Cell Adhesion Strengthening: Contributions of Adhesive Area, Integrin Binding, and Focal Adhesion Assembly

Nathan D. Gallant, Kristin E. Michael, and Andrés J. García

Woodruff School of Mechanical Engineering, Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA 30332-0363

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Mechanical interactions between a cell and its environment regulate migration, contractility, gene expression, and cell fate. We integrated micropatterned substrates to engineer adhesive area and a hydrodynamic assay to analyze fibroblast adhesion strengthening on fibronectin. Independently of cell spreading, integrin binding and focal adhesion assembly resulted in rapid sevenfold increases in adhesion strength to steady-state levels. Adhesive area strongly modulated adhesion strength, integrin binding, and vinculin and talin recruitment, exhibiting linear increases for small areas. However, above a threshold area, adhesion strength and focal adhesion assembly reached a saturation limit, whereas integrin binding transitioned from a uniform distribution to discrete complexes. Adhesion strength exhibited exponential increases with bound integrin numbers as well as vinculin and talin recruitment, and the relationship between adhesion strength and these biochemical events was accurately described by a simple mechanical model. Furthermore, adhesion strength was regulated by the position of an adhesive patch, comprised of bound integrins and cytoskeletal elements, which generated a constant 200-nN adhesive force. Unexpectedly, focal adhesion assembly, in particular vinculin recruitment, contributed only 30% of the adhesion strength. This work elucidates the roles of adhesive complex size and position in the generation of cell–extracellular matrix forces.

INTRODUCTION

Cell adhesion to the extracellular matrix (ECM) is central to development and the organization, maintenance, and repair of tissues by providing anchorage and triggering signals that direct cell survival, migration, cell cycle progression, and expression of differentiated phenotypes (De Arcangelis and Georges-Labouesse, 2000; Danen and Sonnenberg, 2003). Furthermore, abnormalities in adhesive interactions are often associated with pathological states, including blood clotting and wound healing defects as well as malignant tumor formation (Wehrle-Haller and Imhof, 2003; Jin and Varner, 2004). Adhesion to extracellular matrix components, such as fibronectin (FN) and laminin, is primarily mediated by the integrin family of heterodimeric receptors (Hynes, 2002). Integrin-mediated adhesion is a highly regulated process involving receptor activation and mechanical coupling to extracellular ligands (Faull *et al.*, 1993; Choquet *et al.*, 1997; García *et al.*, 1998a). Bound receptors rapidly associate with the actin cytoskeleton and cluster together to form focal adhesions, discrete supramolecular complexes that contain structural proteins, such as vinculin, talin, and α -actinin, and signaling molecules, including FAK, Src, and paxillin (Geiger *et al.*, 2001).

Significant progress has been made in understanding biochemical aspects of integrin-mediated adhesion, particularly

in terms of identifying key adhesive components and signaling interactions. This information has been instrumental in deciphering mechanisms regulating cell morphology, migration, and integration of adhesive and growth factor-activated signals that direct high order cellular functions. In contrast, the mechanical aspects of adhesion remain poorly understood due to a lack of robust, quantitative measurement systems and the inherent complexities of the adhesive process. Cell spreading and migration are often used as indirect indicators of adhesion strength, but these multistep, dynamic processes exhibit complex dependencies on adhesion strength (Palecek *et al.*, 1997) and hence do not provide direct or sensitive measurements. This lack of a quantitative understanding of adhesion strengthening limits the interpretation of functional studies of structural and signaling adhesive components. Furthermore, it is increasingly evident that mechanotransduction between cells and their environment regulates gene expression and cell fate (Wozniak *et al.*, 2003; Engler *et al.*, 2004; McBeath *et al.*, 2004; Mammoto *et al.*, 2004; Polte *et al.*, 2004); therefore, it is essential to have direct measurements of cell–matrix adhesion strength to fully analyze these mechanosensory interactions.

The generally accepted model for adhesion strength, proposed by McClay and Erickson, postulates a two-step process consisting of initial integrin–ligand binding followed by rapid strengthening (Lotz *et al.*, 1989). The strengthening response arises from 1) increases in cell–substrate contact area (spreading), 2) receptor recruitment to anchoring sites (clustering), and 3) interactions with cytoskeletal elements that lead to enhanced force distribution among bound receptors via local membrane stiffening (focal adhesion assembly). Subsequent observations from various systems support roles for each of these mechanisms in adhesion strengthening (Choquet *et al.*, 1997; Hato *et al.*, 1998; Stupack *et al.*, 1999; Maheshwari *et al.*, 2000; Balaban *et al.*, 2001; Galbraith *et al.*,

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Address correspondence to: Andrés J. García (andres.garcia@me.gatech.edu).

Abbreviations used: FN, fibronectin; DPBS, Dulbecco's phosphate-buffered saline; PDMS, poly(dimethylsiloxane).

2002; Giannone *et al.*, 2003; Tan *et al.*, 2003). For example, laser tweezers studies have shown rapid increases in adhesion strength after initial binding that result from the recruitment of the focal adhesion components talin and vinculin (Choquet *et al.*, 1997; Galbraith *et al.*, 2002; Giannone *et al.*, 2003). Recent analyses with elastic substrates demonstrate that focal adhesions function as foci for the generation of strong anchorage in stationary cells and propulsive forces in migrating cells (Balaban *et al.*, 2001; Beningo *et al.*, 2001; Tan *et al.*, 2003). Although these studies support significant contributions from these molecular events to adhesion strengthening, an integrated understanding of the strengthening process remains incomplete. In particular, the relative contributions of each of these mechanisms to overall strengthening have not been elucidated. For example, many of these experimental results are limited to relatively short-term adhesion events (<10 min) and focus on nascent adhesive complexes before robust focal adhesions develop. Furthermore, quantitative relations among the recruitment of integrin receptors and cytoskeletal proteins to adhesive structures, the size and position of these adhesive complexes, and the strengthening response have not been derived. This information is critical for a complete understanding of how adhesive supramolecular complexes operate as functional anchorage units. In the present study, we used a robust hydrodynamic cell adhesion assay and quantitative biochemical methods in combination with micropatterned substrates to control adhesive area to analyze the adhesion strengthening response and to dissect the contributions of adhesive area, integrin binding, and focal adhesion assembly to adhesion strength.

MATERIALS AND METHODS

Cells and Reagents

NIH3T3 fibroblasts (American Type Culture Collection, Manassas, VA) were cultured in DME supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) and penicillin-streptomycin. Wild-type and vinculin-deficient F9 cells were cultured as described previously (Coll *et al.*, 1995; Xu *et al.*, 1998). Cell culture reagents, including human plasma FN and Dulbecco's phosphate-buffered saline (DPBS), were purchased from Invitrogen. Chemical reagents, including hexadecanethiol [$\text{H}_3\text{C}(\text{CH}_2)_{15}\text{SH}$] and anti-FN polyclonal and anti-talin (8D4) antibodies were obtained from Sigma (St. Louis, MO). Integrin-specific antibodies were purchased from Chemicon International (Temecula, CA), whereas anti-vinculin (V284) antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Blocking antibody against human FN (HFN7.1) was acquired from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). AlexaFluor 488- and 594-conjugated secondary antibodies, LIVE/DEAD viability kit, Hoechst-33258, and rhodamine-conjugated phalloidin were purchased from Molecular Probes (Eugene, OR). Cross-linker 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) was acquired from Pierce Chemical (Rockford, IL). Poly(dimethylsiloxane) (PDMS) elastomer and curing agent (Sylgard 184 and 186) were produced by Dow Corning (Midland, MI). Tri(ethylene glycol)-terminated alkanethiol [$\text{HO}(\text{CH}_2\text{CH}_2\text{O})_3(\text{CH}_2)_{11}\text{SH}$] was synthesized as described previously (Capadona *et al.*, 2003).

Micropatterned Surfaces

Microcontact printing was used to pattern self-assembled monolayers of alkanethiols on gold into adhesive and nonadhesive domains (Gallant *et al.*, 2002). Using standard photolithography methods, master templates of microarrays of circular islands (2, 5, 10, and 20 μm in diameter; 75- μm center-to-center spacing) were manufactured on Si wafers. Photoresist was spun onto a Si wafer and exposed to UV light through an optical mask containing the desired pattern to degrade the photoresist. The exposed areas were then etched away, leaving a template mold of recessed wells with the desired patterns. The template was exposed to (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane under vacuum to prevent adhesion of the elastomer to the exposed Si. The PDMS precursors (Sylgard 184/186; 10:1) and curing agent were mixed (10:1), poured over the template in a dish, evacuated under vacuum to remove air bubbles from the elastomer, and cured at 65°C for 12 h. The cured PDMS stamp containing the desired array of circular posts was then peeled from the template.

Glass coverslips (25 mm in diameter) were used as substrates for micropatterned arrays. After cleaning by O_2 plasma etching, coverslips were sequentially coated with optically transparent films of titanium (10 nm) and gold (20 nm) via electron beam evaporation at 2 $\text{\AA}/\text{s}$ deposition rate. For microcontact printing, stamps were cleaned by sonicating in 50% ethanol for 15 min and the flat back of the stamp was allowed to self-seal to a glass slide to provide a rigid backing. Gold (Au)-coated samples were rinsed with 95% ethanol and dried under a stream of N_2 . The face of the stamp was inked with a 1.0 mM ethanolic solution of hexadecanethiol and then quickly blown dry for 30 s with N_2 . The stamp was brought into conformal contact with the Au-coated substrate for 15 s to produce an array of circular islands of a CH_3 -terminated monolayer, to which proteins readily adsorb. Samples were subsequently incubated in a 2.0 mM ethanolic solution of tri(ethylene glycol)-terminated alkanethiol for 4 h to create a nonfouling and nonadhesive background around the CH_3 -terminated islands. Unpatterned reference substrates, on which cells spread normally, were created by immersion of a gold-coated coverslip in a 1.0 mM ethanolic solution of hexadecanethiol. After rinsing in 95% ethanol and drying, substrates were coated with FN (20 $\mu\text{g}/\text{ml}$ in DPBS) for 1 h and then blocked in 1% bovine serum albumin (BSA) for 1 h. Cells were seeded on micropatterned substrates at 225 cells/ mm^2 in DME supplemented with 0.1% FCS. For serum-free experiments, cells were plated in DME supplemented with ITS-A and 1% BSA.

Cell Adhesion Strength

Cell adhesion strength was measured with a spinning disk device (García *et al.*, 1997, 1998a). Micropatterned substrates with adherent cells were mounted on the spinning disk device and spun in DPBS + 2 mM glucose at room temperature for 5 min at a constant speed. After spinning, cells were fixed in 3.7% formaldehyde + 1% Triton X-100, stained with ethidium homodimer, and counted at specific radial positions using a Nikon TE300 equipped with a Ludl motorized stage, Spot-RT camera, and Image-Pro analysis system. Sixty-one fields (80–100 cells/field before spinning) were analyzed and cell counts were normalized to the number of cells present at the center of the disk. The fraction of adherent cells (f) was then fit to a sigmoid curve $f = 1/(1 + \exp[(\tau - \tau_{50})))$, where τ_{50} is the shear stress for 50% detachment and b is the inflection slope.

Integrin Binding and Focal Adhesion Assembly

For integrin staining, adherent cells were incubated in 1.0 mM DTSSP in chilled DPBS for 30 min to cross-link bound integrins to the underlying ECM (García *et al.*, 1999). Unreacted cross-linker was quenched for 10 min by the addition of 50 mM Tris. Uncross-linked cellular components were extracted in 0.1% SDS supplemented with 350 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin. Samples were blocked in 5% fetal bovine serum (FBS) for 1 h and incubated in integrin subunit-specific antibodies followed by fluorochrome-labeled secondary antibodies. For visualization of focal adhesions, cells were extracted in 0.5% Triton X-100 in 50 mM NaCl, 150 mM sucrose, 3 mM MgCl_2 , 20 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM PMSF, 50 mM Tris, pH 6, for 10 min to remove membrane and soluble cytoskeletal components. Extracted cells were fixed in 3.7% formaldehyde for 5 min, blocked in 5% FBS, and incubated with primary antibodies against focal adhesion components followed by a fluorochrome-labeled secondary antibodies or rhodamine-phalloidin and counterstained with Hoechst dye.

Bound integrins were quantified using a cross-linking/extraction/reversal method (García *et al.*, 1999; Keselowsky and García, 2005). Adherent cells were incubated in DTSSP (1.0 mM) for 30 min to cross-link integrins to their bound ligand. After quenching unreacted cross-linker with 50 mM Tris, cells were extracted in 0.1% SDS supplemented with protease inhibitors (350 $\mu\text{g}/\text{ml}$ PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin) to remove uncross-linked cellular components. After washing, proteins cross-linked to the dish were recovered by reversing the cross-linking in 50 mM dithiothreitol (DTT) and 0.1% SDS at 37°C for 30 min. Recovered integrins were quantified by Western blotting. Soluble extracted fractions and whole cell lysates were used as positive controls and reference to normalize for differences in cell number among substrates. In parallel samples, cross-linked integrins were visualized via immunofluorescence staining.

Focal adhesion proteins localized to adhesive complexes were isolated and quantified by a wet cleaving technique (Keselowsky and García, 2005). Cells were washed with DPBS, and a dry nitrocellulose sheet was overlaid onto the cells for 30 s. Cells were then mechanically cleaved by rapidly lifting the nitrocellulose sheet with tweezers. Remaining cellular components were rinsed and scraped in Laemmli sample buffer. Recovered proteins were analyzed by Western blotting. Whole cell lysates served as reference for normalization. Parallel samples were analyzed by immunostaining.

Small Interference RNA (siRNA)

Inhibition of vinculin expression was performed using vinculin-directed siRNA reagents (mouse vinculin, siGENOME SMARTpool siRNA; Dharmacon, Lafayette, CO). NIH3T3 fibroblasts were transfected with siRNA duplexes using DharmaFECT3 (Dharmacon) according to the manufacturer's

Figure 1. Micropatterned substrates to control cell shape and cell-substrate adhesive area. (A) Microcontact printing of self-assembled monolayers. (1) A template is used to cast a PDMS stamp. (2) Stamp is coated with CH₃-terminated alkanethiol, and (3) used to transfer alkanethiol to Au-coated substrate, creating CH₃-terminated domains. (4) The sample is then exposed to tri(ethylene glycol)-terminated alkanethiol to functionalize bare Au areas and render them nonfouling. (5) FN preferentially adsorbs onto CH₃-terminated islands, creating adhesive islands within a nonadhesive background. (B) Schematic showing spherical cell (15 μm in diameter) and array of 5-μm-diameter islands (black circles). By using different adhesive island sizes, available adhesive area can be varied over a 100-fold range. (C) Immunostaining image for FN showing adsorption only onto 10-μm-diameter islands. Bar, 20 μm. (D) Phase contrast image of NIH3T3 fibroblasts localized to 10-μm-diameter islands at 16 h. (E) Immunostaining images showing localization of integrins and talin to 10-μm-diameter adhesive island. Bar, 2 μm.

instructions. Protein expression and adhesion strength levels were evaluated at 72 h posttransfection.

Statistical Analyses

Experiments were performed in triplicate in at least three independent experiments. Data are reported as mean ± SE of the mean, and statistical comparisons using SYSTAT 8.0 were based on analysis of variance and Tukey's test for pairwise comparisons, with a p-value <0.05 considered significant. Curve fits of experimental data to specified functions were conducted using the Marquardt-Levenberg algorithm in SigmaPlot.

RESULTS

Micropatterned Substrates to Control Adhesive Area and Cell Shape

Micropatterned substrates were used to control cell-substrate adhesive area and to eliminate the contributions of changes in cell shape and spreading to cell adhesion strengthening. For example, segregation of adhesive structures to the periphery of spreading cells and changes in cell morphology may influence adhesion strengthening independently from changes in adhesive area. Microcontact printing of self-assembled monolayers of alkanethiols on gold was used to create FN-coated, cell-adhesive domains within a nonfouling/nonadhesive background (Figure 1A). Although this approach has been previously used to control cell spreading (Singhvi *et al.*, 1994; Chen *et al.*, 1997; Lehnert *et al.*, 2004), we applied it here to engineer cell adhesive area while maintaining a constant cell shape. Arrays of circular adhesive islands of varying dimensions (2, 5, 10, 20 μm in diameter) were engineered to examine a 100-fold range in cell-substrate-available adhesive area (Figure 1B). The 75-μm interisland spacing eliminated cell-cell interactions

and ensured that a cell would only interact with a single adhesive island. FN preferentially adsorbed onto the CH₃-terminated circular islands, whereas the tri(ethylene glycol)-terminated regions remained devoid of adhesive protein (Figure 1C). NIH3T3 fibroblasts adhered to FN-coated micropatterned islands (one cell/island) and remained constrained to the adhesive area (Figure 1D). Unlike endothelial cells that undergo apoptosis when grown on small micropatterns (Chen *et al.*, 1997), NIH3T3 cells on all island sizes remained viable for more than 5 d in culture with no evidence of apoptosis, as determined by annexin V expression or DNA fragmentation (our unpublished data). Furthermore, there were no differences in metabolic activity, as determined by reduction of the tetrazolium salt WST-1, among cells cultured on different adhesive island sizes. More importantly, adhesive structures, including complexes of integrin α₅β₁, vinculin, talin, α-actinin, and paxillin, localized to and remained constrained to the micropatterned island (Figure 1E). These micropatterned adhesive domains also limited cell spreading, constraining cells to a nearly spherical morphology (Figure 1D). This well-defined cell shape allows simple and direct calculation of applied detachment forces and the resultant adhesive forces generated by adhesive complexes. Together, these results demonstrate control of cell adhesive area in order to engineer focal adhesion size and position and decouple integrin clustering and focal adhesion assembly from changes in cell morphology.

Integrin-mediated Adhesion Strength Increases Rapidly until Reaching Steady-State Values

Cell adhesion strength to FN-coated micropatterned islands was quantified using a spinning disk device previously characterized by our group (García *et al.*, 1997, 1998a). This system applies a well-defined range of hydrodynamic forces to adherent cells and provides sensitive measurements of adhesion strength. Substrates containing adherent cells were mounted on the device and spun in buffer at a constant speed. Fluid flow over the cells on the disk produces a detachment force that is proportional to the hydrodynamic wall shear stress τ (force/area). The wall shear stress increases linearly with radial position (r) along the disk surface and is given by

$$\tau = 0.8 r \sqrt{\rho \mu \omega^3} \quad (1)$$

where ρ and μ are the fluid density and viscosity and ω is the rotational speed. For this configuration, cells at the center of the sample experience negligible force, whereas cell numbers decrease toward the outside of the disk as the applied force increases. Thus, in a single sample, a linear range of forces is applied to a large cell population (~6000 cells analyzed/sample). After spinning, remaining cells were fixed, stained, and counted at specific radial positions. The fraction of adherent cells (f) was calculated by dividing the number of cells in each field by the number of cells at the center of the array, where negligible forces are applied. The detachment profile (f versus τ) was then fit to a sigmoid curve ($f = 1.0/(1.0 + \exp[b(\tau - \tau_{50})])$) to obtain the shear stress for 50% detachment (τ_{50}). We define τ_{50} as the adhesion strength. Figure 2A shows a typical detachment profile and sigmoid fit ($\tau_{50} = 475$ dyne/cm², $R^2 = 0.92$). The adhesion strength values obtained with this system have been reproducible over a 2-yr period of analysis.

Blocking antibodies against human FN or integrin α₅β₁ eliminated adhesion strength to these micropatterned surfaces (Figure 2B). This result indicates that adhesion in this

Figure 2. Cell adhesion strength measurements. (A) Detachment profile showing fraction of adherent cells versus applied shear stress for cells adhering to 5- μ m-diameter islands for 16 h. Experimental points were fit to sigmoid to obtain the shear stress for 50% detachment (τ_{50}). (B) Antibodies against human FN or $\alpha_5\beta_1$ integrin completely block adhesion to micropatterned islands. (C) Immunostaining for vinculin after the application of high detachment forces illustrating detachment mechanism. Bar, 5 μ m.

system is mediated by $\alpha_5\beta_1$ binding to preadsorbed FN and excludes significant contributions to adhesion strength from other receptors and/or extracellular ligands. To elucidate the mechanism by which cell detachment occurred under the applied force, cells were spun and stained for FN, integrins, and focal adhesion components. Areas at the periphery of the disk, where cells had detached, displayed complete FN staining and minimal traces of residual integrins or

Figure 3. Evolution of cell adhesion strength over time for cells plated on 5- μ m-diameter islands. Adhesion strength exhibits rapid increases until reaching steady state values at 4 h. Steady-state values increase with FN surface density. Exponential curve fits shown ($R^2 > 0.95$).

focal adhesion components (Figure 2C). This result indicates that cell detachment took place at the integrin-FN junction, resulting in removal of the entire cell without gross failure. In contrast, cells treated with latrunculin A, an inhibitor of actin polymerization, displayed significant cytoskeletal debris after detachment (Figure 2C). This residual cytoskeletal debris indicates gross cell rupture at points above focal adhesions due to loss of cellular integrity arising from impaired actin polymerization. Together, these results demonstrate that this system provides sensitive and reliable measurements of $\alpha_5\beta_1$ integrin-FN-mediated adhesion strength.

The kinetics of the adhesion strengthening response was analyzed on micropatterned islands coated with subsaturating (20 ng/cm²) or saturating (200 ng/cm²) FN densities. Islands of 5- μ m diameter were selected because this adhesive area corresponds to the cell-substrate contact area observed at early adhesion times (15 min) for unpatterned substrates (Lotz *et al.*, 1989; García *et al.*, 1998a). Adhesion strength increased rapidly at early time points and reached steady-state values by 4 h (Figure 3). There were no differences in adhesion strength between cycloheximide-treated and control cultures, indicating that the increases in adhesion strength do not involve new protein synthesis. The strengthening kinetics is described accurately by a simple exponential fit, which provides two parameters for characterization, the steady-state adhesion strength and rise time. Steady-state adhesion strength was dependent on FN density, consistent with a simple model in which receptor-ligand bond numbers, and hence adhesion strength, increase with ligand density. There were no differences in rise time between FN densities. Furthermore, the use of micropatterned substrates that maintain nearly constant cell morphology and restrict the size and position of adhesive contacts allows analysis of the evolution of adhesion strength independently from changes in cell spreading. Adhesion strength exhibited a sevenfold enhancement from initial binding (15 min) at 65 ± 6.9 to 447 ± 32 dyne/cm² at steady state. We attribute this strengthening response to integrin recruitment and clustering and focal adhesion assembly, independently from changes in cell spreading. Finally, similar trends in adhesion strengthening have been observed in mouse embryo fibroblasts and other fibroblast lines, indicating that this response is not unique to NIH3T3 cells.

Figure 4. Adhesive area strongly modulates steady-state (16-h) adhesion strength. Values are shown for micropatterned islands (black circles) and unpatterned samples (white circle). A simple hyperbolic curve accurately describes the cell strength–adhesive area relationship.

Adhesion Strength Increases Nonlinearly with Adhesive Area

We next examined the functional dependence of adhesion strength on available adhesive area by evaluating steady state adhesion strength (16 h) for adhesive islands with different dimensions coated with saturating levels of FN. Adhesive area strongly modulated adhesion strength, resulting in significant increases at small adhesive areas and reaching saturation levels (~ 600 dyne/cm²) for the 10- μ m-diameter islands (Figure 4). The dependence of adhesion strength on adhesive area is accurately described by a hyperbolic curve ($R^2 = 0.94$). This relationship indicates that adhesive area is limiting for small areas, but above a critical value of adhesive area ($78.5 \mu\text{m}^2$), additional increments in adhesive area do not significantly influence adhesion strength. A possible explanation for the saturation limit in adhesive area is that another dominant parameter in the strengthening response, such as availability of receptor and focal adhesion molecules, becomes limiting. Remarkably, the adhesion strength plateau for the micropatterned substrates approximated the adhesion strength for unpatterned cells (average spread area $1575 \pm 89 \mu\text{m}^2$). It is important to note, however, that the effective detachment forces applied to micropatterned and unpatterned cells are different because of differences in cell morphology.

Adhesive Area Strongly Modulates Integrin Binding and Focal Adhesion Assembly

The time- and adhesive area-dependent enhancements in adhesion strength can be attributed to integrin recruitment and clustering and/or interactions with cytoskeletal proteins. To derive quantitative relationships between adhesive area and recruitment of integrins and focal adhesion components, immunostaining and biochemical analyses were performed for cells adhering for 16 h to micropatterned islands of various dimensions. Independently of micropattern size, integrins localized to and remained constrained to the adhesive island (Figure 5A). For all island sizes, integrins were preferentially localized to the periphery of the adhesive island. However, for the smaller islands (2 and 5 μ m in diameter), there was a more uniform distribution of integrins across the adhesive island, whereas for the larger islands integrins were segregated into discrete complexes. These complexes, although smaller, are reminiscent of integrin clusters in spread cells (Figure 5A). This transition from

Figure 5. Integrin binding to micropatterned substrates at steady state (16 h). (A) Immunostaining for α_5 integrin subunit showing integrin localization to micropatterned domain. Integrin staining is preferentially located to the periphery of the contact area, especially for the larger islands. Bar, 10 μ m. (B) Western blot (top) and quantification (bottom) of bound integrins (micropatterned islands [black circles], unpatterned substrate [open symbol]). Integrin binding is expressed as the percentage of levels bound in unpatterned cells, which corresponds to 20% of total integrin pool. Integrin binding increases nonlinearly with adhesive area and the relationship is accurately described by a hyperbolic curve.

a uniform distribution of receptors to discrete complexes suggests a “set point” or critical number of bound integrins. For the smaller islands, adhesive area is limiting and integrin binding would require dense packing in order to approach the set point. For larger adhesive islands, adhesive area is no longer limiting and integrins can localize to discrete clusters surrounded by regions with lower integrin packing.

As an independent but complimentary approach, bound integrin numbers were quantified using a cross-linking/extraction/reversal method (García *et al.*, 1999; Keselowsky and García, 2005). Bound integrins were covalently cross-linked to FN using the cell-impermeable bifunctional reagent DTSSP. After detergent extraction of cellular components, including unbound receptors, the cross-linker was cleaved in DTT. Recovered integrins were quantified by Western blotting. We previously demonstrated that this approach specifically isolates bound integrins and provides robust measurements of the number of integrin-FN bonds (García *et al.*, 1999; Keselowsky and García, 2005). The number of bound integrins, normalized to the number of bound integrins for unpatterned cells ($\sim 20\%$ of the total integrin pool), increased linearly with adhesive area for small islands until reaching saturation values for the larger islands and unpatterned substrates (Figure 5B). The relationship between bound integrin number and adhesive area is accurately described by a simple hyperbola ($R^2 = 0.89$). This

Figure 6. Recruitment of vinculin and talin to micropatterned substrates at steady state (16 h). (A) Immunostaining showing protein localization to micropatterned domain. Bar, 10 μm . (B) Biochemical quantification (micropatterned islands [black circles], unpatterned substrate [open symbols]) of recruited vinculin (left) and talin (right). Recruitment is expressed as the percentage of levels recruited in unpatterned cells, which corresponds to 17% of total cellular pool. Recruitment of focal adhesion components increases nonlinearly with adhesive area and the relationship is accurately described by a hyperbolic curve (excluding unpatterned group).

functional dependence indicates that adhesive area limits integrin binding for small adhesive islands, but above a threshold value, integrin binding is independent of adhesive area. Interestingly, the adhesive area for half-maximal binding (77 μm^2) is equivalent to the area for the 10- μm -diameter island (78.5 μm^2). This adhesive area is also the crossover point from uniformly distributed receptors to discrete clusters (Figure 5A).

Vinculin and talin were selected for analysis of focal adhesion assembly at steady state (16 h) because these proteins are commonly associated with focal adhesions and have

been implicated in adhesion strengthening responses (Galbraith *et al.*, 2002; Giannone *et al.*, 2003). In agreement with integrin binding results, vinculin and talin localized to adhesive structures constrained to the micropatterned islands (Figure 6A). These proteins were preferentially localized to the outer rim of the adhesive island, although discrete complexes were evident in the interior of the larger islands. Vinculin and talin recruitment to adhesive structures was quantified using a wet cleaving technique (Keselowsky and Garcia, 2005). A nitrocellulose sheet was overlaid onto cells on micropatterned arrays and rapidly removed to mechanically dissociate cell bodies from basal cell membranes containing adhesive structures. Recovered focal adhesion proteins were analyzed by Western blotting. Levels of recruited vinculin and talin were normalized to recruited levels in unpatterned cells, which corresponded to ~17% of the total cellular pool of these proteins. Both vinculin and talin recruitment increased strongly with adhesive area for the small islands and reached saturation values for the 10- and 20- μm -diameter islands (Figure 6B). Hyperbolic fits described well the relationship between focal adhesion protein recruitment and adhesive area ($R^2 > 0.94$). This functional dependence indicates that adhesive area limits focal adhesion assembly for small areas, but above 78.5 μm^2 , recruitment of focal adhesion proteins is independent of available adhesive area. Interestingly, in contrast to integrin binding, saturated levels of recruited vinculin and talin were ~35% lower than levels in unpatterned, spread cells. The reason for this difference is not known, but we hypothesize that this disparity arises from differences in the state of contractility in these cells. Indeed, Chen and colleagues demonstrated that cell shape modulates RhoA activity (McBeath *et al.*, 2004), which regulates contractility and drives focal adhesion assembly (Chrzanowska-Wodnicka and Burridge, 1996).

Correlations between Mechanical and Biochemical Events in Adhesion Strengthening

By combining the quantitative functional relationships presented above, correlations between mechanical (adhesion strength) and biochemical (integrin binding and focal adhesion assembly) events in adhesion strengthening can be derived for the first time (Figure 7). Although these correlations do not provide causal relationships, there are several notable points. First, the fact that the results for micropatterned and unpatterned cells follow the same relationship indicates that micropatterned substrates provide an appropriate model for investigating adhesive interactions. Second,

Figure 7. Relationships between adhesion strength (steady state, 16 h) and integrin binding or focal adhesion assembly for micropatterned (black circles) and unpatterned (white circles) substrates. Experimental adhesion strength values were fit to an exponential rise curve and fit parameters are shown (linear regression for talin is shown as dashed line).

Figure 8. Serum stimulation increases adhesion strength and induces recruitment of vinculin and talin to adhesive area without altering bound integrin levels. (A) Adhesion strength values for cells cultured under serum-free conditions for 16 h and stimulated with 10% serum for 30 min. (B) Immunostaining and biochemical analyses of the effects of serum stimulation on integrin binding. (C) Recruitment of vinculin and talin to adhesive structures as determined by immunostaining and biochemical analyses. Bar, 5 μ m.

there is very good correspondence between biochemical events and mechanical outputs, suggesting that these processes are tightly coupled. Therefore, mechanical analyses of adhesion strengthening could provide critical information on structure–function relationships in adhesive interactions. Third, the nonlinear nature of these correlations indicate deviations from simple models in which adhesion strength is directly proportional to the number of integrin–ligand bonds or focal adhesion area and provide insights into the development of new hypotheses or refinement of existing models. For example, simple exponential fits accurately describe the experimental data (except for talin, for which a linear regression performs equally well), in excellent agreement with theoretical analyses modeling nonuniform bond loading within the contact area (Evans, 1985; Dembo *et al.*, 1988).

Focal Adhesion Assembly Provides Only 30% of the Strengthening Response

We next examined the relative contribution of focal adhesion assembly to adhesion strengthening. First, the effects of serum stimulation on adhesion strength and focal adhesion assembly were analyzed. Serum stimulation of serum-starved cells drives focal adhesion assembly and stress fiber formation via Rho-mediated contractility (Chrzanowska-Wodnicka and Burridge, 1996; Amano *et al.*, 1997). Cells were cultured under serum-free conditions for 16 h on 5- μ m-diameter islands coated with saturating levels of FN. Cells were then switched to media containing 10% serum for 30 min before spinning and biochemical analyses. Serum stimulation enhanced adhesion strength by 30% (Figure 8A, 410 ± 14 dyne/cm² for serum-starved cells versus 542 ± 19 dyne/cm² for serum-stimulated cultures). This increase in

Figure 9. Role of vinculin in cell adhesion strength. (A) Western blot for vinculin expression in parental F9 cells, cells with vinculin gene deleted (g229), and line reexpressing vinculin (g229 + vinc). (B) Adhesion strength (16 h) is lower for cells with vinculin gene deleted than for parental and mutant reexpressing vinculin lines (* versus F9, $p < 0.004$; † versus g229 + vinc, $p < 0.03$). (C) Cells lacking vinculin (g229) do not exhibit increases in adhesion strength associated with serum stimulation.

adhesion strength did not involve changes in integrin binding, as no differences in bound integrin numbers were observed between starved and stimulated cultures (Figure 8B). In contrast, serum stimulation resulted in significant enhancements in vinculin (400%) and talin (90%) recruitment to adhesive structures (Figure 8C). Similar results were ob-

served for LPA-treated samples (our unpublished data). Furthermore, these serum-induced increases in adhesion strength and focal adhesion assembly were completely blocked by inhibitors of contractility, including blebbistatin and Y-27632 Rho-kinase inhibitor (Shin and García, unpublished observations).

To further analyze the contribution of focal adhesion assembly to adhesion strengthening, the adhesion strength of vinculin-deficient cells was evaluated on micropatterned (5- μ m-diameter) substrates coated with 200 ng/cm² FN. Vinculin-deficient cells (clone g229) exhibited adhesion strength values that were 20% lower than those for the parental F9 cells (Figure 9, A and B, 292 ± 10.1 versus 362 ± 7.2). Reexpression of full-length vinculin in vinculin-deficient cells (clone M16) rescued the deficits in adhesion strength. Furthermore, deletion of vinculin abolished the serum-induced increases in adhesion strength for serum-starved cells (Figure 9C). The slight but not significant increases in adhesion strength for the vinculin-deficient cells may be attributed to other focal adhesion proteins, including talin and α -actinin (Volberg *et al.*, 1995), which may compensate for the loss of vinculin.

Finally, the contribution of vinculin to the adhesion strength of NIH3T3 cells was examined by inhibiting vinculin expression via siRNA approaches. Transfection with siRNA duplexes reduced vinculin levels by 60–70% compared with control and mock-transfected cells (Figure 10A), whereas no differences were detected in talin expression. More importantly, knockdown of vinculin expression eliminated vinculin localization to focal adhesions (Figure 10B). Knockdown of vinculin expression also reduced adhesion strength by 25% compared with controls (Figure 10C). These results are in excellent agreement with the strengthening analysis for the vinculin-null lines. Together, these results demonstrate that focal adhesion assembly provides ~30% of the strengthening response and suggest that integrin binding and clustering provide the bulk of the enhancements in adhesion strengthening.

Figure 10. siRNA knockdown of vinculin in NIH3T3 fibroblasts reduces adhesion strength by 25%. (A) Western blot for vinculin expression showing 70% knockdown after siRNA treatment. (B) siRNA knockdown of vinculin expression eliminates vinculin localization to focal adhesions. Arrowhead denotes vinculin localization to focal adhesions in control cells. (C) Knockdown of vinculin expression reduces cell adhesion strength compared with untreated cells.

Figure 11. Mechanical analysis of adhesion strength. (A) Force balance for a cell (radius R) adhering to micropatterned adhesive island (diameter $2a$). Hydrodynamic shear flow results in shear force (F_s) and torque (T_s), which are balanced by tangential (F_{tan}) and normal tensile (F_T) and compressive (F_c) forces at the adhesive interface. For equilibrium, the force produced by an adhesive patch at the leading edge is equal to F_T and is related to the surface shear stress (τ). Table lists predicted F_T for the experimental results (τ_{50}) showing a nearly constant force of 200 nN. (B) Microscopic model of bond loading in the adhesive patch. Adhesive patch is divided into five segments. Bonds in a particular segment can occupy one of three states: 1) uniformly distributed, 2) clustered, and 3) focal adhesion (FA)-associated bonds. Adhesive force produced by segment i (F_i) is given by the force rule shown. Plot depicts resultant adhesive patch force as a function of bond number showing enhancements in adhesive force with integrin clustering and cytoskeletal interactions.

"Adhesive Patch" as Functional Adhesive Unit

A simple engineering analysis was conducted to assist in the interpretation of our experimental data for adhesion strengthening. First, a "macroscopic" model was developed to calculate the forces produced by adhesive structures to resist the applied hydrodynamic forces. The model considers a spherical cell (radius R) adhering to a micropatterned island (diameter $2a$) (Figure 11A). For mechanical equilibrium, the applied hydrodynamic shear force (F_s) and torque (T_s) must be balanced by a tangential force (F_{tan}) and tensile (F_T) and compressive (F_c) forces acting normal to the adhesive interface. Because of the use of micropatterned substrates to produce a well-defined cell shape, expressions for F_s and T_s as a function of surface shear stress τ can be easily obtained from the solutions for a sphere in shear flow (Goldman *et al.*, 1967). In line with previous analyses (Evans, 1985; Dembo *et al.*, 1988; Ward and Hammer, 1993), cell detachment is expected to occur via peeling of the leading edge of the cell. For membrane peeling, bond loading is highly nonuniform along the contact area—bond forces are maximal at the periphery and decay rapidly toward the center of the cell. Therefore, we assumed that the tensile force F_T , which represents the resultant of the bond forces in the adhesive area, acts at the leading edge of the adhesive island. By solving the equations of equilibrium, the following expression for the resultant bond force F_T as a function of adhesion strength τ was derived:

$$F_T = 32 R^2 \tau \sqrt{1 + \left(\frac{0.8 R}{a}\right)^2} \quad (2)$$

This expression was used with the experimental values for τ_{50} and geometry to compute the resultant bond force F_T (Figure 11A). Remarkably, the resultant bond force is nearly constant at 200 nN for all island sizes. This result indicates

that the force exerted by an adhesive patch at the leading edge of the cell is constant and independent of adhesive area. The increases in adhesion strength (τ_{50}) with adhesive area (Figure 4) can be simply explained by increases in the distance of the adhesive patch from the center of the adhesive area—effectively increases in the lever arm of the resultant bond force. This analysis supports the concept of a functional adhesive unit that provides a maximum adhesive force of ~200 nN.

We next formulated a "microscopic" model to gain insights into the contributions of integrin binding and focal adhesion assembly to adhesive patch function. This analysis is based on a model developed by Ward and Hammer, 1993 to examine the effects of focal contact formation on adhesion strength. The adhesive patch was divided into five segments ($i = 1-5$), which contained the bonds that provided adhesive forces (Figure 11B). Because the smallest pattern that we analyzed experimentally was 2 μm in diameter, we modeled a 1-μm adhesive patch (200-nm segments) containing a maximum of 3000 bonds (estimated from integrin binding analysis). Three cases were considered: 1) uniformly distributed bonds—bonds were equally distributed among patch segments; 2) clustered bonds—bonds localized to the outermost segment until a saturation number was reached (1000), and then the next segment was filled; 3) focal adhesion-associated bonds—bonds were distributed as in the clustered case but a fraction of them were assigned as "focal adhesion-associated" bonds (see below). The force produced by each segment (F_i) was then calculated using the rule

$$F_i = f B_i [\chi + (1 - \chi) \cdot \exp(1 - i)] \quad (3)$$

where f is the individual bond strength, B_i is the number of bonds in segment i , and χ is the fraction of bonds associated with focal adhesions. Based on previous analyses of mem-

brane peeling (Dembo *et al.*, 1988; Ward and Hammer, 1993), bond loading for both uniformly distributed and clustered bonds was assigned an exponential decay with segment number. Focal adhesion-associated bonds were treated as “rigid”—all bonds must break simultaneously. Finally, the forces produced by all segments were added to calculate the overall force for the adhesive patch.

We ran simulations to compute the adhesive patch force as a function of total bond number using published values for the individual bond force ($f = 100$ pN) (Li *et al.*, 2003) and fraction of bonds coupled to focal adhesions ($\chi = 0.33$) (Coussen *et al.*, 2002) (Figure 11B). For uniformly distributed bonds, adhesive force increases linearly with bond number. This is in excellent agreement with our experimental observations for initial adhesion strength (García *et al.*, 1998a,b). Integrin clustering alone or in combination with focal adhesion association resulted in exponential increases in adhesion force with bond number, consistent with our experimental results (Figure 7). Integrin clustering alone enhanced adhesion force over uniform bond distribution (1.6-fold enhancement for 3000 bonds) by localizing bonds to the periphery of the adhesive area and enhancing the torque resisting the applied hydrodynamic forces. Focal adhesion association further enhanced the effects of clustering alone (30%) by altering bond loading distribution. Notably, the predicted enhancements in adhesion strength arising from association with focal adhesion components agree well with the experimental results. Furthermore, the predicted values for adhesive patch force are similar to the values derived from the macroscopic model and experiments (100–200 nN). Overall, these simulations agree well with our experimental observations and assist in explaining how adhesive structure components operate as functional mechanical structures.

DISCUSSION

We integrated a robust hydrodynamic adhesion assay, quantitative biochemical approaches, and micropatterned substrates to analyze the adhesion strengthening response and to dissect the contributions of adhesive area, integrin binding, and focal adhesion assembly. By engineering cell-substrate adhesive area, the effects of integrin binding and focal adhesion assembly on adhesion strength were isolated from gross changes in spreading and cell shape. This experimental system provided sensitive and robust measurements of integrin-mediated adhesion strength over a wide range of conditions (time, adhesive area, and ligand density), and generated direct measurements of long-term (steady-state) adhesion strength. These measurements complement and greatly expand results from other approaches, such as laser tweezers, AFM, and centrifugation assays, which are limited to initial adhesion in terms of the magnitude of detachment forces that can be applied. Furthermore, the present work provides new insights on how adhesive area, integrin binding, and focal adhesion are integrated to generate functional mechanical complexes.

Adhesion strength exhibited rapid increases (~ 170 dyne/cm² h) until reaching steady-state values at 4 h. This fast strengthening response is consistent with previous observations from centrifugation and laser tweezers experiments (Lotz *et al.*, 1989; Choquet *et al.*, 1997). For 5- μ m-diameter islands, the strengthening response resulted in a sevenfold enhancement in adhesion strength. This increase in adhesion strength was attributed solely to integrin clustering and focal adhesion assembly. In contrast, cells on unpatterned substrates displayed a 12-fold enhancement in strength, re-

flecting additional contributions from spreading and segregation of adhesive structures to the periphery of the spread cell. Available adhesive area strongly modulated adhesion strength, exhibiting linear increases for small areas (<75 μ m²). Consistent with the strong dependence of adhesion strength on adhesive area, McClay and colleagues showed a positive correlation between initial adhesion strength (<15 min) and areas of cell-substrate close contact (Lotz *et al.*, 1989). Furthermore, several groups have demonstrated a relationship between traction forces and focal adhesion area (Balaban *et al.*, 2001; Galbraith *et al.*, 2002; Tan *et al.*, 2003). Notably, we demonstrate that above a threshold adhesive area (78.5 μ m²), adhesion strength and focal adhesion assembly (vinculin and talin recruitment) reach a saturation limit and further increases in adhesive area do not influence these mechanical and biochemical outcomes. In addition, this adhesive area value corresponds to the transition in integrin binding from relatively uniform bound receptors to spatially segregated complexes. This saturation limit does not seem to arise from limiting numbers of FN ligand, integrin receptors or vinculin and talin molecules, as the recruited numbers for these molecules are well below the available surface density or total cellular pool. It is possible that another adhesion molecule becomes limiting above this threshold adhesive area. Alternatively, this critical point could reflect a “set point” for the adhesive interaction. The existence of a set point suggests a higher level of integrated control of the adhesion variables (integrin binding and focal adhesion assembly), different from control at the level of the focal adhesion structure. An important attribute of a set point is that it allows for robust adaptive responses to external stimuli, such as applied forces and soluble factors. For example, the set point could be shifted to increase focal adhesion area and organization to modulate traction forces during mechanical stimulation or growth factor-induced cell migration (Davies *et al.*, 1994; Girard and Nerem, 1995; Greenwood *et al.*, 2000; Riveline *et al.*, 2001).

Adhesion strength exhibited nonlinear increases with bound integrin numbers and vinculin and talin recruitment, and the relationship between adhesion strength and these biochemical events was accurately described by exponential functions. The exponential dependence between adhesion strength and bond clusters is in excellent agreement with theoretical models for cell adhesion (Evans, 1985; Dembo *et al.*, 1988; Ward and Hammer, 1993). These models propose nonuniform bond loading along the adhesive interface, with the adhesive clusters farthest from the center of the cell providing the highest adhesive forces. Indeed, a simple mechanical equilibrium analysis revealed that the increases in adhesion strength with adhesive area could be explained by an adhesive patch localized to the periphery of the adhesive area. This analysis yielded a constant 200-nN force for the adhesive patch, independently of adhesive area. We interpret this value to be an estimate of the maximum adhesive force for a functional adhesive “unit,” which comprises bound integrins and associated cytoskeletal elements. Once this maximum force is exceeded, the adhesive patch breaks and the cell detaches; other adhesive complexes within the adhesive area cannot support the applied load because their effective moment arm is shorter. The 200 nN force is in good agreement with estimates for the peeling force required to detach adherent myotubes (Ra *et al.*, 1999). This value, however, is 10-fold higher than adhesive and propulsive forces measured on elastic substrata (Balaban *et al.*, 2001; Beningo *et al.*, 2001; Tan *et al.*, 2003). This difference suggests that adhesion complexes operate in a force regime well below their adhesion strength and underscores the fact that migra-

tion assays provide measurements of contractile and traction forces and not measures of adhesion strength.

Unexpectedly, focal adhesion assembly contributed only 20–30% of the adhesion strength at steady state as determined in three independent systems. Serum stimulation of quiescent NIH3T3 fibroblasts resulted in significant recruitment of vinculin and talin to adhesive structures and a concomitant 30% increase in adhesion strength, whereas bound integrin levels remained unchanged. Vinculin-deficient F9 cells displayed adhesion strength values that were 20% lower than those for the parental cell line. Furthermore, vinculin was responsible for the adhesion strengthening response to serum stimulation in these cells. Knockdown of vinculin expression in NIH3T3 cells also reduced adhesion strength to levels comparable to those obtained by these other approaches. These results indicate that focal adhesion assembly, in particular vinculin recruitment, contributes only 30% of the adhesion strengthening response. We attribute the bulk of the enhancements in adhesion strengthening to integrin binding and clustering. In fact, a simple force simulation indicates that, compared with the adhesion force produced by uniformly distributed bonds, integrin clustering provides a 60% enhancement in adhesive force, whereas focal adhesion assembly increases adhesion strength above integrin clustering by only 30%. These findings contrast with studies indicating a strong correlation between adhesive forces and focal adhesion area (Balaban *et al.*, 2001; Beningo *et al.*, 2001; Tan *et al.*, 2003). However, the contributions of integrin clustering and focal adhesion assembly were not separated in these analyses. Although this study supports a limited role for focal adhesion assembly on cell adhesion strength, it does not discount a central role for focal adhesions in regulating signaling interactions and establishing the direction/orientation of traction forces. Finally, it is important to point out that the relative contributions of the various steps to adhesion strengthening may vary among cell types or culture conditions due to differences in expression levels of particular adhesive components and/or differences in cytoskeletal and adhesive structures. In addition, the present analysis presents a “snap shot” in time or average measurement of a highly dynamic process.

The experimental framework presented provides a robust system to analyze cell adhesion strengthening responses for a wide range of experimental conditions. The combination of micropatterned substrates to control cell adhesive area size and position and the hydrodynamic adhesion assay provides sensitive and reproducible measurements of mechanochemical events at the adhesive interface. The present work focusing on the effects of adhesive area, integrin binding, and focal adhesion assembly establishes a baseline for the analysis of the function of structural and signaling adhesive components and the regulation of adhesive interactions.

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