Article

The Cytoskeleton Regulates Cell Attachment Strength

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ABSTRACT Quantitative information about adhesion strength is a fundamental part of our understanding of cell-extracellular matrix (ECM) interactions. Adhesion assays should measure integrin-ECM bond strength, but reports now suggest that cell components remain behind after exposure to acute force for radial shear assays in the presence of divalent cations that increase integrin-ECM affinity. Here, we show that focal adhesion proteins FAK, paxillin, and vinculin but not the cytoskeletal protein actin remain behind after shear-induced detachment of HT1080 fibrosarcoma cells. Cytoskeletal stabilization increased attachment strength by eightfold, whereas cross-linking integrins to the substrate only caused a 1.5-fold increase. Reducing temperature—only during shear application—also increased attachment strength eightfold, with detachment again occurring between focal adhesion proteins and actin. Detachment at the focal adhesion-cytoskeleton interface was also observed in mouse and human fibroblasts and was ligand-independent, highlighting the ubiquity of this mode of detachment in the presence of divalent cations. These data show that the cytoskeleton and its dynamic coupling to focal adhesions are critically important for cell adhesion in niche with divalent cations.

INTRODUCTION

Integrin-mediated adhesion to extracellular matrix (ECM) occurs via complex molecular clusters called focal adhesions (FAs) that enable cells to transduce forces and signals to and from the cell's surroundings. Proteins within FAs are intrinsically dynamic, with average integrin bond lifetimes on the order of seconds (1); thus, cell adhesion can only be achieved by the continuous binding, disengaging, and rebinding of many integrins to and from ECM, i.e., avidity. Single-molecule studies indicated that integrin binding affinity for ECM is highly influenced by niche conditions, i.e., cation type and concentration (2). Given the broad scope of cation-mediated cell processes (3), such reductionist experiments might be preferable; however, integrin affinity and avidity are internally regulated within FAs (4), and thus their response to cations has been demonstrated to differ in situ. For example, $\alpha_5\beta_1$ integrin dominated adhesion of fibrosarcoma cells supported highest attachment strength in the presence of both Mg^{2+} and Ca^{2+} (5) unlike in single-molecule experiments (2); conversely, Ca^{2+} decreased attachment strength of fibroblasts (5). Although integrin affinity may be regulated differently in situ versus at the single-molecule level, the cytoskeleton has been identified as an important contributor to adhesion strength. In the presence of cations, cells detach by a peeling mechanism when subjected to shear, i.e., cells detach piecewise beginning with the side of the cells exposed to shear and subsequently undergo cytoskeletal remodeling (5,6). Because cytoskeletal remodeling is cell type, cation type, and ligand specific, these data suggest that shear force assays could reveal differences in cytoskeletal dynamics.

A variety of methods have been developed to quantify cell adhesion in situ after cell attachment. These range from bead binding assays, e.g., biomembrane force probes and optical tweezers, to whole cell-ECM interactions, e.g., micropipette aspiration and centrifugal or shear force assays. Most of these methods apply force to dissociate bonds shortly after attachment (seconds to hours) and over extremely short periods of time (milliseconds to minutes) (7,8). Besides the differences in duration of applied force, the environmental conditions during measurement also differ. Centrifugation assays are commonly performed at 4°C, whereas other adhesion assays specify room or physiological temperature. For those assays performed at nonphysiological temperature, significant changes in protein function, e.g., folding, metabolism, etc., could alter adhesion at the cell level. For example, ATP- regulated actin polymerization and depolymerization rates are significantly lowered at subphysiological temperatures (9). Recent work has also suggested that these temperature-sensitive cytoskeletal changes could play a crucial role in cell adhesion strength when integrins are in a high affinity state due to the cations present (5). The importance of cytoskeletal dynamics is also bolstered by recent observations, which challenge the current paradigm that force-induced cell detachment of integrin-mediated cell adhesion is limited by integrin bond strength, i.e., that attachment strength is a direct measure of integrin-ECM bond strength,

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e.g. (10–12). Although some quantitative assessments of cell adhesion are in agreement with this paradigm (13,14) others have detected cell components, i.e., integrins and other FA proteins, that remain on the substrate after cell detachment (5,15,16). Although these recent data would suggest that detaching cells break their connection to the ECM higher up than at the integrin-ECM interface, i.e., somewhere between the FA-cytoskeleton, the frequency of occurrence of this detachment mechanism and its functional impact on detachment strength has yet to be determined.

Here, we analyze the molecular mechanisms that control cellular detachment under an externally applied force. These data reveal that detaching cells leave a footprint containing FA proteins behind. Drug- and temperature-induced stabilization of the cytoskeleton significantly increases attachment strength. As this increased strength is several-fold higher than attachment strength of cells with integrins cross-linked to the substrate, this demonstrates that the cytoskeleton can disconnect from the FAs during cellular detachment. Our data further suggest that this disconnection mechanism represents a cellular function, which can differ between cell type and state, resulting in cell type specific differences in apparent attachment strength.

MATERIALS AND METHODS

Cell culture and reagents

Mouse NIH 3T3 fibroblast cells, human WI38 fibroblast cells, and human HT1080 fibrosarcoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in their respective media, noting typical formulations from Life Technologies (Carlsbad, CA) (a comprehensive table of the media conditions can be found in (5)). All cells were cultured at 37°C in a humidified incubator containing 5% carbon dioxide. Integrins were cross-linked by DTSSP (3,3′-dithiobis(sulfosuccinimidylpropionate)) at 1 mM (Thermo Scientific, Carlsbad, CA). The F-Actin stabilizer phalloidin oleate (PO) was added at 100 μM (Sigma, St. Louis, MO). The inhibitors cytocholasin D and latrunculin A (Sigma) were added at 0.5 μM and 1 μM, respectively. All chemicals were added in serum free conditions (0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ and 4.5 mg/ml dextrose) for times indicated. Unless otherwise noted, cell culture products purchased were from Life Technologies.

Cell adhesion strength

25-mm glass coverslips (Fisher Scientific, St. Louis, MO) were sonicated with ethanol and pure water before being used for incubation of 10 μg/ml human fibronectin (isolated from serum (17)) or 20 μg/ml type I collagen (rat tail, BD Biosciences, Franklin Lakes, NJ) for 60 min at room temperature. Under regular conditions cells were allowed to attach for 24 h at 37°C and 5% CO_2. The coverslips were then mounted on a custom-built spinning disc device and dipped into the temperature-controlled spinning buffer (37°C). As spinning buffer, phosphate buffered saline (PBS) (without magnesium and calcium or with 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ (Cellgro, Manassas, VA). All spinning buffers contained 4.5 mg/ml Dextrose. Once immersed into the spinning buffer, coverslips were spun for 5 min at defined angular velocities and fixed with 3.7% formaldehyde immediately after spinning.

Quantification of adhesion strength

Shear stress τ by radial fluid motion over the surface of the coverslip was calculated according to (11) such that:

$$\tau = \frac{4}{5} r \sqrt{\rho \mu \omega^3}, \quad (1)$$

where r is the radial position from the center of the disk, ρ is the buffer density, μ is the buffer viscosity, and ω is the rotational speed. The viscosity and density of PBS are very similar to water (18,19) and because the viscosity is highly temperature-dependent, values were obtained as a function of temperature (20). To obtain quantitative information of adhesion strength, whole 25 mm coverslips were imaged at 10× magnification on a Nikon Ti-S microscope (Tokyo, Japan; ~1000 individual images stitched together with Metamorph 7.6 software and custom macros (Molecular Devices, Sunnyvale, CA)) and analyzed using a custom written MATLAB program (The MathWorks, Natick, MA). In brief, the user defines the outer circle of the coverslip from a stitched overview image and the software then finds the position of each nucleus relative to the center of the coverslip. Cell densities as a function of radial position and subsequently shear, are stored and combined with other measurements, e.g., those obtained at different revolutions per minute. A sigmoidal fit is used to quantify values of adhesion strength and determine the statistical error of the fit. Additionally, to determine cell alignment, cell morphology was analyzed similarly as a function of shear for each cell when stained for actin cytoskeleton.

Immunofluorescence staining and focal adhesion analysis

Fixed cells were incubated for 10 min with 0.25% Triton X-100 followed by 1% albumin overnight at 4°C for blocking. Primary paxillin antibody (1:2000, ab32084, Abcam (Cambridge, MA)) was applied for 2 h at room temperature, and then a secondary AlexaFluor 488-conjugated antibody (1:2000, Invitrogen) was applied for 1 h or rhodamine phalloidin (1:2000 Invitrogen) and Hoechst 33342 (3.2 μM, Invitrogen (Carlsbad, CA)) for 30 min at room temperature. The cells were subsequently mounted with Fluoromount-G (Southern Biotech, Birmingham, AL). All buffers used contained 1 mM $MgCl_2$. The samples were imaged by using a CARV II confocal (BD Biosciences) Nikon Eclipse Ti-S microscope equipped with a motorized, programmable stage using a Cool-Snap HQ camera (Photometrics, Tucson, AZ) and controlled by Metamorph 7.6 (Molecular Devices). A custom written MATLAB (The MathWorks) program was used to quantify cell area and focal adhesion number and size.

Green fluorescent protein (GFP) imaging

3T3 fibroblasts were transfected with GFP-Paxillin using Lipofectamine 2000 (Life Technologies). The full-length cDNA of Paxillin fused with enhanced GFP was subcloned into pCDNA3 vector (Invitrogen).

Western blotting and focal adhesion isolation

Focal adhesions were isolated using an established protocol (21). In brief, cells were exposed to ice-cold 2.5 mM triethanolamine inducing a hypotonic shock for 3 min. By rigorous pipetting with cold water containing protease inhibitor tablets (complete mini EDTA-free, Roche (Basel, Switzerland)), the remaining cell bodies were removed by hydrodynamic