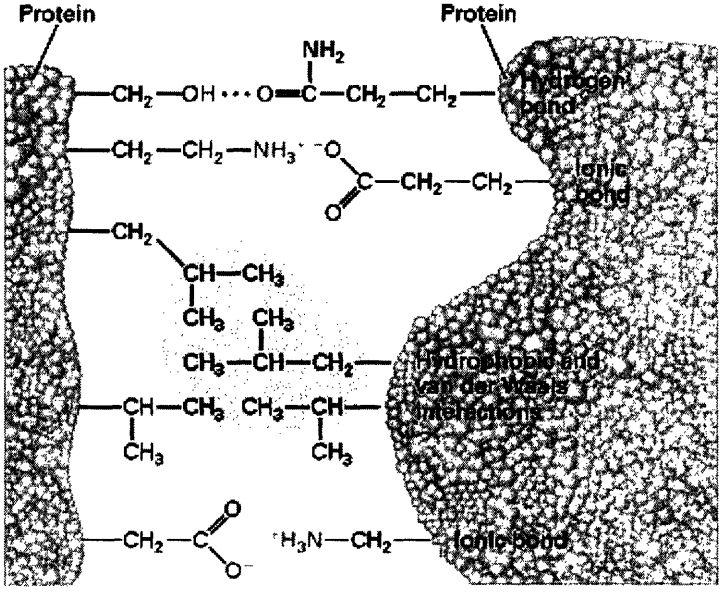


Figure 6 Noncovalent interactions in binding. These determine shape, specificity, and strength of molecular bonding (Lodish *et al.* 2000)

2.1 Chemical Equilibrium

It should be recognized that the behavior of single molecular bond seen in experimentation can be deceiving. In truth there are hundreds of bonds in equilibrium, constantly forming and disassociating between favorable energy states. The rate and location of specific reactions occur will determine what takes place in the cell. Take a simple reaction where capital letters represent molecules:

$$A + 2B + 3C + ... = Z + 2X + 3Y + ... \quad (1)$$

The rates are just:

$$\text{Rate}_{forward} = k_f [\text{A}][\text{B}]^2[\text{C}]^3... \quad (2)$$

$$\text{Rate}_{reverse} = k_r [\text{Z}][\text{X}]^2[\text{Y}]^3... \quad (3)$$

The constants are related by:

$$K_{eq} = \frac{[\text{Z}][\text{X}]^2[\text{Y}]^3...}{[\text{A}][\text{B}]^2[\text{C}]^3...} \quad (4)$$

$$K_{eq} = \frac{k_f}{k_r} \quad (5)$$

These rates and constants describe how reactions, including binding, take place. For a binding reaction, K_{eq} is known as the dissociation constant K_D.

2.2 Bond Energetics

Reactions utilize kinetic and potential energy to form and dissociate bonds. Consider the landscape below (Figure 7):

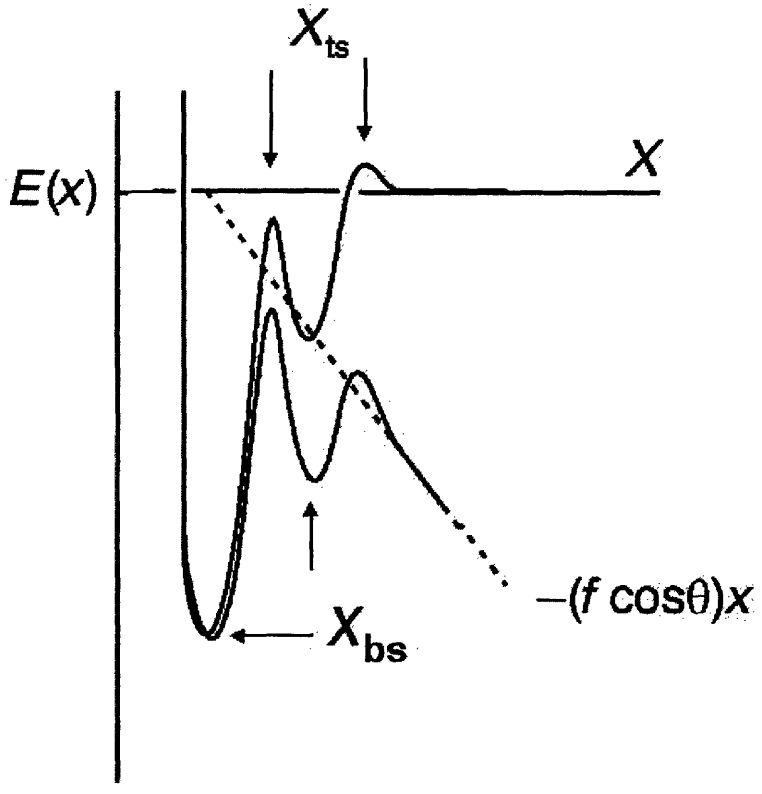


Figure 7 Energy Landscape. Shows the bound states and transition states as a function of separation. External force shifts the barriers down and inward (taken from Merkel *et al.* 1999).

This general landscape contains two bound states and two transition states. When a bond is in its deeply bound state, its energy level is at the minimum of the first well. If *E(x)* is the threshold barrier to bond dissociation, E_b, then the bond is broken anywhere to the right of the last barrier. This barrier, the activation energy, results from the energy required to move the molecules close enough and in the right configuration to react.

An explanation for this how this landscape comes about can be within the non-covalent interactions. Each receptor-ligand bond contains many interactions, all with their own energy landscapes. Some add and some repel, in the same or different energy levels. There must be one maximally favorable bound energy level where the most interactions sum to result in the strongest bond.

Evans has theorized that the bonds are comprised by a complex energy landscape, consisting of one deeply bound barrier followed by a series of small energy barriers. Although in equilibrium, a bond with no force applied to it will apparently last forever, contrary to reaction theory. The small energy barriers presented in Evans' work significantly extend bond lifetime because they are large enough to prevent the thermal energy fluctuations responsible for equilibrium from dissociating the bond. The deeply bound energy barrier provides the strength of the bond when force is applied. This configuration implies if even a small amount of force is applied for a long period of time, it can break by using thermal assistance to cross the small barriers. The molecule pair is bound throughout the landscape; the unbound state exists on the right side of the last barrier.

2.3 Recent Theory

Each energy landscape has some number of wells and barriers in a one dimensional figure. Consider the following, (Figure 8) with j energy barriers and i wells.

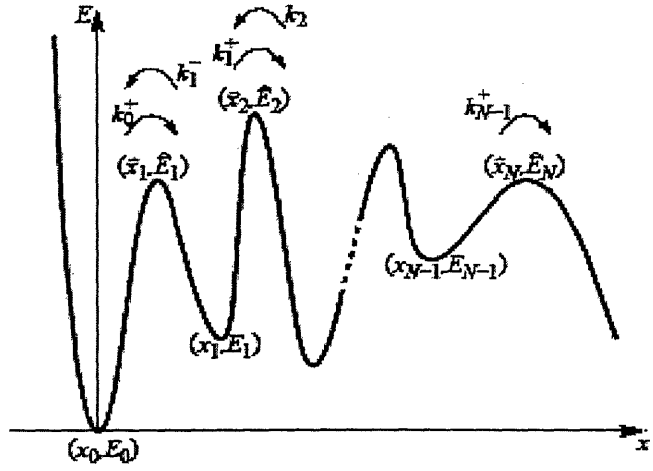


Figure 8 A more general landscape, with N wells and barriers, *i* and *j* symbolize the energy wells and barriers respectively (taken from Derenyi *et al.* 2004).

The terminology is straightforward (Derenyi *et al.* 2004). The relation between *i* and *j* is simply that $i + 1 = j$. An * or ^ indicates a barrier variable. Reaction rates are given by k^+ and k^-, which are described by the following equations from Kramers:

$$k_i^- = \omega_0 \alpha_i \alpha_i^* e^{-(E_i^* - E_i)/k_b T} \tag{6}$$

$$k_i^+ = \omega_0 \alpha_i \alpha_{i+1}^* e^{-(E_{i+1}^* - E_i)/k_b T} \tag{7}$$

The term ω_0 is the frequency of escape attempts. Both α_i and α_j^* describe the geometry of the wells and barriers, respectively. The energy differences (E_i^* - E_i) must exceed the thermal energy (k_bT) in order to cross a barrier.

Force is related to this chemistry through the energy required to break the bond.

$$E_b(f) = E_b - f x_b \tag{8}$$

$$f_\beta = k_b T / x_b \tag{9}$$

Bell proposed that the rate of barrier passage must be exponential, and Evans used

equation (9) to demonstrate that under any pulling force the bond will disassociate much more rapidly. The force that may occur spontaneous will be tiny because k_bT and x_b can be extremely small at room temperature, 4.1 pN nm and ~.1nm, respectively (Flyvbjerg *et al.* 2002).

Another interesting theory is that although intuitive, there is no reason to believe that the deepest well must be first. An intermediate well may be the well wherein the bond is strongest. There are several energy landscapes that correctly model a force loading rate that are possible, which makes identifying the proper one impossible in some cases (Derenyi *et al.* 2004).

3. High resolution force spectroscopy

Single laminin-membrane bonds were previously in Dr. Gilbert's laboratory by high-resolution force spectroscopy (HRFS) on the surface of wild-type myoblasts (C2C12). In brief, experiments were carried out with the Molecular Force Probe assembly (Asylum Instruments, CA) an atomic force microscope modified to provide pico-newton scale force measurements between two opposing surfaces. The measurement of intermolecular forces using high-resolution force spectroscopy involves a micro-fabricated cantilever, possessing a small tip with a contact area of several nanometers. A piezo-electric crystal is used to raise or lower the cantilever and to maintain a constant bending of the cantilever, whereas a laser beam is reflected from the top of the cantilever towards a photo-detector that detects any bending of the cantilever. Internal optical lever sensitivity (IOLS) values were calibrated using force-curve data from fresh mica sheets mounted on a slide. In order to translate cantilever deflection (x) into force (F), a value

for the cantilever spring constant (k_c) was determined ($F = k_c x$) for each cantilever tip, employing thermal vibration analysis. Laser intensity (SUM) values were on the order of 8 V for each tip used in these experiments. Internal optical lever sensitivity (IOLS) values were calibrated using force-curve data from freshly cleaved mica surfaces mounted on a slide. The cantilever spring constant was 0.10 N/m and retraction distance was maximally 2 microns.

4. Laminin-dystroglycan force domains as a function of compliance

Single molecule adhesive bond force was determined by high-resolution force spectroscopy in calcium containing PBS, measuring the abrupt force changes, which occurred with cantilever retraction from the membrane surface following attachment. Force-distance curves were obtained by tracking the displacement of the cantilever tip upon retraction from the surface of the cell. Incubation of C2C12 cells with specific DG antibody resulted in a near complete (> 90%) blockade of the attachment of laminin-functionized tips to C2C12 cells. Incubation of C2C12 cells with antibody to B1 integrin, on the other hand, had little effect on the frequency or characteristics of attachment or the distribution of forces. In addition, where LG4-5, the DG specific binding domain of laminin, was used to functionalize the cantilever tips, the bond forces recorded were similar to those obtained with whole laminin-functionalized cantilever tips. The combined data thus provided evidence that the single bond forces recorded were due to laminin-DG bonds.

Quantitative evaluation of the set of individual laminin-membrane receptor bonds was obtained by histogram analysis. The distribution of bond breakages was shown as a

function of force and as a function of distance from the point of initial cantilever contact to the point of bond breakage, i.e. deformation distance (Figures 9, 10). The relationship between force and the distance at which bond disruption occurred was further demonstrated in a scatter plot in Figure xx. These data revealed two mechanical domains on the membrane in approximately equal proportions: 1) bonds which detached at >500 nm membrane deformation, which were smaller in magnitude (38.2 +/- 9.1 pN) and highly homogenous, and 2) bonds which detached at <500 nm membrane deformation, which were significantly greater in magnitude (92.2 +/- 37.9 pN, $p < 0.05$) and considerably more heterogeneous. There was no difference between the bond populations in regard to the number of bonds disrupted prior to separation of the cantilever from the membrane surface.

Figure 9 Membrane deformation > 500 μm

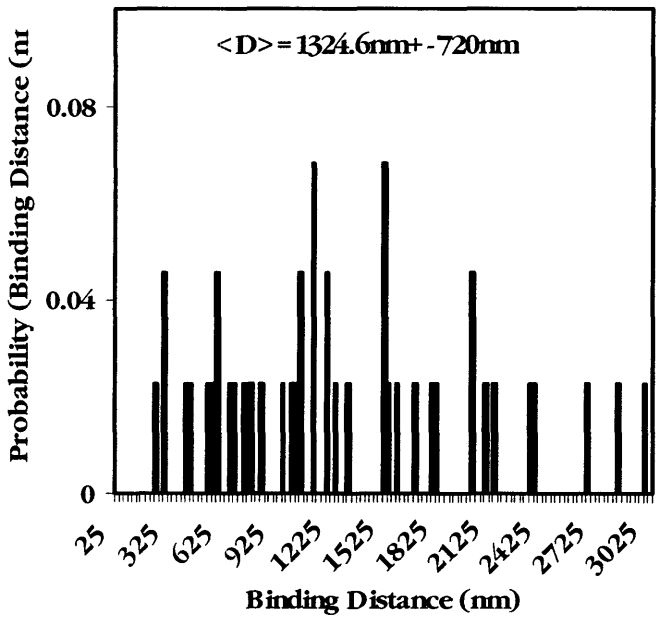


Figure 10 Membrane deformation < 500 μm

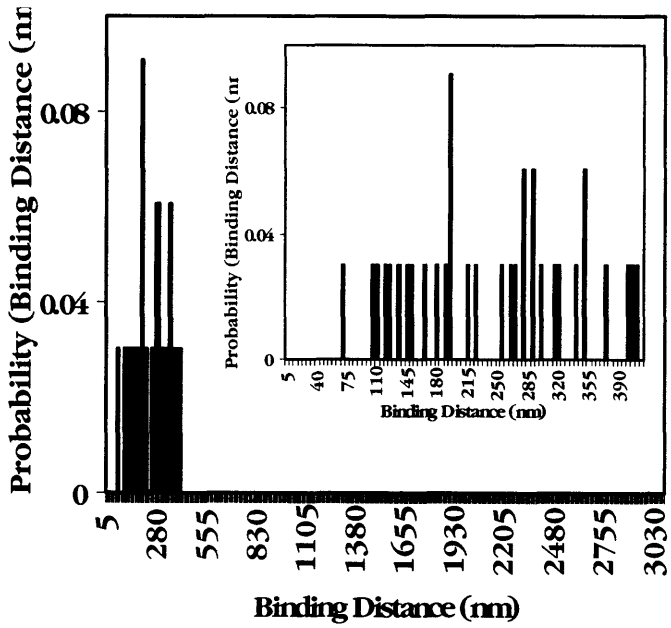


Figure 11 Membrane deformation > 500 μm

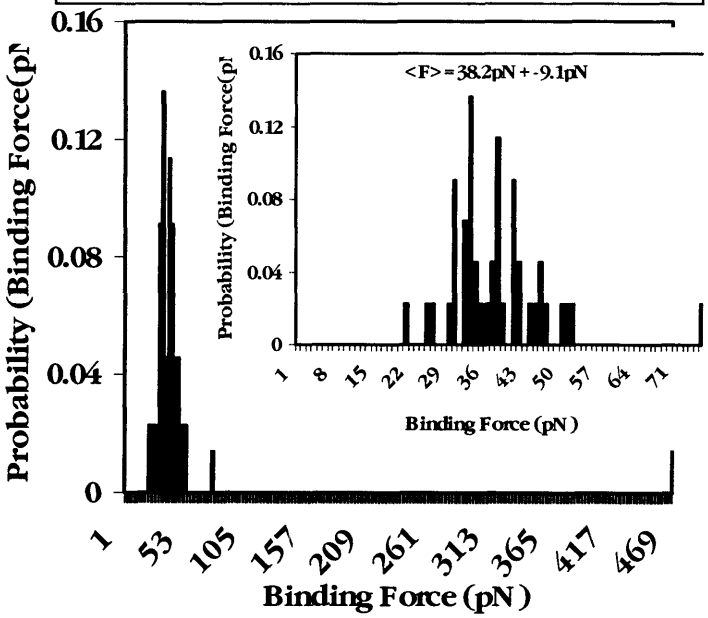
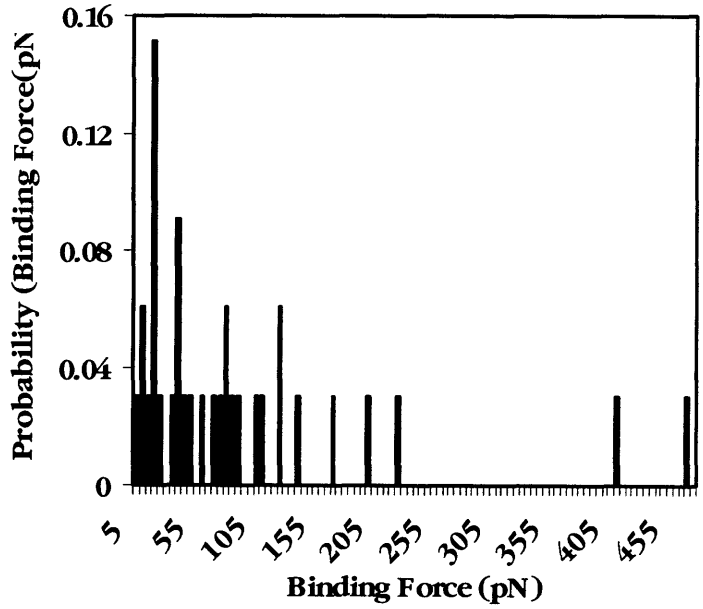


Figure 12 Membrane deformation < 500 μm



5. Development of model accounting for the tethering effect of the cytoskeleton on plasma membrane compliance and bond force

Many previous modelers have neglected the membrane in their models. The logic behind this is that the cell's membrane and substrate effects can be subtracted from the experimental recordings to give a value for local properties. Further, this allows for the measurement of specific receptor sites (Rudd *et al.* 2002). This is a sound theory as long as the membrane and cytoskeleton deform with consistency. The cytoskeleton is typically treated as incompressible because its stiffness so far exceeds that of the other contributors. The assumption that the cytoskeleton's effect can dependably be subtracted out is substantial. Differing sections of cells vary in stiffness, which has been shown and linked to cell migration (Nagayama *et al.* 2001). The membrane also does not deform consistently. Besides its varying elastic deformability, the membrane demonstrates a binary behavior of stiff and slack conditions. Therefore, both the membrane and the cytoskeleton need to be included in the model.

The membrane deforms in a nonlinear elastic manner and can be expressed as follows (McElfresh *et al.* 2002):

$$\varepsilon / m = S \cdot J + B \cdot H^2 \tag{10}$$

Where ε the strain measured, m is the mass, S and B expressing the stretching and bending modulus and are constants. J describes the local change in area, and H, the mean curvature (Steigmann 1999).

To incorporate the slack and stiff conditions of membrane attachment, the key terms need to be rearranged. The membrane and cytoskeleton will both have bending and stretching behavior in each condition, so therefore there will be four terms necessary.

$$\varepsilon / m = S_{slack} \cdot J + B_{slack} \cdot H^2 + \tau(S_{stiff} \cdot J^* + B_{stiff} \cdot H^{*2}) \quad (11)$$

This equation relates back to force simply. Strain, ε, is proportional to stress through Young's Modulus. Force is the stress multiplied the area. This makes the assumption that the slack condition is the just the absence of being membrane tethering. The weighting term, τ , should be considered binary, and variable for zero and one only. When the membrane is tethered, $\tau = 1$; if not tethered then only the slack condition prevails. To clarify Eq. (11) is a more detailed description of Eq. (10), not solely the addition on two terms.

The compliance of the membrane changes how the force is loaded onto the bond. In the stiff, tethered condition, the bond is relatively loaded much more quickly than when there is compliant membrane.

6. Conclusion

The relationship between membrane compliance and bond force can be qualitatively discussed considering tethering. When a membrane is anchored to the cytoskeleton the result is a relatively higher loading rate on the bond. The result is a distribution of low rupture force levels when the membrane is slack and a more precise distribution of high rupture force levels when it is not.

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