SMD simulations, a moving harmonic potential (spring) is used to induce motion along a reaction coordinate. The free end of the spring is moved at constant velocity, while the protein atoms attached to the other end of the spring are subject to the steering force. The force applied is determined by the extension of the spring and can be monitored throughout the entire simulation.


The work reviewed here involved many researchers from our own and other groups. We apologize to all researchers whose pioneering work could not be reviewed because of space limitation. We thank M. Gao, B. Isralewitz, S. Izrailev, H. Lu, J. C. Gumbart, and members of the Theoretical and Computational Biophysics Group for their contributions and helpful discussions; our long-time collaborator, V. Vogel, for guidance and inspirations; collaborators D. P. Corey, D. Craig, A. Krammer, O. Mayans, and M. Wilmanns; and J. Fernandez and P. Marszalek for a wonderful experimental-theoretical collaboration. The molecular images in this paper were created with the molecular graphics program VMD (69) and Tachyon. This work was supported by funds of the NIH (grant no. P41 RR05969 and grant no. 1 R01 GM073655) and the Humboldt Foundation (K.S.). The authors also acknowledge computer time provided by the NSF through the Large Resource Allocations grant MCA95028.

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REVIEW

Forces and Bond Dynamics in Cell Adhesion
Evan A. Evans1,2* and David A. Calderwood3

Adhesion of a biological cell to another cell or the extracellular matrix involves complex couplings between cell biochemistry, structural mechanics, and surface bonding. The interactions are dynamic and act through association and dissociation of bonds between very large molecules at rates that change considerably under stress. Combining molecular cell biology with single-molecule force spectroscopy provides a powerful tool for exploring the complexity of cell adhesion, that is, how cell signaling processes strengthen adhesion bonds and how forces applied to cell-surface bonds act on intracellular sites to catalyze chemical processes or switch molecular interactions on and off. Probing adhesion receptors on strategically engineered cells with force during functional stimulation can reveal key nodes of communication between the mechanical and chemical circuitry of a cell.

T he physical role of a cell adhesion bond is to hold a cell to other cells or to tissue substrata while supporting the forces involved in cell function. Complicating this task, a single adhesion bond effectively resists force only for time periods less than that needed for its spontaneous dissociation under thermal activation. Thus, the diversity in the mechanochemistry of adhesion bonds reflects how mechanical force applied to a bond between a pair of interacting molecules alters activation energy barriers along kinetic pathways, or switches pathways, that lead to dissociation. Viewed ideally as illustrated by Fig. 1, applying adhesion stress through the local material structure to a bond is conceptually like pulling on the chemical interaction with a mechanical spring that mimics the compliance properties of structures attached to the binding site. Stretching this equivalent spring produces a force that lowers the chemical activation barrier to increase the frequency of bond dissociation while, at the same time, the spring potential defines an “energy well” that captures the dissociated states and regulates the likelihood of rebinding. Focusing our discussion on adhensive interactions in soft tissues and organs of...
eukaryotic cell systems, the important insights derived from the simple view in Fig. 1 are that bond survival effectively decreases exponentially with the level of pulling force and that deformations of soft structures even under small forces suppress rebranding after dissociation.

With a precipitous reduction in lifetime under stress and little likelihood of rebinding, bonds in cell adhesion are therefore being continually created, loaded over some period of time, then failing. Even for cells in tissues seemingly under static stress, forces build up transiently on the individual bonds that connect cells, albeit very slowly (maybe at miniscule rates of only ~1 pN/s) until the bonds break and shift their loads to other bonds. The balance of stress is achieved through recruitment of new bonds driven by cytoskeletal movements, resulting in a “bubbly” dynamic process of bond loading, failure, and formation. By comparison, at the other extreme, the initial attachment of an immune system cell in the vasculature can apply force to bonds at an incredibly fast rate (for instance, ~10^4 pN/s), which is then followed by quick release of the cell or rapid activation.

Fig. 1. Conceptual view of force propagation to a bond and its impact on the chemical energy landscape governing bond kinetics. (A) Pulling on structural connections to a molecular bond creates a mechanical “springlike” potential (dashed blue curve) that alters the chemical energy of interaction or “landscape” (solid and dashed red curves) along the reaction coordinate defined by the pulling direction (37). The slope of the spring potential at the origin of interaction is the pulling force f, the product of the effective spring constant k_x of the structural linkages with the increase in their separation x_{separation} under pulling. Of greatest impact on bond survival, the spring potential reduces the height of the activation energy barrier governing the off-rate kinetics located at x_b by ~k_BT/f. Brought to our attention years ago by Bell (38), the change in Arrhenius factor predicts a large exponential-like reduction in bond survival time, t_{off}(f) = t_{off0} \exp(-f/f_0), relative to its apparent unstressed lifetime t_{off0} (39). The response scale for this accelerated dissociation is the level of force, f_0 = k_BT/\kappa_0, that drops the barrier by one unit of thermal energy k_BT (40).

The reduction in bond lifetime with increase in force is illustrated in (B) by the behavior expected in a force-clamp test at different forces. When the anchored molecules unbind, the dissociated states are confined near the displaced minimum of the spring potential, as indicated in (C), from which strong thermal excitations can cause them to rebind. However, there is little likelihood of rebinding when the depth of the spring potential in (C) exceeds the binding energy E_b for forces \( f > \kappa_0 \Delta x \). Consequently, molecules anchored by soft structures (small \( \kappa_0 \) ) rebind very infrequently even under low stress, which suggests why bond recruitment in cell adhesion usually involves bringing the constituents together by large-scale cytoskeletal movements as when forming the immunological synapse in T lymphocyte adhesion (41).
connections and behave as transient connectors when binding just their outer-tip domains, which may be important for the dynamics of recognition and patterning of cells in development. On the other hand, deep-trans bonding of all domains produces strong attachments even under very slow loading, as demonstrated by the force responses of attachments between full-length cadherins (Fig. 3B). With little sensitivity to stress rate, such “persistent” connections may enable formation of durable structures like desmosomes in lateral junctions of epithelial cells, although opposing models based on dense networks of tip interactions have also been proposed (7). Last, integrins mediate perhaps the most diverse range of adhesive interactions in eukaryote biology, exhibiting widely different levels of attachment strength and lifetime. Representing one extreme of their dynamical response, many integrin interactions are long-lived and provide the persistent strength needed, for example, to hold together tissues, to transmit force during muscle contraction, and to arrest circulating immune cells on activated endothelium and enable their migration (8). A prominent example of persistent strength and the insensitivity to stress rate is demonstrated by the force responses of attachments to the integrin α1β1 (Fig. 3B). Yet, representative of the opposite extreme, other integrin interactions are short-lived and behave as transient connectors that require fast loading for strength, as when integrins at the leading edge of a spreading cell form new attachments to an extracellular matrix (9) or when integrins initiate capture of lymphocytes in the systemic circulation (10), mimicking the response of a selectin as demonstrated by force responses of attachments to the integrin α4β1 (Fig. 3A). Because of the diversity in integrin mechanical response and the important role of their cytoskeletal connections in adhesion, we will center the remainder of our discussion around integrin bonds.

**Integrin Bonds: The Archetype of Multifunctional Adhesive Design**

Integrins are composed of noncovalently-associated α and β subunits. Each subunit is a type I trans-

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**Fig. 2.** Dynamic strength of a bond: the “nanorheology” of a molecular interaction. The important dynamical corollary to the Bell exponential model (Fig. 1) for the off rate of bonds under stress is that the strength and lifetime of a bond become interrelated properties governed by the stress rate (42), \( r_t = \frac{\Delta f}{kt} \), and thereby the speed \( v_t \) at which the molecular linking structures are separated, that is, \( r_t = \frac{v_t}{K_{\text{diss}}}. \) When loaded by an increasing force, the failure rate of an idealized bond grows exponentially with time, predicting that the bond will break “most often” at a force \( f^* \), increasing by 1 unit of the thermal-activation force \( f_0 \) for each e-fold exp (1) \( \sim 2.72 \) times increase in the loading rate. The increase in strength and decrease in survival expected for bonds at different force rates (called force ramps) are sketched in (A). Derived from tests at several force rates, the most frequent rupture forces for the idealized bond follow a straight line when plotted against logarithms of the force rates as sketched in (B), that is, \( f^* = f_0 \log (t_{\text{off}}f_0). \) Key to the dynamics, the kinetic scale for force rate, \( r_b^* = f_0/t_{\text{off}}f_0 \), defines the loading speed above which the bond is driven “far from equilibrium,” dissociating faster than its apparent spontaneous off rate \( 1/t_{\text{off}}f_0 \), and thus resists force. The most frequent “lifetime” \( t^* \) of the bond is precisely its strength divided by the loading rate, \( t^* = f^*/r_b. \) So, while bond strength grows sluggishly with increased loading speed, bond survival falls extremely rapidly, as illustrated in (C).

**Fig. 3.** Transient and persistent cell adhesion bonds. The distinction between these two types of adhesion bonds reflects major differences in their stress rate requirements \( r_b^* = f_0/t_{\text{off}}f_0 \) for onset of strength and their scales \( f_0 \) for amplification of strength under increasing stress rate (Fig. 2). Consistent with the labels, the examples of transient connectors in (A) require fast loading for strength, whereas the examples of persistent connectors in (B) are strong even under slow loading. Also intriguing is that the transient connectors show much larger amplification of strength at high-loading speeds than persistent connectors, which suggests a fundamental feature of the way in which weak biomolecular bonds are chemically designed to achieve strength (43). Taken from in vitro tests of single recombinant receptor and ligand interactions when immobilized on a force probe and microsphere target (44), the responses for transient connectors in (A) are demonstrated by the dynamic strengths of P-selectin (PS) bonds to a reactive N-terminal segment of mucin PS glycoprotein ligand-1 (PSGL-1) (45) and by the dynamic strengths of integrin α4β1 bonds to a two-domain construct of VCAM-1 (46). Although similar to the β1-integrin interaction at high force rates, the strength of the PS interaction is switched on at a fast loading rate. Called a “catch bond” (4), this unusual response represents a mechaenochemical switch triggered by force rate to turn off a fast dissociation pathway and lock in a slow dissociation pathway that resists force (45). Also taken from in vitro tests, the responses for “persistent connectors” in (B) are demonstrated by the dynamic strengths of homophilic full-length cadherin bonds (47, 48) and by the dynamic strengths of integrin αβ2 bonds to ICAM-1 (46). Feedback from chemical pathways inside cells is known to reinforce cell adhesion mediated by integrin bonds. We found a significant parallel upward shift [†, blue-dashed line in (B)] in dynamic strengths of β2-integrin bonds when testing ICAM-1 bonds to the β2 integrin (LFA-1) in situ at the surface of cytokine-stimulated white blood cells; this suggests a range of affinity states for the β2 integrin and demonstrates the “inside-out” feedback at the single-molecule level. The units for force and force rate are pN and pN s, respectively.
membrane glycoprotein with a relatively large multidomain extracellular projection and a single membrane-spanning helix, usually ending with a short (20 to 70 amino acid), largely unstructured cytoplasmic tail (8). Humans produce 18 α and 8 β subunits that combine to form at least 24 different heterodimers, each of which binds to a specific overlapping repertoire of extracellular matrix ligands such as fibronectin, collagen, laminin, or fibrinogen and to cell surface counterreceptors like the Ig-superfamily proteins intercellular adhesion molecule–1 (ICAM-1) or vascular cell adhesion molecule–1 (VCAM-1) (8). Many of the advances in our understanding of the mechanisms by which integrins bind ligand have come from x-ray crystallography of the extracellular domains of α1β1, and α6β1, as well as structures of complexes between ligands and isolated α subunit ligand-binding A domains [reviewed in (11, 12)]. These studies have revealed the architecture of integrin extracellular domains, explained the well-established requirement for divalent cations in integrin-ligand binding, and demonstrated that both the α and β subunits participate in binding ligands containing an Arg-Gly-Asp peptide or a related tripeptide motif. Very important in their function, conformational changes in the integrin extracellular domains play a major role in regulating the affinity of integrins for their extracellular ligands through a process termed integrin activation (8, 11, 12).

Although ligand binding is mediated by the large extracellular domains, the integrin cytoplasmic tails play a key role in cellular control of their adhesive interactions and the subsequent dynamic cellular responses such as cell spreading or migration. Interactions of the short cytoplasmic tails, and of the β tails in particular, with intracellular cytoskeletal and signaling proteins figure prominently in the regulation of integrin activation (13). Furthermore, after the binding of an extracellular ligand, complex multiprotein assemblies of cytoskeletal, scaffolding, and signaling proteins are recruited to the integrin cytoplasmic face, where they both link integrins to the actin cytoskeleton and convey signals into the cell (14, 15). Hence, by binding both extracellular and intracellular ligands, integrins provide a transmembrane conduit for the bidirectional transmission of mechanical force and biochemical signals across the plasma membrane to regulate cell adhesion, migration, proliferation, and death.

**Integrin Anchoring in Cell Adhesion**

The mechanical properties of adhesive attachments to cells are most often attributed to the ligand/receptor interaction. However, formation, strength, and survival of a cell adhesive attachment also depend on how molecular connections below the membrane surface—those anchoring the receptor to the cell cytostructure—respond to force. Integrins generally function in specialized complexes involving assemblies of many adhesion molecules and cytoskeletal signaling adaptors (15). These integrin clusters come in various forms, for example, focal adhesions, focal complexes, fibrillar adhesions, or podosomes, which are defined according to their size, shape, subcellular localization, molecular constituents, and organization (15). While some clusters are widespread, others, for example, the immunological synapse or costamers, show strict cell-type specificity. The differences in size and composition of adhesion sites presumably reflect the link to the cytoskeleton and integrin signaling. Nonetheless, despite their various specialized roles in mediating transient or stable adhesion, reorganizing the extracellular matrix, and activating specific signaling pathways, these adhesions share a number of common features. They are sites at which integrins connect intracellular actomyosin-generated cytoskeletal contractility to extracellular ligands and where external forces can be transmitted to the cytoskeleton, for example, sites where they can initiate biochemical signals. Like the exterior ligand/receptor interaction, intracellular molecular bonds are also time-dependent connections whose formation and persistence change considerably with application of force. As illustrated in Fig. 4, the abrupt reduction in interfacial stiffness and the onset of fluid-like tether flow often observed when pulling on an integrin bond suggest that the pulling force can disrupt the molecular-scale complex anchoring the integrin tails to the cytoskeleton.

The many molecules present in adhesion sites imply many, potentially parallel, mechanisms for linking an integrin to the cytoskeleton (14, 15). Nonetheless, several proteins have been identified as prime candidates for direct integrin-ligand linkages, including talin, filamin, α-actinin, and tensin [which, along with integrin-associated adaptor and signaling molecules such as vinculin, paxillin, focal adhesion kinase, and Src-family kinases, activate and/or respond to kinase,
phosphatase, and small guanosine triphosphatase signaling cascades (16, 17)). Each of these large actin-binding proteins also contains a binding site for integrin β subunit cytoplasmic tails (15, 16, 18, 19). Of these, talin, an antiparallel homodimer composed of 250-kD subunits, has received the most attention. Acting as a “hub” in the linkage between integrin β tails and the cytoskeleton, talin interacts with a constellation of focal adhesion proteins—including the integrin β-chain tail, vinculin, focal adhesion kinase, phosphatidylinositol phosphate kinase type 1 γ, and F-actin (20)—and plays important roles in activation of integrin receptors (21), in formation of the initial linkage between ligand-occupied receptors and the cytoskeleton (9), and in the subsequent reinforcement of the linkage (22). As discussed below, the use of cell lines deficient in these linker proteins (9, 19) or expressing mutations that selectively disrupt an integrin-linker, a linker-linker, or a linker-cytoskeletal interaction (9, 19, 21, 23) holds considerable promise for elucidating the roles of specific molecular interactions during cellular response to force.

The Future: Mapping the Communication Between Mechanical and Chemical Circuitry of a Cell

All cells sense and respond to applied forces in a cell-type–specific manner to regulate a broad range of processes from cell migration to stem cell differentiation, tissue formation, and tumorigenesis (24, 25). Although a variety of systems are employed to sense force and convert it into biochemical signals, adhesion molecules (and integrins in particular) are known to play an important role in this mechanosensory process and in how cells respond to the applied stress (26, 27). Although other adhesion receptors, such as the selectins and cadherins, are also regulated through cytoskeletal interactions, a defining feature of integrins is that integrin/ligand and integrin/cytosplasmic connections transmit and receive feedback (through conformational changes) to enhance or reduce their strengths of attachment (12, 28, 29). Along with the prominent clustering of receptors [increasing what is referred to as “avidity” (30)], feedback from inside the cell also acts directly on the integrin adhesion bond, greatly amplifying its mechanical strength as demonstrated by the force responses of attachments to the β integrin LFA-1 on the surface of a cytokine-stimulated white blood cell (Fig. 3B). Because many of the enzymes and signaling molecules involved in this feedback are closely associated with the receptor-cytoskeletal linkage, the effect of pulling on, or even detaching, a receptor from the cytoskeleton as described in Fig. 4 is likely to alter interactions among these proteins, which suggests a physical mechanism for communication between the mechanical (stress-bearing) circuitry and chemical circuitry of a cell. Pulling forces can catalyze cellular processes in many ways (26, 29), including (i) conformational transitions (from limited to full denaturation and unfolding) that expose otherwise cryptic sites to promote new protein–protein interactions or that expose sites with specific posttranslational modification, (ii) reorganization or segregation of specific molecules in an adhesive complex, and (iii) even liberation of a constituent so that it can interact with more distant complexes.

Because of the numerous molecules present in adhesion complexes, the experimental challenge is to sort through the many (possibly parallel) intracellular signaling pathways that likely emanate from integrin linkages to the cytoskeleton. Current investigations with engineered knock-out or overexpressing cells are making substantial progress in identifying the proteins important for integrin-mediated responses to force (22, 31), which are enhanced by innovative in vitro assays suggesting ways that the integrin-associated proteins can act as force sensors (32). Moreover, potentially aiding in this quest, probing individual cell adhesion complexes with ultrasensitive force techniques (Fig. 4) provides an unexpected opportunity to assay the kinetics of molecular connections (Fig. 5) hidden beneath the cell membrane (maybe even deep in the cell, if linked to a long structural filament). Taking advantage of structural and functional assays that identify key mutations selectively targeting integrin-cytoskeletal linkages and signaling pathways, the exciting prospect is to use cell-surface force spectroscopy and engineered cell lines as a material science tool to explore and characterize key nodes in the “mechanical circuitry” that connect receptor tails to the cytoskeleton and to examine how forces applied to these nodes communicate physical cues from outside the cell to catalyze or trigger specific steps in cell signaling and regulation inside the cell. Even bolder, the next step should be to integrate precision techniques like single-molecule force spectroscopy with high-resolution optical techniques like single-molecule fluorescence (33) or novel methods that image the real-time dynamics of coupling between integrins, actin, and other components of adhesion and signaling at the cellular level (34, 35). Together, such integrated approaches can provide access to the molecular machinery by which adhesion molecules transmit force and biochemical signals into and out of the cell during cell migration, tissue remodeling, and differentiation.

References and Notes
Advanced computational methods like the "steered molecular dynamics" described in the companion review by Sotomayor and Schulten (36) provide valuable tools for investigating how variations in chemical structure affect activation energy barriers and pathways governing bond strength.

In typical laboratory tests of single adhesion bonds, constructs of the ligand and receptor molecules are chemically immobilized on solid surfaces at very low surface densities, for example, a ligand to the face of an ultrasmall force probe and its receptor to a solid target held by a feedback-stabilized piezo translator. The target is then repeatedly moved to/from contact to the probe face, during which time the deflection of the probe is tracked at high precision and multiplied by its "spring" constant to report the force history. Bond events are identified by the cycles showing periods of probe stretch ending in precipitous recoil, as sketched in Fig. 2A.


"near-sighted" (6). In many applications, especially in the life sciences, collecting the light far away from the sample is mandatory.

In the 1990s, the first concrete and feasible concepts emerged showing that in fluorescence microscopy the diffraction barrier can be broken even with propagating light and regular lenses—that is, in the far-field (7, 8). A hallmark of these concepts was use of the molecular states of the fluorescent marker not just for signal generation, but also for overcoming the limits set by diffraction (9). They radically departed from the far-field superresolution strategies prevalent at the time, such as confocal (10, 11) and multiphoton microscopy, because they implied that a resolution far below λ, in fact diffraction-unlimited resolution, is possible without eliminating diffraction per se. Meanwhile, other powerful approaches (12–14) have emerged, bolstering far-field fluorescence nanoscopy as an emerging field of science. Here, we will review this field with emphasis on the breaking of the diffraction barrier. I will refrain from overly discussing technical implementations, unless I deem them inherent to the concept; particularly, imaging speed, sensitivity, and cost-efficiency are constantly improving as new technology becomes available. Rather, I will show that all fluorescence nanoscopy concepts realized so far have used a bright and a dark state of the fluorescent marker to record sub-λ features sequentially in time. I will classify these concepts according to the states used and show that they differ on whether the sequential recording of the marker occurs molecule by molecule or in molecular ensembles.

Pushing the Diffraction Barrier

Since the mid-20th century, several concepts aimed at pushing the diffraction limits by reducing the focal spot size. Confocal fluorescence microscopy is one of them. Using pointlike illumination and detection, its effective spot is described by |

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38. Because of thermal activation, the appropriate unit for the force constant of an adhesive bond is pN/nm, the force per unit length gained in the direction of force when the bond breaks. Thus, for some time, the only fluorescence nanoscopy concepts realized so far have used a bright and a dark state of the fluorescent marker to record sub-λ features sequentially in time. I will classify these concepts according to the states used and show that they differ on whether the sequential recording of the marker occurs molecule by molecule or in molecular ensembles.

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