Activation of beta 1 but not beta 3 integrin increases cell traction forces

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\section*{A R T I C L E   I N F O}

Article history:
Received 13 August 2012
Revised 3 January 2013
Accepted 28 January 2013
Available online 8 February 2013

Keywords:
Integrin
Adhesion
Cytoskeletal tension
Cell traction force
Micropost array
Cell spreading

\section*{A B S T R A C T}

Cell-generated traction forces induce integrin activation, leading to focal adhesion growth and cell spreading. It remains unknown, however, whether integrin activation feeds back to impact the generation of cytoskeletal tension. Here, we used elastomeric micropost arrays to measure cellular traction forces in wildtype and integrin-null cells. We report that activation of $\beta_1$ but not $\beta_3$ integrin, by either increasing density of immobilized fibronectin or treating with manganese, elicited fibroblast spreading and cytoskeletal tension. Furthermore, this force generation required Rho kinase and myosin activity. These findings suggest that integrin activation and cell traction forces comprise a bi-directional signaling unit of cell adhesion.

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\section*{1. Introduction}

The binding of integrins to extracellular matrix (ECM) initiates cell adhesion, which can be described as a series of processes including cell spreading against the underlying matrix, assembly of focal adhesions (FAs), and generation of actomyosin-mediated cytoskeletal tension against these adhesions \cite{1}. Each of these processes appears to be linked through several pathways. For example, the degree of cell spreading against a micropatterned substrate regulates RhoA activity and cytoskeletal tension \cite{2,3}, and this cytoskeletal tension is important for adhesion assembly \cite{4,5}. Conversely, it has been shown that the clustering of integrins required for adhesion assembly is critical to support cell spreading and tension generation \cite{6,7}. Because cell spreading, adhesion assembly, and cytoskeletal tension each have been shown to regulate many cellular functions including proliferation, differentiation, and migration, understanding how these processes are regulated is an important question.

Integrin receptors undergo conformational activation from a low affinity to high affinity state \cite{8,9}, and these changes in integrin activity may contribute to the regulation of cell spreading and FA assembly. Indeed, direct activation of integrins via manganese (Mn\textsuperscript{2+}) \cite{10} or conformation-modulating antibodies \cite{11} appears to enhance cell spreading and adhesion assembly \cite{12,13}. Although numerous studies have linked integrin activation to FA growth and superior cell adhesion and spreading on ECM, it is unclear whether integrin activation can also directly regulate cytoskeletal tension generation.

In this study, we found that $\beta_1$ integrin activation via increased fibronectin (FN) density or Mn\textsuperscript{2+} leads to enhanced generation of cellular traction forces. We measured these forces by culturing cells on FN-functionalized arrays of uniformly spaced elastomeric microposts, a system we developed previously to enable studies of traction force dynamics \cite{5,14}. Our data indicate that the activation state of integrins is intimately connected to basic adherent cell behaviors like contractility, which has implications for improving our understanding of the regulation of cell shape, mechanics, and function.

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http://dx.doi.org/10.1016/j.febslet.2013.01.068

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Abbreviations: $\beta_1$KO, $\beta_1$-integrin null mouse embryonic fibroblast; $\beta_3$KO, $\beta_3$-integrin null mouse embryonic fibroblast; BSA, bovine serum albumin; ECM, extracellular matrix; FA, focal adhesion; FN, fibronectin; LPA, lysophosphatidic acid; mPAD, micropost array detector; PDMS, polydimethylsiloxane; ROCK, Rho kinase; wtMEF, wildtype mouse embryonic fibroblast

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2. Materials and methods

2.1. Cell culture

Wildtype and $\beta_1$ integrin-null MEFs were provided by Dr. Richard Assouan (University of Pennsylvania) and Dr. Richard Hynes (MIT), respectively. $\beta_1$ integrin-null MEFs were maintained as previously described [15]. All cells were cultured in 10% FBS/DMEM (Atlanta Biologicals).

2.2. Reagents and antibodies

Reagents were obtained as follows: fibronectin (BD); vitronectin (Sigma); lysophosphatidic acid (Avanti Polar Lipids); Y27632 (Tocris Bioscience); blebbistatin (Calbiochem); FN blocking antibody 16G3 (20 μg/ml; gift of Dr. Martin Schwartz, University of Virginia); $\beta_1$ integrin blocking antibody BMC5 and rat control IgG (10 μg/ml; Chemicon); anti-$\beta_1$, integrin (BD); anti-GAPDH (Ambion); anti-active-$\beta_1$ integrin (clone 9EG7, BD); anti-vinculin (hVin1, Sigma–Aldrich); adenosivial sh-$\alpha_5$ integrin and scrambled sequence (gift of Dr. Rebecca Wells, University of Pennsylvania).

2.3. Cell attachment assay

Plates were coated overnight at 4 °C with FN in triplicate (BD Biosciences) and blocked with 50 μg/ml BSA/PBS. Cells were seeded, gently rinsed after 1 h with warm PBS, and quantified using CyQuant (Invitrogen Molecular Probes).

2.4. Substrate preparation

Micropost array detectors (mPADs) were fabricated using PDMS-based replica-moldling as previously described [5,16]. Microcontact printing FN on these or flat substrates, with either continuous or 625 μm² islands, was performed as described previously [17]. FN concentrations of 0.0625 or 4.0 μg/ml FN in 50 μg/ml BSA are designated as low or high FN density, respectively.

2.5. Western blotting

Cells were lysed in Laemmli sample buffer (Bio-Rad), separated via SDS–PAGE, transferred to PVDF, immunoblotted, and detected using Chemiluminescence (Invitrogen Molecular Probes).

2.6. Immunofluorescence, cell imaging, and quantitative analysis of focal adhesions and strain energies

For immunofluorescence, cells were fixed with 3.7% paraformaldehyde (Electron Microscopy Sciences), permeabilized with 0.1% Triton X-100, and labeled using primary and then secondary antibodies. Quantitative analyses of adhesions and cell area were performed using a custom-developed MATLAB program [18]. For mPAD experiments, cells were labeled with CellTracker Green CMFDA (Invitrogen Molecular Probes). Quantitative analyses of cell area and total cell strain energies on mPADs were performed as previously described [5].

2.7. Knockdown of $\alpha_5$ integrin

MEFs were infected with adenovirus encoding either shRNA directed against $\alpha_5$ integrin or a scrambled sequence [19] at a MOI of 50. Cells were trypsinized at 48 h post-infection and seeded on mPAD substrates.

2.8. Statistical analysis

For each box-and-whisker plot, 15 or more cells per condition were imaged and analyzed across 3 or more experiments. Statistical comparisons between experimental conditions used either Mann–Whitney-U tests or Wilcoxon signed-rank tests, as indicated in individual figure legends. For all tests, statistical significance was assigned at $P$-value ≤ 0.05 (ns: non-significant, *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001).

3. Results

3.1. Integrin activation enhances cell spreading and traction force

We first confirmed that increasing the density of immobilized FN and exposure to Mn²⁺ enhanced integrin activation [20,21] in our system. Wildtype mouse embryonic fibroblasts (wtMEFs) were plated on substrates coated with a range of FN densities, cultured in the presence or absence of 1 mM Mn²⁺ for 1 h, and then immunostained for activated $\beta_1$ integrin. In the absence of Mn²⁺, cells cultured on low FN density exhibited small peripheral $\beta_1$ integrin-positive adhesions (Fig. 1A). In contrast, Mn²⁺ treatment of cells on low FN resulted in increases in spread cell area and the number and size of $\beta_1$ integrin-positive adhesions (Fig. 1A and B). On high FN densities, cells displayed greater cell spreading and adhesion number and size relative to untreated cells on low FN, and Mn²⁺ treatment did not promote additional spreading (Fig. 1A and B). As a functional measure of integrin-mediated adhesion, we show that cell attachment was improved by increasing FN density and/or Mn²⁺ treatment (Fig. 1C). Together, these data confirm that shifting the equilibrium towards ECM-engaged integrin, by either increasing FN density or conformational activation of integrin by Mn²⁺, promotes cell attachment, spreading, and adhesion assembly.

We next examined whether integrin activation impacts cytoskeletal tension, by using elastomeric micropost array detector substrates (mPADs) to measure cell traction forces [5], wtMEFs attached to and spread on the posts (Fig. 1D – top left panel). Cell spreading correlated with FN density on mPADs similarly to flat substrates, and the deficiency in spreading on low FN was rescued by the addition of Mn²⁺ (Fig. 1D – top right panel and E). Importantly, we observed that Mn²⁺-induced integrin activation triggered enhanced cell traction forces on low FN, and increasing FN density also increased traction force generation (Fig. 1D – bottom panels and F). Moreover, this enhanced traction force production was blocked by the addition of a FN blocking antibody, 16G3 (Fig. 1G), demonstrating that Mn²⁺-triggered forces require the formation of new integrin–FN bonds. These data show that increasing the amount of ECM-engaged integrin leads to a net increase in traction forces.

3.2. $\beta_1$KO MEFs have defects in Mn²⁺-induced spreading and traction force generation.

Although Mn²⁺ activates integrins indiscriminately, we hypothesized that specific integrin subtypes might be important for mediating the changes in cell spreading and traction force generation in our system. Therefore, we tested the responses of MEFs carrying a deletion of the $\beta_1$ gene. As originally reported [15], expression of $\beta_1$ integrin is undetectable in these cells, as illustrated here by the absence of reactivity in a anti-$\beta_1$ Western blot (Fig. 2A). $\beta_1$KOs showed reduced attachment to FN (Fig. 2B), relative to the robust attachment curves seen for wtMEFs (Fig. 1C). To assess whether $\beta_1$KO cells could respond to Mn²⁺, we assayed whether Mn²⁺ could induce cell spreading and FA assembly on low FN. While $\beta_1$KOs...
failed to spread in the presence of Mn\(^{2+}\) (Fig. 2C–E), enhanced FA growth was still observed (Fig. 2D).

We then investigated the effect of β1 knockout on Mn\(^{2+}\)-induced cytoskeletal tension. Although β1KO cells exhibited strong basal...
Fig. 2. β1KO MEFs have defects in Mn²⁺-induced spreading and traction force generation. (A) Western blotting for β₁ integrin expression in wtMEFs and β1KO MEFs. The two bands correspond to a partially glycosylated precursor protein and the mature protein. (B) β1KO attachment curves across varied FN densities in the presence or absence of Mn²⁺. Error bars indicate standard error of the mean for 4 independent experiments done in triplicate. (C) Vinculin immunofluorescence for wtMEFs and β1KOs in the presence or absence of Mn²⁺ on low FN. Scale bar = 20 μm. (D) Quantification of spread cell area, average number of FAs per cell, and average FA area (n ≥ 25 cells/condition). (E and F) β1KO spread cell area (E) and total strain energy (F) on low or high FN density, at baseline or after Mn²⁺ treatment. (G and H) wtMEF spread cell area (G) and total strain energy (H) on low FN, following 30 min of pretreatment with either β₁ blocking antibody BMC5 or control IgG antibody (baseline) then 1 h of Mn²⁺ (Mn²⁺). (D–H) Mann–Whitney-U test (ns: non-significant, *P < 0.05, **P < 0.01, ***P < 0.001).
contractility compared to wildtype MEFs, the β1KOs failed to mount increased traction forces in response to either increased FN density or Mn²⁺ treatment (Fig. 2E and F). In fact, Mn²⁺ treatment resulted in a statistically significant decrease in traction forces on low FN (Fig. 2F).

Loss of β₁ integrin disrupts numerous integrin heterodimers, including the principal fibronectin receptor α₅β₁ integrin. To test whether α₅β₁ integrin was specifically required for Mn²⁺-induced cytoskeletal tension, we treated wtMEFs with a function-blocking α₅β₁ integrin antibody, BMCS. Inhibition of α₅β₁ integrin trended toward decreased cell spreading on low FN mPADs and did not prevent the spreading response to Mn²⁺ treatment (Fig. 2G). In spite of the trend toward decreased spreading, basal contractility showed an unexpected increase in response to BMCS (Fig. 2H), whereas MEFs treated with an isotype-matched control IgG showed similar baseline contractility to untreated cells (Fig. 2H vs. 1F). Nonetheless,
upon treatment with Mn\(^{2+}\), BMC5-treated cells showed a statistically significant decrease in strain energy that paralleled the response observed in \(\beta 1\)KOS cells (Fig. 2H vs. F). Moreover, we observed a similar loss of strain energy in response to Mn\(^{2+}\) treatment when \(\alpha 5\) integrin was depleted by RNA interference (Supp. Fig. 1A and B). Taken together, these data suggest that the increased cell traction force upon stimulation of integrin activation requires \(\beta 1\) integrin and is likely mediated by \(\alpha 5\beta 1\).

3.3. \(\beta 1\) Integrin-dependent tractions require spread cell shape, ROCK, and myosin activity

To better understand the requirement for \(\beta 1\) integrin in generating traction forces, we investigated whether these forces were mediated by non-muscle myosin II activity and whether \(\beta 1\)KOSs were competent to respond to other contractility agonists. We tested the role of myosin activity in Mn\(^{2+}\)-induced traction by pretreating wtMEFs with Y27632 and blebbistatin, pharmacological inhibitors of Rho kinase (ROCK) and myosin, respectively. Both blebbistatin (Fig. 3A) and Y27632 (Fig. 3B) prevented Mn\(^{2+}\)-induced traction forces. Prior work from our group demonstrated that cellular contractility can also be blocked by culturing cells on small micropatterned FN islands [5]. Here we observed that micropatterned islands (625 \(\mu\)m\(^2\)) of high FN prevented cells from mounting traction forces in response to Mn\(^{2+}\) stimulation (Fig. 3C), consistent with a role for actomyosin contractile machinery in mediating Mn\(^{2+}\)-dependent traction forces.

Restricting cell spreading by micropatterning results in a generalized defect in coupling extracellular stimuli with traction force production, a relationship we have reported in multiple systems [5,16]. We therefore tested whether the defect in traction force production in \(\beta 1\)KOSs could be attributed to a generalized defect in organizing or activating actomyosin contractility or was specific to Mn\(^{2+}\). Treatment of cells with lysophosphatidic acid (LPA), a strong agonist for Rho-mediated myosin activation, triggered a sustained increase in traction force in both wtMEFs and \(\beta 1\)KOSs within 1 min (Fig. 3D and E), indicating that the coupling between soluble agonists and contractility remains intact in the \(\beta 1\)KOSs.

3.4. \(\beta 1\) Integrin is dispensable for Mn\(^{2+}\)-induced traction forces

Our results suggest that, in the absence of \(\beta 1\) integrin, activation of integrins induces FA assembly, but fails to induce cell spreading or traction forces on low FN. Because fibroblasts also use \(\beta 3\) integrin to bind FN, we next evaluated whether \(\beta 1\) integrin activation could contribute to either enhanced cell spreading or traction forces following Mn\(^{2+}\) treatment. To do so, we first plated wtMEFs on vitronectin, a preferential ligand for \(\beta 3\) integrin [22]. Upon treatment with Mn\(^{2+}\), wtMEFs on vitronectin increased spread cell area (Fig. 4A) but generated no additional net traction force (Fig. 4B).

These data suggest that \(\beta 3\) integrin engagement with ECM ligand is not sufficient to mediate Mn\(^{2+}\)-induced traction forces but could support cell spreading. To test the role of \(\beta 3\) integrin in the response to Mn\(^{2+}\) more directly, we examined the effect of Mn\(^{2+}\) on \(\beta 3\)-null MEFS (\(\beta 3\)KOSs) [23]. Similar to wtMEFs, \(\beta 3\)KOSs treated with Mn\(^{2+}\) demonstrated an increase in spread cell area and FA growth and number (Fig. 4C and D). Likewise, the \(\beta 3\)KOSs increased cell traction forces after Mn\(^{2+}\) treatment (Fig. 4E). Thus, \(\beta 3\) integrin activation can promote cell spreading; however, it is dispensable for Mn\(^{2+}\)-induced traction force generation.

4. Discussion

Cellular traction forces play an integral role in cell adhesion to matrices. These forces regulate FA assembly, presumably by acting directly on integrins to activate them through “inside-out” signaling [4,5]. Additionally, myosin-mediated contractility regulates adhesion through recruitment of signaling proteins to FAs [24,25]. Signaling downstream from these FA proteins impacts proliferation [26], differentiation [27], migration [28,29], and other higher-level cellular functions. Here, we show “outside-in” signals that promote integrin activation (increased FN density, Mn\(^{2+}\)) also trigger traction force generation. This finding clarifies how the cell might sense matrix density through ECM-modulated integrin affinity that directly adjusts cytoskeletal tension to benefit the microenvironment.

Integrin activation could modulate cell traction forces through several possible mechanisms. Here, we reveal that traction forces induced by integrin activation require Rho kinase and myosin activity, suggesting that Rho GTPases could be involved. Alternatively, integrin adhesion complexes can nucleate actin polymerization via Arp2/3 in a manner dependent on the density of ligated integrins [30,31] and independently of Rho GTPases [32]. Actin polymerization creates protrusive force [33] that can drive cell motility [34] and may contribute to the integrin-mediated forces reported in this work.

Different integrin subtypes often have overlapping functions, with some instances where distinct integrins produce unique effects on cells. For example, both \(\alpha 5\beta 1\) and \(\alpha 6\beta 1\) integrins bind FN, but drive divergent migratory behavior; \(\alpha 5\beta 1\)-FN adhesion promotes thin cell protrusions and random cell migration whereas \(\alpha 6\beta 1\)-FN adhesion supports persistent migration with broad lamellipodia [35]. In fact, it has been proposed that these effects are mediated through a change in the balance of Rho/Rac signaling [35]. Here we show that in normal cells, Mn\(^{2+}\) stimulates cell traction forces in a \(\beta 1\) integrin-dependent manner, whereas in cells deficient in \(\alpha 5\) or \(\beta 1\), Mn\(^{2+}\) stimulation leads to a decrease in traction forces. While the mechanism of decreased traction forces in \(\alpha 5\) or \(\beta 1\) deficient cells is as yet unknown, it is interesting to speculate that \(\alpha 5\beta 1\)-specific signaling, such as to Rac1 GTPase, may play a role.

Our finding that activation of \(\beta 1\) integrin contributes to enhanced traction forces is consistent with previous studies in which force production on fibronectin substrates is disrupted by \(\alpha 5\beta 1\) blocking antibody in fibroblasts [36] or myocytes [37]. It is interesting that the same \(\beta 1\) integrin subtype that induces intracellular forces also undergoes conformational activation in response to extracellular forces and uniquely displays catch-and adhesion strength-reinforcing behavior upon force transmission [38–41]. In view of these previous studies, our results suggest that feedback between force sensing and traction force generation may be a necessary component to the cell mechanotransduction system. Further studies on the regulation of basic cell functions by integrins will help shed light on how cells manage complex behavior in response to mechanical and adhesives cues.

Acknowledgements

We thank R. Hynes for his kind gift of \(\beta 3\) integrin-null MEFS, R. Assoin for the wildtype MEFS, M. Schwartz for the FN blocking antibody, R. Wells for the adenosival sh-\(\alpha 5\), M. Yang and M. Lynch for assistance with mPADs, and J. Eyckmans and C. Choi for helpful discussions. This work was supported in part by grants from the National Institutes of Health (EB00262 and GM74048), the RESBIO Technology Resource for Polymeric Biomaterials, and Center for Engineering Cells and Regeneration of the University of Pennsylvania. D.M.C. acknowledges financial support from the Ruth L. Kirschstein National Research Service Award, and R.A.D. was supported by the National Science Foundation.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.01.068.

References
