Cross-Linking of Cell Surface Receptors Enhances Cooperativity of Molecular Adhesion

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ABSTRACT Cooperativity of molecular adhesion has been proposed as a mechanism for enhanced binding strength of adhesion molecules on the cell surface. Direct evidence for its mechanism, however, has been lacking until now. Atomic force microscopy (AFM) was used to measure the adhesive strength between concanavalin A (Con A) coupled to an AFM tip and Con A receptors on the surface of NIH3T3 fibroblast cells. Cross-linking of receptors with either glutaraldehyde or 3,3′-dithio-bis(sulfosuccinimidylproprionate) (DTSSP) led to an increase in adhesion that could be attributed to enhanced cooperativity among adhesion complexes. An increase in loading rate due to greater stiffness of fixed cells also contributed to the twofold increase in binding strength. These results show that receptor cross-linking can greatly contribute to a total increase in cell adhesion by creating a shift toward cooperative binding of receptors.

INTRODUCTION

Dynamic changes in cell detachment force play an important role in the movement of migrating cells (Galbraith and Sheetz, 1998) and axonal growth cones (Suter and Forscher, 1998; Suter et al., 1998). Changes in adhesive strength also occur during changes in cell state, such as the metastasis of cancerous cells and T-cell activation after adhesion with antigen-presenting cells. During cell migration and in cell-cell interactions, surface receptors from the integrin family form clusters at focal adhesion sites that link the cytoskeleton with extracellular ligand (Burridge et al., 1988). Theoretical (Ward and Hammer, 1993; Ward et al., 1994) and experimental studies (Hermanowski-Vosatka et al., 1988; Detmers et al., 1987; Hato et al., 1998) have suggested that the formation of these receptor aggregates enhances cell adhesion. A possible mechanism for the increased adhesion is a shift toward cooperative binding between individual complexes. For example, cell surface receptors in a cluster could share a more even distribution of load, thus allowing the receptors to support a greater detachment force before simultaneous breakage of the complexes (Fig. 1 *A*). In a random distribution of receptors the full load would be exerted on fewer bonds at a time and the two surfaces would peel apart with less applied force (Fig. 1 *B*).

Chemical fixation of surface receptors could also enhance cooperative binding, because cross-linkage of membrane protein to the cytoskeleton and other proteins would prevent lateral diffusion in the phospholipid bilayer. Immobilized membrane proteins would be less likely to move away from the site of interaction and hence load would be evenly distributed during detachment. In this case, chemical fixation could enhance adhesion in the absence of receptor clustering.

To determine if receptor cross-linking could increase cell adhesion by enhancing cooperative binding, we acquired atomic force microscope (AFM) force measurements before and after chemically cross-linking adhesion receptors on the surface of fibroblast cells. Previously, the AFM has been employed to study the interaction of various ligand-receptor systems, including avidin/biotin, antibody/antigen, and p-selectin/carbohydrate pairs (Florin et al., 1994; Hinterdorfer et al., 1996; Dammer et al., 1995; Fritz et al., 1998). Typically, the receptor is coupled to the AFM cantilever, and the ligand is attached to the substrate via a long spacer. We coupled Con A to an AFM cantilever via a biotin-avidin linkage. The concanavalin A (Con A) receptors were on the surface of a NIH3T3 cell line. Here we present direct evidence that cross-linking of surface receptors causes a shift toward cooperative binding and hence an increase in receptor binding strength.

MATERIALS AND METHODS

Cell culture and preparation

Force measurements were acquired on a NIH3T3 fibroblast cell line (courtesy of B. Buehler). Cells were maintained in continuous culture at 37°C and 5% CO_2 in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Irvine Scientific, Santa Ana, CA), penicillin (50 U/ml; Gibco BRL), and streptomycin (50 μg/ml; Gibco BRL). Confluent cells were released from wells by a 1-min incubation in 0.05% trypsin/0.5 mM EDTA 1–5 days before use and then plated on 35 × 10 mm tissue culture dishes. Measurements were carried out at room temperature in RPMI medium prepared without glucose and supplemented with 100 μg/ml bovine serum albumin (BSA) and 0.1 mM $MnCl_2$.

Cells were chemically fixed with either glutaraldehyde or DTSSP to cross-link receptors. For glutaraldehyde fixation, cells were treated with 1% glutaraldehyde (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) (20 mM PO_4^{3-}, 50 mM NaCl, pH 7.2) for 1 min, rinsed three times with PBS, and resuspended with glucose-free RPMI supplemented with 0.1 mg/ml BSA and 0.1 mM $MnCl_2$. To limit fixation to membrane surface

Received for publication 25 October 1999 and in final form 8 March 2000.
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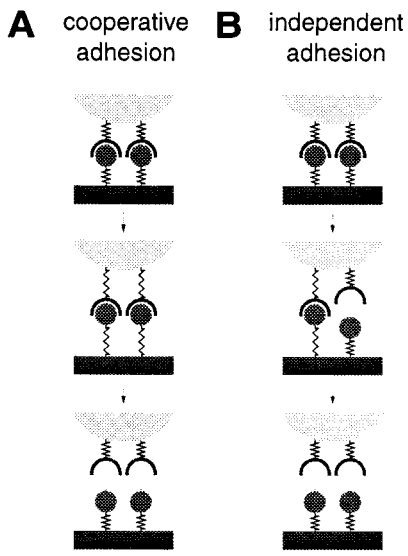

FIGURE 1 Schematic of possible mechanisms for cell detachment. (*A*) In cooperative molecular adhesion multiple ligand-receptor bonds break simultaneously for enhanced cell detachment force. (*B*) During independent molecular adhesion, bonds rupture sequentially, and a lower total detachment force is exerted on the cell. The term "cooperativity" in this report refers to the simultaneous breakage of multiple bonds and not to a change in receptor binding affinity.

proteins, cells were fixed with 2 mM DTSSP in PBS at 37°C for 30 min. The disulfide bond of DTSSP (Pierce, Rockford, IL) was reduced by the application of 20 mM dithiothreitol.

Western blots

To verify chemical cross-linking of Con A receptors we performed Western blots, using peroxidase-labeled Con A on cell membrane extracts prepared from glutaraldehyde-fixed (0.1%) or untreated NIH3T3 fibroblast cells. In brief, harvested cells suspended in 15 mM HEPES buffer with 5 mM EDTA, 5 mM EGTA, and protease inhibitors were homogenized (Brinkman Polytron) at 3000 rpm for 15 s. The homogenate was centrifuged at 100,000 $\times$ *g* for 1 h at 4°C to isolate the membrane fragments. Approximately 5 μg of the membrane protein, resuspended in 50 mM HEPES, 5 mM EDTA, and 5 mM $MgCl_2$, was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After protein transfer to polyvinyl pyrrolidine fluoride membranes (BioRad, Hercules, CA), immunoblotting was carried out with Con A conjugated with horseradish peroxidase (ICN, Aurora, OH) at 1 μg/ml in 0.2% Tween (v/v) in PBS supplemented with 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. Protein bands were revealed with a chemiluminescent substrate (SuperSignal, Pierce).

Con A histochemistry

To visualize the distribution of Con A receptors on the surface of NIH3T3 cells, cells were grown on polylysine-coated glass chambers (P35G-0-14-C-GM; MatTek, Ashland, MA). Adhering cells were then exposed for 5 min to Con A conjugated to Oregon Green 488 (Molecular Probes, Eugene, OR; 100 μg/ml diluted in balanced salt solution (BSS; 145 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5 mM Trizma base, 10 mM HEPES, pH 7.4)). After several washes in BSS, cells were fixed in 3% formaldehyde in BSS (pH 7.4) at 4°C for 30 min, washed in BSS containing 1% BSA and 1% glycine, and then viewed with a Zeiss (Jena, Germany) Axiovert 135 microscope equipped with a 40$\times$ objective and a fluorescein filter set. Negative controls were stained with Oregon Green 488-labeled Con A pretreated with 100 mM α-D-mannopyranoside for 30 min at 25°C.

Force apparatus

An atomic force apparatus was constructed to acquire force measurements (Fig. 2). Cells or agarose beads were localized using an inverted optical system attached to the AFM. The position of the AFM tip relative to the substrate was set by a piezo translator with a strain gauge position sensor (Physik Instrumente, Waldbronn, Germany). The interaction between the AFM tip and the substrate was determined from deflection of the AFM cantilever. A focused laser spot from a pigtailed diode laser (Oz Optics, Ontario, Canada) was reflected off the back of the cantilever onto a two-segment photodiode to monitor the cantilever's deflection. The photodiode signal was then preamplified, digitized by a 16-bit analog-to-digital converter (Instrutech Corp., Port Washington, NY), and processed by an Apple Power Macintosh computer. All force scan measurements were recorded at room temperature, using unsharpened Si_3N_4 cantilevers (MLCT-AUHW; Thermomicroscopes, Sunnyvale, CA). Cantilevers were calibrated by thermal fluctuation analysis according to the method of Hutter and Bechhoefer (1994) and had a spring constant of $\sim$0.010 N·m^{-1}.

Functionalization of AFM tips

Cantilevers were coated with biotinylated bovine serum albumin (biotin-BSA) and then coupled with avidin bound to biotin-Con A. In brief, cantilevers were washed in acetone for 5 min, UV irradiated for 15 min, and then immersed overnight in 50–100 μl of biotin-BSA (Sigma; 0.5 mg/ml in 100 mM $NaHCO_3$, pH 8.6) in a 37°C humidified chamber. After several rinses in PBS, biotin-BSA-coated tips were then coupled with avidin (Neutravidin; Pierce, Rockford, IL; 0.5 mg/ml in 100 mM $NaHCO_3$) during a 10-min incubation. Unbound avidin molecules were washed away by several PBS rinses before a subsequent incubation of the biotin-BSA/avidin complexes with biotinylated Con A (Sigma; 1 mg/ml in 100 mM $NaHCO_3$, pH 8.6). Before being used in acquiring force measurements, Con A-functionalized tips were rinsed with PBS.

Bead preparation

For force measurements on beads, D-mannose cross-linked 4% agarose beads were obtained from Sigma. Agarose beads were washed with PBS and resuspended in glucose-free RPMI supplemented with 0.1 mg/ml BSA and 0.1 mM $MnCl_2$, which was the same solution used for measurements. Culture dishes were coated with Con A (Sigma; 1 mg/ml in $NaHCO_3$, pH 8.6) at 37°C overnight in a humidified chamber and then rinsed. Just before measurements were acquired, beads were placed on the surface of the coated dishes.

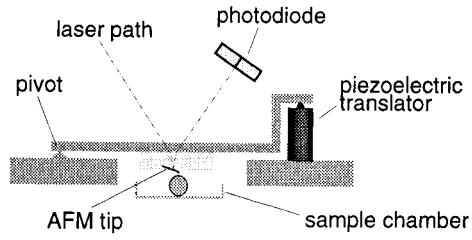

FIGURE 2 Schematic of the atomic force apparatus.

RESULTS AND DISCUSSION

Con A adhesion force measurements on D-mannose beads

To test for successful functionalization of the cantilever tip with Con A we first set out to acquire force measurements on agarose beads coupled with D-mannose, a specific ligand for Con A. Use of the beads enabled us to examine Con A adhesion in a well-defined system. The force measurements were carried out with Con A-functionalized cantilever tips positioned over the center of the bead. The deflection of the cantilever was then recorded on approach to the bead and upon subsequent retraction of the cantilever from the bead. Using the measured spring constant of the cantilever, we could convert the deflection of the cantilever directly into force.

Fig. 3 *A* shows a typical scan for an AFM force measurement acquired with a Con A-functionalized tip on a D-mannose agarose bead. Once the cantilever made contact with the bead there was a gradual bend in the cantilever and a sloping in the approach trace as the tip pressed against the surface of the elastic bead. Upon retraction the adhesive force between the functionalized tip and D-mannose on the bead surface resulted in a deflection of the cantilever tip toward the bead. With increasing bend of the cantilever, the tension on the adhesive bonds between cantilever and bead increased until the bonds yielded. The sawtooth-like appearance of the retract trace was attributed to multiple interactions between Con A on the tip and D-mannose on the bead surface that were sequentially broken during retraction of the cantilever. At the last step of the unbinding there was a sharp transition back to the baseline as the last molecular bonds were broken. The specificity of the adhesive force was confirmed by blocking with 100 mM α-D-mannopyranoside (Fig. 3 *B*).

Ligand-receptor unbinding on cells

Measurements of Con A receptor binding were acquired on living cells by the same method as described previously for the agarose beads. To determine the distribution of Con A receptors on the surface membrane, cells were briefly exposed to Con A conjugated to Oregon Green 488. Fig. 4 *A* shows that Con A receptors were distributed throughout the cell surface. The staining was specific, as preincubation of Oregon Green-labeled Con A with methyl α-D-mannopyranoside (100 mM), a high-affinity ligand, blocked staining of fibroblast cells (data not shown).

For force measurements, a Con A-functionalized cantilever tip was carefully positioned on top of a cell (Fig. 4 *B*). The deflection of the cantilever was then recorded on approach to the cell and upon subsequent retraction of the cantilever from the cell. Fig. 5 shows a series of AFM force measurements obtained with a Con A tip and National Institutes of Health 3T3 cells. In these experiments the applied force was minimal, ~250 pN. Frequently there were multiple jumps in force in the retract trace. These jumps

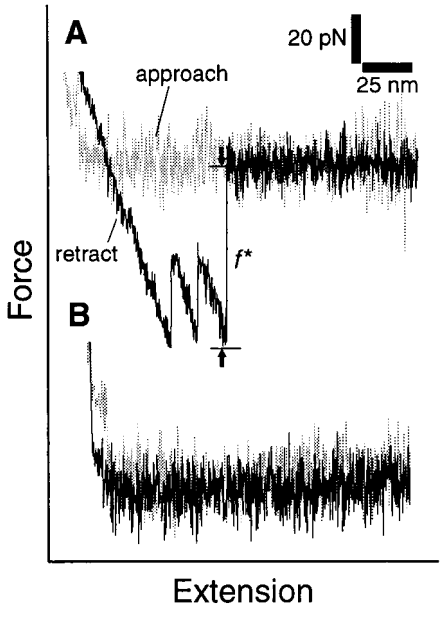

FIGURE 3 Force-extension curves obtained with a Con A-functionalized AFM cantilever in measurements on D-mannose-coupled agarose beads. (*A*) No α-D-mannopyranoside. (B) With 100 mM α-D-mannopyranoside added. *f** is the unbinding force of the last adhesion unit to break.

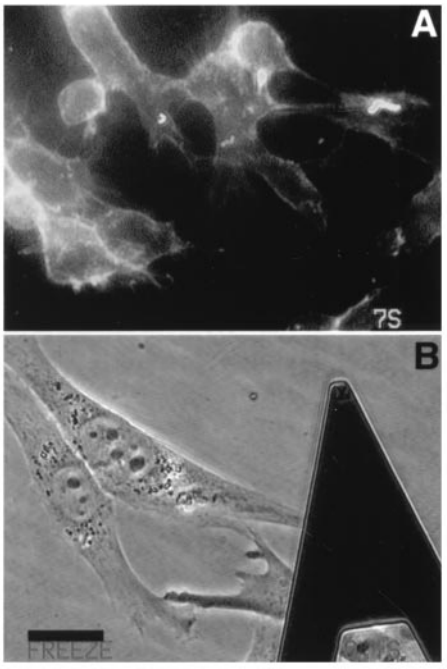

FIGURE 4 (*A*) Fluorescent micrograph of NIH3T3 cells stained with Con A labeled with Oregon Green 488. (*B*) Phase-contrast micrograph of an AFM cantilever and NIH3T3 cells. Bar = 25 μm.

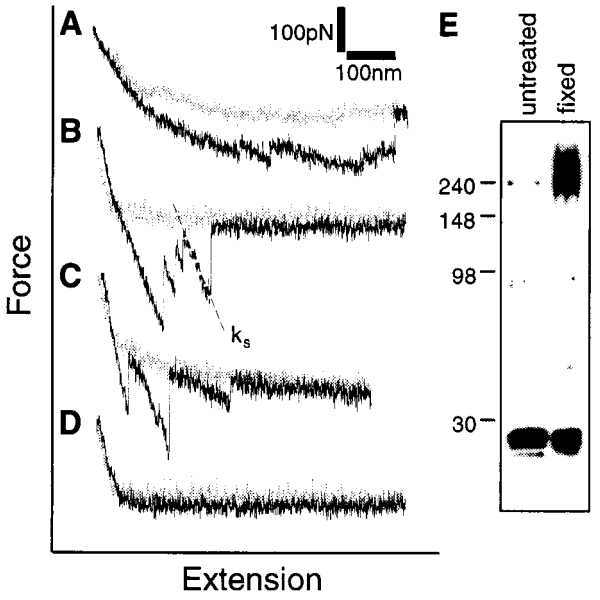

FIGURE 5 Force versus extension curves acquired from Con A-functionalized AFM tips interacting with Con A receptors on the surface of NIH3T3 cells that were (*A*) not fixed, (*B*) fixed with glutaraldehyde, and (*C*) fixed with DTSSP. The dotted line in *B* is the slope of the force versus displacement curve from which system compliance, k_S, was determined. α-D-Mannopyranoside (100 mM) was able to block adhesion events in glutaraldehyde-fixed cells (*D*) and in untreated and DTSSP-treated cells (data not shown). (*E*) Peroxidase labeled Con A recognized low-molecular-mass bands (<30 kDa) in cell membrane extracts from both untreated and glutaraldehyde-fixed cells. The presence of high-molecular-mass bands (>150 kDa) in fixed cell extracts indicated that Con A receptors were being cross-linked with other proteins.

involved the breakage of one or more Con A/Con A receptor bonds. After each breakage, the tension in the system was reduced and the cantilever relaxed. The average rupture force for untreated cells was 86 ± 2.6 pN (SEM; $n = 375$) and was consistent with measurements by others (Gad et al., 1997). Fig. 5 *A* shows a typical measurement carried out with an untreated cell in which stretching occurred over an extension range of ~500 nm before final separation. Thus the receptors seemed to be anchored to a flexible membrane/cytoskeleton that stretched with the applied force until the tip separated from the cell surface. Such cell tethers have also been observed in studies of adherent particles in various cell systems (Hochmuth et al., 1973; Dai and Sheetz, 1995; Shao and Hochmuth, 1996).

We have attributed our force measurements on cells to the dissociation of the Con A receptor bond. Potential sites of failure also exist at 1) the junction between biotin-BSA and the cantilever tip, 2) biotin/avidin linkages such as the biotin-BSA/avidin bond and the avidin/biotin-Con A bond, 3) the Con A receptor anchorage to the cell membrane, and 4) the membrane tethers. We determined that breakage occurred between Con A and Con A receptors based on the following arguments. First, both the absorption of biotin-BSA to the cantilever and the avidin/biotin bond are much stronger than the measured rupture forces (Florin et al., 1994). Second, it is unlikely that the receptors were being extracted from the membrane, as the stability of transmembrane proteins in a cell membrane has been estimated to be on the order of 70 kcal/mol (Haltia and Freire, 1995). Assuming that the potential changes linearly over the entire thickness of the membrane (~3 nm), the force needed to extract a transmembrane protein is ~160 pN, which is much larger than the measured rupture forces. Receptors could also be anchored by a lipid tail. The energy required to pull a lipid from a bilayer is ~16*kT*, and an estimate of the required force is only 23 pN (Helm et al., 1991), which is sufficiently close to the experimental noise and therefore would have been rejected. Finally, it is unlikely that membrane failure was being measured, as hundreds of cycles of binding and unbinding could be acquired with a single functionalized tip. If portions of the membrane were removed from the cell surface, then the contaminated tip would not be reusable. Thus rupture force measurements must have been between the Con A-functionalized tip and Con A receptors on the surface of the cells.

Receptor cross-linking enhances adhesion strength

Cells were chemically fixed to test the effect of cross-linking on the strength of binding. To verify fixation-formed chemical linkages between Con A receptors and other proteins, Western blots of membrane extracts from untreated and glutaraldehyde-fixed cells were stained with a Con A probe (Fig. 5 *E*). Both untreated and fixed cells had low-molecular-mass bands that confirmed the presence of Con A receptors. Additional high-molecular-mass bands were detected in glutaraldehyde-fixed membrane protein extracts, indicating that Con A receptors were forming chemical linkages with other proteins and possibly among themselves. Such linkages would serve to immobilize the receptors in the intact cell.

Cells were fixed with either glutaraldehyde or DTSSP to test the effect of receptor cross-linking on the strength of binding. The chemically fixed cells (Fig. 5, *B* and *C*) were stiffer than the untreated cells, as evident from the analysis of the approach trace and from the observed membrane elongation upon cantilever retraction. In Fig. 5, *B* and *C,* membrane elongation measurements were ~200 nm and 270 nm for glutaraldehyde and DTSSP-fixed cells, respectively. To demonstrate binding specificity, 100 mM methyl-α-D-mannoside was added to glutaraldehyde-fixed cells. The absence of a hysteresis between the approach and retract traces demonstrated null adhesion (Fig. 5 *D*). Adhesion was also blocked in unfixed and DTSSP fixed cells (data not shown).

On average the magnitude of the force jumps was larger in fixed cells than in untreated cells. Fig. 6 shows force

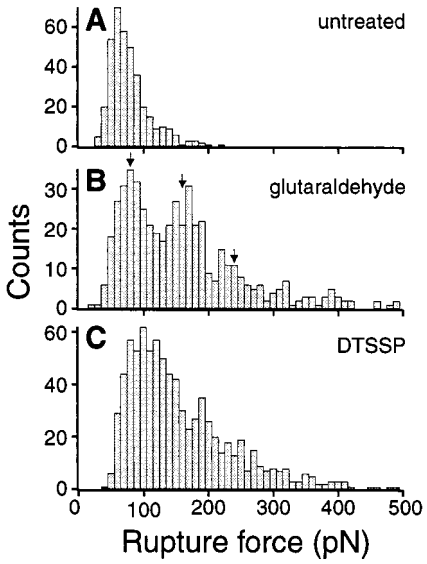

FIGURE 6 Histograms of rupture force between Con A-functionalized AFM tips and Con A receptors on (*A*) untreated cells, (*B*) glutaraldehyde-fixed cells, and (*C*) DTSSP-fixed cells. Arrows in *B* indicate quantized peaks at 80, 160, and 240 pN after fixation of cells in glutaraldehyde.

histograms obtained at a constant scan speed for untreated, glutaraldehyde-fixed, and DTSPP-fixed cells. After fixation the force distribution was shifted toward higher values. The average rupture force increased almost twofold from 86 ± 2.6 pN in untreated cells to 173 ± 6.1 pN after glutaraldehyde fixation. After treatment with DTSSP, a membrane-impermeable and cleavable cross-linker, the average rupture force was 166 ± 3.2 pN. The observed increase in rupture force was not a result of chemical modification of the receptor, as rupture force (133 ± 4.3 pN) and cell elasticity values could be brought closer to untreated cell values after dithiothreitol (DTT) cleavage of disulfide bonds in the DTSSP cross-links. The incomplete reversal could be a consequence of endogenous cross-linking within the system due to stimulation from the experimental protocol.

If the increase in adhesion was due to enhanced cooperativity, one might expect peaks in the force histogram at integer multiples of a unitary rupture force. In the force histogram for glutaraldehyde-fixed cells, quantal peaks were observed at 80, 160, and 240 pN (Fig. 6 *B*). Lesser defined peaks were visible in the histogram of DTSSP-treated cells. This, however, could be a result of a lesser degree of cross-linking with the DTSSP. The appearance of quantal peaks provided strong evidence for receptor cooperativity.

Effect of loading rate on adhesion strength

Chemical fixation of cells decreases the compliance of the system (cell and cantilever) and therefore could also increase ligand/receptor rupture force. In AFM force measurements, the compliance of the system and the scan rate of the force apparatus determine the loading rate of the measurement. In turn, an increase in loading rate has been shown to increase bond strength (Evans and Ritchie, 1997; Fritz et al., 1998; Merkel et al., 1999). By measuring the average slope of the retraction traces of the force measurements (see Fig. 5 *B*), we determined that the system compliance k_S decreased after fixation. Before cell fixation, the system compliance and loading rate of the measurement were $\sim 4.2 \times 10^4$ N/m and 420 pN/s, respectively. After fixation with glutaraldehyde, k_S increased to 26.6×10^4 N/m, and the loading rate was increased by more than sixfold to ~2700 pN/s. DTSSP fixation increased the loading rate to a lesser degree, bringing it to ~1700 pN/s. The difference in loading rates, however, could reflect the restriction of DTSSP action to surface receptors and not the cytoskeleton.

To determine if changes in loading rate could contribute to the increased Con A/Con A receptor adhesion, we obtained force measurements for the breakage of individual Con A and D-mannose complexes over a range encompassing the observed loading rates of unfixed and fixed cell measurements (Fig. 7). For these experiments the adhesive strength was determined at different cantilever retraction speeds and hence different force loading rates. Measurements were acquired from D-mannose agarose beads, because the low system compliance of unfixed cells did not allow for measurements to be acquired at loading rates achieved for fixed cells. Conditions were such that an applied force of <100 pN restricted adhesion to less than 30% of the trials and increased the probability that adhesion would be mediated by a single con A bond to >80% (Merkel et al., 1999). Fig. 7 *A* shows force histograms from measurements acquired at different loading rates: 415 pN/s and 4980 pN/s. The corresponding average rupture forces were 82 ± 2.9 pN and 125 ± 4.8 pN, respectively. Immediate data points in the force versus loading rate relation are given in Fig. 7 *B*. A 52% increase in rupture force was observed over this range of loading rates. In the cell measurements, however, there was an almost twofold change in rupture force. Thus the D-mannose bead measurements demonstrated that the increase in loading rate after cell fixation could contribute to the observed change in force in cells; however, it might not completely account for the total increase. Moreover, these measurements were insightful because they provided a reference value for the strength of a Con A bond and suggested that the force distribution obtained from the untreated cells and the first peak distribution obtained from the fixed cells stemmed from the breakage of a single Con A bond.

In experiments in which the loading rate was varied, increases in average rupture force were attributed to a shift in a single peak distribution. Thus, if the increase in average rupture force in fixed cells were a result of increased loading rate, one might expect a single peak distribution, similar to that of untreated cells, but the distribution would be

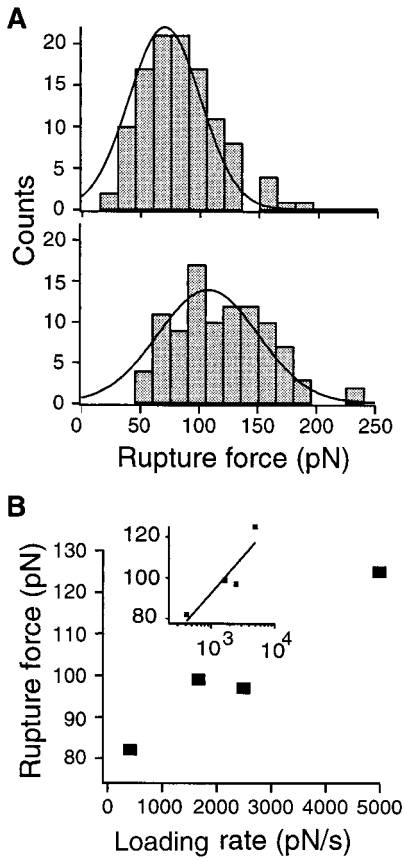

FIGURE 7 (*A*) Force histograms acquired from measurements carried out with a Con A-functionalized tip on a D-mannose-linked agarose bead. Loading rates were at 415 pN/s (*top*) and 4980 pN/s (*bottom*). Each histogram was generated from ~100 measurements. The center of the force histogram was determined by a Gaussian fit. (*B*) Rupture force f^* dependence on loading rate r_f. According to Bell's model, the dissociation rate of a complex is amplified by an applied force (Evans and Ritchie, 1997; Bell, 1978). The relationship between loading rate and rupture force is given by

$$f^* = \frac{k_B T}{x_\beta} \ln\left(\frac{x_\beta}{k^\circ k_B T}\right) + \frac{k_B T}{x_\beta} \ln r_f$$

where k° is the dissociation rate constant in the absence of applied force, x_β is a parameter that characterizes the relationship between force and dissociation rate, k_B is Boltzmann's constant, and T is temperature. Bell's model parameters x_β and k°, obtained from the plot of f^* versus $\log(r_f)$ (*inset*), were 0.27 nm and 0.17 s^{-1}, respectively.

shifted toward higher values. Consistent with this idea, the center of the first peak in the Con A/Con A receptor histograms shifted from 68 pN to 83 pN after chemical fixation (Fig. 5). However, the shift only contributed a small amount toward the total increase in average rupture force (86 pN to 173 pN). Most of the fixative-induced increase was due to the appearance of quantal peak distributions at higher force values, and this was most likely due to chemical cross-linking of Con A receptors. Thus, after glutaraldehyde fixation, increased loading rate contributed to the overall increase in average rupture force; however, most of the increase could be attributed to cooperative binding events that appeared as quantized peak distributions at higher rupture forces.

In summary, by using the AFM to acquire direct measurements of binding strength on the surfaces of living and fixed cells, we have shown that cross-linking of receptors leads to enhanced adhesion. Moreover, the increased adhesion is a product of enhanced cooperativity between receptors, such that there is a greater probability of simultaneous breakage of multiple bonds. Changes in loading rate that result from cross-linking may also contribute to increased binding strength.

Cooperative binding could be an important physiological mechanism for modulating cell adhesion. For example, at an immunological synapse formed by an antigen-presenting cell and an activated T lymphocyte, the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and LFA-1 cluster at the site of contact and may help to promote enhanced adhesion (Monks et al., 1998; Grakoui et al., 1999). During the formation of the synapse, ICAM-1 molecules form dimers that may in turn be cross-linked to other ICAM-1 dimers via the cytoskeleton. This cross-linkage may help to promote cooperative binding of the receptors and hence contribute to the observed increase in adhesion strength. In addition, cross-linking of the cytoskeleton and/or surface receptors could increase the system compliance and hence affect the loading rate applied to individual bonds for stronger rupture forces during deadhesion.

We thank R. D. Heathcote for comments on the manuscript and B. Evans for her assistance with Western blots.

This work was supported by grants from the Whitaker Foundation, the American Cancer Society, and the National Institutes of Health (1 R29 GM55611-01) to VTM.

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