Protein–protein unbinding induced by force: single-molecule studies
John W Weisel*, Henry Shuman† and Rustem I Litvinov*

Experiments in which two specifically interacting protein molecules are dissociated by external force have yielded new insights into mechanisms involved in cell adhesion, leukocyte rolling, regulation of integrin activity, antigen–antibody interactions and other protein-mediated reactions contingent upon molecular recognition. Another important aspect of force-induced protein–protein unbinding studies is the new information being gleaned about the thermodynamics and kinetics of bond rupture.

Addresses
*Department of Cell & Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6058, USA
e-mail: weisel@cellbio.med.upenn.edu
†Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6085, USA

Abbreviations
AFM atomic force microscopy
BFP biomembrane force probe
ConA concanavalin A
ICAM-1 intercellular adhesion molecule-1
LFA-1 leukocyte function-associated antigen-1
LT laser tweezers
PSGL-1 P-selectin glycoprotein ligand-1
SGCT sodium glucose co-transporter
vWF von Willebrand factor
WASP Wiscott–Aldrich syndrome protein

Introduction
Protein–protein unbinding studies are a part of the major field of investigation termed receptor–ligand interactions or molecular recognition. During the past decade, important new information about receptor–ligand interactions at the single-molecule level has complemented conclusions based on conventional methods, which measure the properties of large ensembles of molecules or observe the behavior of whole cells. The main advantages of studying individual receptor–ligand pairs are: minimized cooperative and/or clustering effects; the possibility of probing conformational transitions of individual molecules, such as activation/inactivation; revealing the structural and functional heterogeneity of seemingly identical molecules; knowing the number of molecules involved in reactions; quantifying directly the magnitudes and working distances of forces in ligand–receptor interactions to elucidate the relationships between molecular structure and the thermodynamics of bond dissociation. Watching individual events and distributions rather than observing average values may reveal rare but physiologically important functional fluctuations [1*,2].

The study of protein–protein interactions has been dominated by a static viewpoint, such that the emphasis is on molecules in solution under equilibrium conditions, whereas their real-life biological interactions generally occur on surfaces under nonequilibrium conditions; the latter is the focus of the papers summarized in this review. The study of the mechanics of protein interactions is necessary to understand the many cellular functions and properties, such as rolling, motility, adhesion, deformability and so on, that are mediated by specific receptor and ligand molecules, and controlled by mechanical forces produced by either external (shear flow) or internal (cytoskeletal rearrangement, motor proteins) sources. In addition, the study of protein–protein unbinding by an applied force has turned out to be a precise and unique tool for analyzing protein structure and function, as well as mechanisms of their regulatory changes [3].

Theory
The results of mechanical rupture (pulling) experiments have been analyzed using two distinct theoretical methods [4]. The first, called the energy landscape model, is based on Kramers’ rate theory and leads to general predictions about the distribution of rupture forces [5*]. In this model, it is assumed that an applied load changes the energy of the transition state as well as the equilibrium of the bound and unbound states, thereby altering the kinetics of association and dissociation [6]. An important conclusion from the model is that the distribution of rupture forces depends on the loading rate (i.e. the rate that the applied force is increased) and that this dependence provides information not obtained from equilibrium experiments [7*]. Pulling experiments in which the loading rate is changed over many orders of magnitude are called dynamic force spectroscopy. One important open question is whether these force spectra can be used to uniquely identify the dissociation mechanism [8*]. A recent advance in nonequilibrium thermodynamics [9*], based on an insight from Jarzynski [10], further suggests that the complete free energy diagram in the direction of the applied load can be inferred by correctly averaging repeated traces of the force versus
time (trajectories) acquired at constant pulling velocity. Although the method has not yet been applied to protein–protein interactions, it was verified for the mechanical unfolding of RNA [11**].

The second method uses molecular dynamics simulations to investigate the disruption of specific pairs of molecules in atomic detail [12]. Ideally, this type of model provides new insights into the mechanisms of protein–ligand interactions [13*,14,15]. However, meaningful simulations require complete X-ray crystal structures of the proteins and huge computational resources. Even with the most powerful current computers, the simulations only last ~10 ns, roughly three orders of magnitude less than typical pulling experiments. One of Jarzynski’s predictions is that his equality holds even very far from equilibrium or at very high loading rates. Recent computational and theoretical advances allow us to be more confident that dynamic force spectroscopy may provide important information on how proteins bind and unbind.

**Principles and methodology**

Force-induced receptor–ligand unbinding studies are always performed at an interface. Molecular binding and rupture result from controlled touching and separation of two surfaces, one bearing receptors and another coated with ligand. The different techniques used to perform these kinds of experiments differ from each other mainly by the surfaces to which the proteins are bound, as well as by the methods of generating, sensing and measuring mechanical forces. The techniques used during the past several years for studies of single-protein molecular mechanics are: atomic force microscopy (AFM), also termed scanning force microscopy (SFM) [16]; laser tweezers (LT) [17]; biomembrane force probe (BFP) with pipette suction [18]; and hydrodynamic methods [19*]. The forces generated and sensed during single-molecule experiments are in the range of several piconewtons (1 pN = 10⁻¹² N) to about one nanonewton (1 nN = 10⁻⁹ N), although no single instrument can adequately cover the entire range of forces. The magnitudes of these forces correspond to the range of noncovalent interactions, but they are insufficient to break a covalent bond [20].

The interacting receptor and ligand molecules both have to be firmly bound to their underlying surfaces to ensure that the measured forces reflect protein–protein unbinding rather than the detachment of molecules from the surface [21]. When experiments are performed with purified proteins, they should be coupled to surfaces either covalently or at least via biotin–(strept)avidin interactions, which are stronger than any known noncovalent protein–protein adhesion. Covalent linkages are made either directly using bifunctional reagents [22**,23] or via spacer molecules [24*,25], which is seemingly preferable because of the higher flexibility and better spatial orientation of surface-bound proteins. However, stretching of polymeric linkers changes the unbinding profile by giving rise to delayed nonlinear receptor–ligand rupture force signals [24*].

As in ensemble experiments, discrimination between specific and nonspecific interactions is a major concern, requiring carefully chosen control experiments. Two important control experiments are replacing one of the reacting partners with an inert protein (e.g. albumin) or applying specific inhibitors that abolish the interaction in a dose-dependent manner. Irrespective of the origin of nonspecific surface-to-surface attraction, several studies have shown that nonspecific protein–protein adhesion strength may reach the value of several tens of piconewtons, partly overlapping the forces produced by specific receptor–ligand interactions (Figure 1) [26,27]. These weaker nonspecific protein–protein interactions may have physiological significance in cell adhesion in cooperation with the specific interactions [22**].

An important question that is difficult to answer unequivocally is whether one or multiple pairs of molecules interact during pulling experiments. Two pieces of evidence, both based on statistics, are commonly used to determine whether single-molecule interactions are detected. The first requires that only a limited fraction of touching cycles between the surfaces result in binding [19*,27]. For example, when only ten IgG molecules were bound per bead and the protein A surface density was also very low, the probability of formation of more than one IgG–protein A pair at a particular scanning velocity was calculated to be at most 9% [27]. Secondly, histograms of the distribution of rupture forces can show a series of quantized peaks that are multiples of a single value [23,28]. Although there are other important indirect criteria supporting individual molecule interactions [29*], reasonable conclusions must be substantiated by combinations of data obtained independently.

Protein–protein unbinding studies performed on live cells with exposed receptor molecules have several limitations and uncertainties. One of them is the chance of uprooting receptor molecules from the membrane, which depends on pulling time and force as well as on membrane tension [30,31]. A related problem is mechanical compliance and membrane tethering, combined with interactions of receptors with the cytoskeleton [30,32]. Receptor clustering also can potentially affect quantification of single-molecule receptor–ligand interactions, because it was shown that chemical cross-linking of receptors led to increased ligand binding strength as a result of receptor cooperativity [33,34*]. Similarly, self-association of selectins led to shear resistance and changed unbinding parameters for P-selectin and its ligand, P-selectin glycoprotein ligand-1 (PSGL-1) [35]. Nevertheless, properly performed receptor–ligand binding studies with proteins naturally residing...
on metabolically active cells are physiologically more relevant and most promising.

Some recent results of enforced single-molecule protein–protein unbinding, along with a partial description of the experimental conditions, are summarized in Table 1. It can be seen that the majority of peak rupture forces measured over a wide range of loading rates are in the interval from several tens up to \( \sim 150 \) pN. The forces measured at similar loading rates appear to be independent of underlying surfaces, coating chemistry and measuring techniques. However, there is an obvious difference in the rupture forces for different protein–protein pairs. These results imply that the different rupture forces reflect multiple mechanisms of receptor–ligand dissociation, rather than diversity of experimental conditions.

### Applications

The strength of cell attachment to substrata and/or to another cell is a good example of how the mechanical characteristics of single molecules determine cell function. That is why integrins, selectins and cadherins, which mediate cellular interactions, were among the first proteins studied at the single-molecule level using force-induced unbinding methodology. Cell adhesion and aggregation are strongly influenced by the mechanical plasticity of cells, by the direction and rate of applied external forces, and by the mutual accessibility of the receptor and ligand molecules [4,36,37].

The regulation of binding-site exposure was directly demonstrated in a study in which the interactions of fibrinogen with the integrin \( \alpha_{IIb}\beta_3 \) were studied on live platelets with different degrees of activation [22**]. Using
Table 1

<table>
<thead>
<tr>
<th>Receptor and receptor-bearing surface</th>
<th>Ligand and ligand-bearing surface</th>
<th>Measuring technique</th>
<th>Reported loading or shear rate/stress</th>
<th>Rupture forces (pN)</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Adhesion molecules</strong></td>
<td></td>
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</tr>
<tr>
<td>GPIIb-IIIa (αIIbβ3) Covalently bound to latex beads</td>
<td>Fibrinogen Free in solution</td>
<td>Hydrodynamic flow 0.6–2.9 N/m² shear stress</td>
<td>70–150 (15.6%) 150–230 (16%) 230–310 (17%)</td>
<td>[19]*</td>
<td></td>
</tr>
<tr>
<td>αIIbβ3 On native adherent platelets</td>
<td>GSSSGRQGDSPA Covalently bound to tips via glutaraldehyde</td>
<td>AFM 12 nN/s</td>
<td>~93</td>
<td>[28]</td>
<td></td>
</tr>
<tr>
<td>αIIbβ3 On resting or activated native adherent platelets Covalently bound to modified silica beads via glutaraldehyde</td>
<td>Fibrinogen Covalently bound to latex beads via carbodiimide</td>
<td>LT 20 nN/s</td>
<td>60–150, with a peak at 80–100</td>
<td>[22]**</td>
<td></td>
</tr>
<tr>
<td>αVβ3</td>
<td>GRGDSP</td>
<td>AFM 30 nN/s¹</td>
<td>42 ± 4</td>
<td>[50]</td>
<td></td>
</tr>
<tr>
<td>αVβ1</td>
<td>GRGDSP</td>
<td>AFM 30 nN/s¹</td>
<td>32 ± 2</td>
<td>[50]</td>
<td></td>
</tr>
<tr>
<td>αVβ3</td>
<td>Osteopontin</td>
<td>LT 20 nN/s</td>
<td>50 ± 2</td>
<td>[50]</td>
<td></td>
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<tr>
<td>αVβ3</td>
<td>Echistatin</td>
<td>AFM 30 nN/s</td>
<td>97 ± 15</td>
<td>[50]</td>
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<tr>
<td>All on adherent osteoclasts partly fixed with paraformaldehyde</td>
<td>All adsorbed on tips via noncovalently bound PEG</td>
<td>LT 20 nN/s</td>
<td>60–150, with a peak at 80–100</td>
<td>[22]**</td>
<td></td>
</tr>
<tr>
<td>GP Ib-IX (αIIIX)</td>
<td>von Willebrand factor (vWF)</td>
<td>LT Not reported</td>
<td>6.5 ± 0.8 8.8 ± 0.3 11.4 ± 2.1 11.5</td>
<td>[51]</td>
<td></td>
</tr>
<tr>
<td>αIIIX</td>
<td>Ultralarge vWF</td>
<td>LT 20 nN/s</td>
<td>60–150, with a peak at 80–100</td>
<td>[22]**</td>
<td></td>
</tr>
<tr>
<td>αIIIX</td>
<td>A1 domain of vWF</td>
<td>LT 20 nN/s</td>
<td>60–150, with a peak at 80–100</td>
<td>[22]**</td>
<td></td>
</tr>
<tr>
<td>P-selectin Bound to silanized glass cover slips via biotin–avidin</td>
<td>PSGL-1 Bound to silanized tips via biotin–avidin</td>
<td>AFM 168 nN/s¹</td>
<td>159 ± 30</td>
<td>[52]</td>
<td></td>
</tr>
<tr>
<td>P-selectin Bound to cantilever as Fc-chimera via anti-Fc-Ab</td>
<td>PSGL-1 On intact neutrophils</td>
<td>AFM 250 nN/s¹</td>
<td>175</td>
<td>[44]*</td>
<td></td>
</tr>
<tr>
<td>PSGL-1 and other selectins’ ligands On neutrophils</td>
<td>P-, E-, L-selectins or peripheral node addressin Adsorbed on latex beads</td>
<td>Hydrodynamic flow 0.5–5.0 dyn/cm² shear stress</td>
<td>37–250</td>
<td>[42]</td>
<td></td>
</tr>
<tr>
<td>L-selectin Covalently bound to glass spheres via PEG</td>
<td>PSGL-1 Covalently bound to glass spheres via PEG</td>
<td>AFM 168 nN/s¹</td>
<td>159 ± 30</td>
<td>[52]</td>
<td></td>
</tr>
<tr>
<td>LFA-1 (αLβ1) On murine T-cell hybridoma cells (3A9) coupled to cantilever via ConA</td>
<td>ICAM-1 Expressed by fibroblasts FT 16.11 or adsorbed in soluble form</td>
<td>AFM 0.02–50 nN/s</td>
<td>20–320</td>
<td>[40]**</td>
<td></td>
</tr>
<tr>
<td>β1-integrins On native mouse 3T3 fibroblasts</td>
<td>Fibronectin Adsorbed on glass</td>
<td>LT 0.005–0.1 nN/s</td>
<td>13–28</td>
<td>[53]</td>
<td></td>
</tr>
<tr>
<td>αLβ1 On K562 cells activated with mAb TS2/16 and attached to tips via ConA</td>
<td>Fibronectin fragment 7-10 Adsorbed on tissue culture dishes</td>
<td>AFM 1.8–2.0 nN/s</td>
<td>69 ± 15 93 ± 1.5</td>
<td>[41]**</td>
<td></td>
</tr>
<tr>
<td>VE-cadherin Covalently bound to SiOH plates via PEG</td>
<td>VE-cadherin</td>
<td>AFM 6 nN/s¹</td>
<td>33</td>
<td>[24]*</td>
<td></td>
</tr>
<tr>
<td>Glycoprotein csA On Dictyostelium discoideum AX20214 strain cells attached to tips via lectin</td>
<td>csA On Dictyostelium discoideum AX20214 strain cells spread on polystyrene</td>
<td>AFM 12 nN/s¹</td>
<td>23 ± 8</td>
<td>[54]</td>
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</table>
Table 1 Continued

<table>
<thead>
<tr>
<th>Receptor and receptor-bearing surface</th>
<th>Ligand and ligand-bearing surface</th>
<th>Measuring technique</th>
<th>Reported loading or shear rate/stress</th>
<th>Rupture forces (pN)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucin Covalently bound to tips via 11-C spacer molecules</td>
<td>Mucin Mucin gel deposited on mica</td>
<td>AFM</td>
<td>1–132 nN/s(^{1})</td>
<td>100–4000</td>
<td>[55]</td>
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<td><strong>Immunoglobulins</strong></td>
<td></td>
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<tr>
<td>Anti-ferritin mouse mAb Covalently bound to silicon wafers via glutaraldehyde</td>
<td>Ferritin Covalently bound to tips via glutaraldehyde</td>
<td>AFM</td>
<td>Not reported</td>
<td>49 ± 10</td>
<td>[56]</td>
</tr>
<tr>
<td>Polyclonal anti-HSA Ab Covalently bound to tips via PEG</td>
<td>Human serum albumin (HSA) Covalently bound to mica via PEG</td>
<td>AFM</td>
<td>54 nN/s(^{1})</td>
<td>240 ± 48</td>
<td>[57]</td>
</tr>
<tr>
<td>Anti ICAM-1 mouse mAb F10.2 Covalently linked to tips via PEG</td>
<td>ICAM-1 Adsorbed on mica in a soluble truncated form (sICAM-1)</td>
<td>AFM</td>
<td>54–191 nN/s(^{1})</td>
<td>100 ± 50</td>
<td>[26]</td>
</tr>
<tr>
<td>Ab against γ-glutamyl-transpeptidase (γ-GT) Ab PAN3 against a segment of SGCT Both covalently bound to tips via PEG</td>
<td>γ-GT SGCT Both on brush border membrane vesicles adsorbed on gold surfaces</td>
<td>AFM</td>
<td>6 nN/s(^{1})</td>
<td>131 ± 44</td>
<td>[45]</td>
</tr>
<tr>
<td>Polyclonal antibodies #8 against ryanodine receptor 1 (RYR 1) Covalently linked to tips via PEG</td>
<td>RYR 1 Adsorbed on mica</td>
<td>AFM</td>
<td>2 nN/s</td>
<td>42 ± 6</td>
<td></td>
</tr>
<tr>
<td>Mouse antibody against β subunit of human chorionic gonadotropin (hCG) Covalently bound to silicon wafers via glutaraldehyde</td>
<td>hCG Staphylococcal protein A</td>
<td>BFP</td>
<td>50–60 pN/s</td>
<td>19 ± 1</td>
<td>[25]</td>
</tr>
<tr>
<td>Anti-β3 mAb (F11) Adsorbed on tips via noncovalently bound PEG</td>
<td>αvβ3 On adherent osteoclasts partly fixed with paraformaldehyde</td>
<td>AFM</td>
<td>30–1500 nN/s(^{1})</td>
<td>127 ± 16</td>
<td>[50]</td>
</tr>
<tr>
<td>Rabbit anti-mouse IgG Covalently linked to synthetic resin beads via dextran</td>
<td>Staphylococcal protein A Covalently co-linked along with lectin to synthetic resin beads via dextran</td>
<td>BFP</td>
<td>0.4–5.3 nN/s</td>
<td>25–44</td>
<td>[27]</td>
</tr>
<tr>
<td>Rabbit, mouse, bovine or goat IgG Covalently linked to latex beads</td>
<td>Protein A Covalently linked to a glass surface</td>
<td>LT</td>
<td>3 nN/s</td>
<td>37 ± 3</td>
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<tr>
<td>Plant lectin from <em>Ricinus communis</em> Plant lectin from <em>Viscum album</em> Bovine galectin-1 IgG All covalently linked either to agarose beads directly or to tips via amylose</td>
<td>Asialofetuin</td>
<td>AFM</td>
<td>3 nN/s</td>
<td>43 ± 5</td>
<td></td>
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<tr>
<td></td>
<td>Covalently linked either to agarose beads directly or to tips via amylose</td>
<td></td>
<td></td>
<td>65 ± 9</td>
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<tr>
<td></td>
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<td></td>
<td>45 ± 6</td>
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<td><strong>Cytoskeletal and motor proteins</strong></td>
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<tr>
<td>Actin from rabbit skeletal white muscle</td>
<td>Heavy meromyosin prepared from rabbit skeletal white muscle myosin Adsorbed on glass</td>
<td>LT</td>
<td>12 pN/s</td>
<td>9.2 ± 4.4</td>
<td>[46](^{*})</td>
</tr>
<tr>
<td>Attached to gelsolin-coated latex beads</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bundles of actin from rabbit skeletal muscle</td>
<td>Heavy meromyosin prepared from rabbit skeletal muscle myosin Attached to polyacrylamide beads via biotin-avidin</td>
<td>AFM</td>
<td>≈140 pN/s(^{1})</td>
<td>14.8 ± 4.0</td>
<td>[60]</td>
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<tr>
<td>Adsorbed on positively charged polyacrylamide beads</td>
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</table>
receptor–ligand adhesion strength as a measure of single receptor activity, it has been shown that \( \alpha_{IIb}\beta_3 \) activation is an all-or-none phenomenon, that is, each integrin molecule resides on the platelet in either completely on or off conformation [22**]. The strength of fibrinogen–\( \alpha_{IIb}\beta_3 \) binding was shown to be dependent on the ‘age’ of bonds; ‘young’ complexes formed within minutes were capable of dissociation under hydrodynamic force, whereas ‘older’ complexes could not be ruptured [19*]. Probing interactions of individual integrins on the surface of live cells with ligands containing the RGD (Arg-Gly-Asp) binding motif revealed a considerable extension of the flexible membrane surface under load [28], as well as a paradoxical increase in the number of cells that remain adherent when larger pulling forces were applied [34*].

The interaction of the platelet integrin GPIb\( \alpha \) with von Willebrand factor (vWF) was shown to have kinetic characteristics similar to those of binding reactions of selectins [38*]. Dynamic force spectroscopy studies revealed that the rupture forces of both integrins and selectins with their protein ligands depend on applied force loading rates. When the range of applied force loading rates was wide enough and reached \( 10^4 \) pN/s or more, two regimes were observed as different slopes in the peak force versus logarithm of loading rate curves. They were interpreted as reflecting two sequential energy barriers along the unbinding pathway. Changing experimental conditions revealed that these barriers had different susceptibility to divalent cations [39**] and may have different physiological significance [40**,41**]. The first, inner barrier for the \( \alpha_\text{IIb}\beta_3 \)–fibronectin pair, observed at high forces, was sensitive to RGD deletion, but not to deletion in the synergy site of fibronectin. The second, outer barrier, operating at lower, physiologically relevant forces, was affected by both RGD and synergy binding regions, and was characteristic of the site of integrin activation [41**]. In a study of leukocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1), it was shown that, in the slow loading regime, Mg\(^{2+} \), known to stabilize the LFA-1–ICAM-1 interaction, increased the unbinding forces, whereas the fast loading regime was susceptible to an inhibitory effect of EDTA. Hence, the outer activation barrier was attributed to an equilibrium dissociation constant, whereas the inner barrier was thought to determine resistance to pulling forces [40**]. These thermodynamic characteristics, along with kinetic parameters and bond elasticity, fully control the dynamic process of cell adhesion and leukocyte rolling [13*,42,43*], and may account for their impairment in pathologically altered cells [44*].

The binding strength between antigen and antibody has been used as a tool to probe conformational states of a single protein molecule in several studies. AFM tips functionalized with antibodies were used to produce two images, the first, an adhesion image of individual ICAM-1 molecules, compared to the second, topographical one [26]. In another AFM study, antibodies were used to confirm proper membrane orientation in cell-derived vesicles and to probe the conformational changes of an

### Table 1

**Table 1 Continued**

<table>
<thead>
<tr>
<th>Receptor* and receptor-bearing surface</th>
<th>Ligand* and ligand-bearing surface</th>
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<th>Reported loading or shear rate/stress</th>
<th>Rupture forces (pN)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microtubules Adsorbed to glass</td>
<td>Two- or one-headed kinesin</td>
<td>LT</td>
<td>5.0 ± 1.68 pN/s</td>
<td>3.3–10</td>
<td>[49**]</td>
</tr>
<tr>
<td>Actin from rabbit muscle</td>
<td>Bovine Arp2/3 complex with VCA</td>
<td>LT</td>
<td>50.5 ± 17.3 pN/s</td>
<td>6–7</td>
<td>[48]</td>
</tr>
<tr>
<td>Attached to gelsolin-coated latex beads</td>
<td>Arp2/3 adsorbed on a VCA-coated surface</td>
<td>LT</td>
<td>50.5 ± 17.3 pN/s</td>
<td>6–7</td>
<td>[48]</td>
</tr>
</tbody>
</table>

* The term ‘receptor’ is relevant by definition only to integrins and selectins, whereas other interacting proteins were named ‘receptor’ or ‘ligand’ arbitrarily. 1 Calculated from reported values of a transducer spring constant and retraction velocity.
antibody-binding epitope of sodium glucose co-transporter (SGCT) during D-glucose transport [45*].

Direct measurement of the strength of rigor bonds between actin and myosin [46*,47], studying detachment of the actin–Arp2/3 complex from the VCA domain of N-WASP (Wiscott–Aldrich syndrome protein) [48*] and using binding strength in the molecular analysis of kinesin motility ([49**]; see also Update) are additional but not the last examples (see Table 1) of diverse and important biological applications of force-induced individual protein–protein unbinding studies.

Conclusions and perspectives

Most of the results of those experiments listed in Table 1 that were carefully designed and executed show that rupture forces for adhesion proteins are characteristic of each ligand–receptor pair. The usual range for typical proteins appears to be ~100 pN +/- 50 pN, but there may be exceptions. Although loading rates under most physiological conditions have not been determined, it is likely that these forces were measured with loading rates that cells might really experience, for example in flowing blood. It should be noted that the experiments described in some papers listed here are incomplete, lacking important controls or have problems with methodology or interpretation, reflecting the difficulty of these studies. The results of all single-molecule protein–protein unbinding induced by force experiments still need to be interpreted with caution. Future research in this field should take into account the cautions presented in this review.

Although studies of protein–protein unbinding induced by force are still in their infancy, there have been important theoretical and experimental breakthroughs, but much remains unknown. The theory behind nonequilibrium events needs to be further tested and related to equilibrium events. More studies will explore the details of kinesin motility. [49**]; see also Update) are additional but not the last examples (see Table 1) of diverse and important biological applications of force-induced individual protein–protein unbinding studies.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


A comprehensive review including discussions of the basic physics of the experimental techniques used to measure single-molecule mechanics and the theory underlying the energy landscape model. The applications discussed are stretching of single DNA molecules, force-induced dissociation of single bonds and unfolding of multidomain proteins.


5. Howard J: Mechanics of Motor Proteins and the Cytoskeleton. Sunderland, Massachusetts, USA: Sinauer Associates Inc; 2001. The first six chapters of this excellent text provide the background needed to understand the literature on single-molecule mechanical measurements, including theories of protein–protein binding and unbinding.


This review covers current status of dynamic force spectroscopy studies in combination with experimental data on force-induced receptor–ligand unbinding.


A theoretical analysis of single-molecule bonds with complex energy landscapes, showing that different landscapes may yield the same dynamic force spectra.


The authors show how equilibrium free energy profiles can be extracted from pulling force measurements using the formalism of Jarzynski [10].

The probability of effective binding events was directly proportional to the Jarzynski between equilibrium and nonequilibrium processes. Different adhesion behaviors of leukocytes were modeled as a function of their dynamic properties and receptor-mediated cell adhesion. The simulation model described provides the means to elucidate how the structures of receptor and ligand molecules correlate with adhesion. The state diagram for cell spontaneous to force-induced bond dissociation was shown to be directly dependent on the time of bond formation.

Optically trapped fibrinogen-integrin bonds were shown to be directly dependent on the time of bond formation.

Optically trapped fibrinogen-coated surfaces were used to determine the force dependence of the rupture of glycoprotein Ib-IIIa-fibrinogen bonds between latex spheres. The breaking force of fibrinogen–integrin bonds was shown to be directly dependent on the time of bond formation.


Force measurements on the molecular interactions between ligand (RGD) and human platelet $a_{IIb}b_3$ receptor system. Surface Sci 2001, 491:433-443.

Mechanical anchoring strength of L-selectin, $\beta_{2}$ integrins, and CD45 to neutrophil cytoskeleton and membrane. Biophys J 1999, 77:587-596.


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Cells expressing LFA-1 were immobilized on AFM tips via concanavalin A (ConA) and interacted with ICAM-1 expressed by fibroblasts or physically adsorbed to the surface of a tissue culture dish in a soluble truncated form. The results of rupture force measurements at loading rates covering three orders of magnitude revealed two loading regimes of different physiological significance.

41. Li F, Redick SD, Erickson HP, Moy VT: Force measurements of the $\alpha_4\beta_1$ integrin-fibronectin interaction. Biophys J 2003, 84:1-11.

Cells expressing $\alpha_4\beta_1$ were attached to ConA-coated AFM tips and touched repeatedly to a surface bearing a fragment derived from the cell-binding fibronectin domain. Two force loading regimes, slow and fast, obtained with a loading rate range of three orders of magnitude, had different susceptibility to deletions in fibronectin.


43. Chen S, Springer TA: Selectin receptor-ligand bonds: formation limited by shear rate and dissociation governed by the Bell model. Proc Natl Acad Sci USA 2001, 98:950-955. Neutrophils were rolled over a P-selectin-coated surface at different shear rates and shear stresses (regulated by viscosity at the same shear rate). Bond formation between neutrophils and the surface was a function of shear rate, whereas bond dissociation was a function of shear stress and hence the force on the bond. The Bell model was shown to fit well the experimental parameters of unbinding.

44. Hanley W, McCarty O, Jadhav S, Tseng Y, Wirtz D, Konstantopoulos K: Single-molecule characterization of P-selectin/ligand binding. J Biol Chem 2003, in press. The forces and unstressed off-rates of P-selectin–PSGL-1 interactions on neutrophils differed from those of P-selectin binding to a novel ligand on colon carcinomas. This provides a mechanistic basis for the differential abilities of neutrophils and carcinomas to tether and roll on P-selectin substrates under blood flow conditions.

45. Wielert-Badl S, Hinterdorfer P, Gruber HJ, Lin JT, Badt D, Wimmer B, Schindler H, Kinne RK: Single molecule recognition of protein binding epitopes in brush border membranes by force microscopy. Biophys J 2002, 82:2767-2774. The observed specific recognition signals with anti-γ-glutamyl-transpeptidase (γ-GT) antibody proved the accessibility of γ-GT and were indicative of proper membrane orientation in the vesicles. Then, the normal unbinding force profile for SGCT and its antibody was determined; it changed in the presence of either α-glucose or phosphoril, a specific inhibitor of SGCT. The data demonstrated that the specific antibody-binding epitope of the SGCT, located in the segment consisting amino acids 606–630, undergoes conformational changes during α-glucose transport.

46. Nishizaka T, Seo R, Tadakuma H, Kinosita K Jr, Ishiwata S: Characterization of single actomyosin rigor bonds: load dependence of lifetime and mechanical properties. Biophys J 2000, 79:962-974. Bead-coupled actin filaments were attached at two or more points to a surface coated with heavy meromyosin (HMM) and then pulled via the optically trapped latex bead to disrupt the bond that was the closest to the bead. The measured average unbinding force of the actin–HMM rigor bond in the absence of ATP was a few times larger than the known sliding force.


48. Fujiwara I, Suetusgu S, Uemura S, Takenawa T, Ishiwata S: Visualization and force measurement of branching by Arp2/3 complex and N-WASP in actin filament. Biophychem Biophys Res Commun 2002, 293:1550-1555. The Arp2/3 protein complex and WASP family proteins containing the active VCA domain are involved in regulation of actin polymerization. Bead-attached actin filaments and free Arp2/3 were allowed to reach the VCA-coated surface, with subsequent unbinding induced by pulling the optically trapped bead and controlling its displacement. It was suggested that the measured unbinding force reflected the detachment of the actin–Arp2/3 complex from the VCA-coated surface, rather than the rupture of actin–Arp2/3 bonds.

49. Uemura S, Kawaguchi K, Yajima J, Edamatsu M, Toyoshima Y, Ishiwata S: Kinesin-microtubule binding depends on both nucleotide state and loading direction. Proc Natl Acad Sci USA 2000, 98:3977-3981. The rupture force distribution for microtubules and two-headed kinesin, unlike that for one-headed kinesin, was bimodal in the presence of an ATP analog, with the larger values representing double-headed binding. The results obtained in the presence of ADP and in a nucleotide–free state were analyzed with regard to the modern mechanistic model of kinesin motility.


62. Vincikier A, Gervasoni P, Zaugg F, Ziegler U, Lindner P, Groscurth P, Feldmann W, Grandbois M, Andre S, Benoit M, Wehle AK, Pluckthun A, Semenza G: Single-molecule recognition of protein–protein interactions in the presence of ADP and in a nucleotide–free state. In an extension to a previous study [40**, the authors proposed a model in which an equilibrium exists between single- and double-headed binding, and detection from the microtubule occurs during either single- or double-headed binding. They conclude that the double-headed binding state is predominant at equilibrium in the absence of load not only in the AMP-PNP state but also in the nucleotide–free state.