Mechanosensing machinery for cells under low substratum rigidity

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Wei WC, Lin HH, Shen MR, Tang MJ. Mechanosensing machinery for cells under low substratum rigidity. Am J Physiol Cell Physiol 295: C1579–C1589, 2008.—Mechanical stimuli are essential during development and tumorigenesis. However, how cells sense their physical environment under low rigidity is still unknown. Here we show that low rigidity of collagen gel downregulates β₁-integrin activation, clustering, and focal adhesion kinase (FAK) Y397 phosphorylation, which is mediated by delayed raft formation. Moreover, overexpression of autoclustered β₁-integrin (V737N), but not constitutively active β₁-integrin (G429N), rescues FAKY397 phosphorylation level suppressed by low substratum rigidity. Using fluorescence resonance energy transfer to assess β₁-integrin clustering, we have found that substratum rigidity between 58 and 386 Pa triggers β₁-integrin clustering in a dose-dependent manner, which is highly dependent on actin filaments but not microtubules. Furthermore, augmentation of β₁-integrin clustering enhances the interaction between β₁-integrin, FAK, and talin. Our results indicate that contact with collagen fibrils is not sufficient for integrin activation. However, substratum rigidity is required for integrin clustering and activation. Together, our findings provide new insight into the mechanosensing machinery and the mode of action for epithelial cells in response to their physical environment under low rigidity.

Actuation of integrin leads to the recruitment and organization of a number of different cytoskeletal proteins (α-actinin, talin, paxillin, etc.) and signaling molecules [focal adhesion kinase (FAK), Src, etc.] into focal adhesion sites (9, 18, 30, 59, 61). Among numerous focal adhesion complex proteins, FAK is a key regulator (26). It transmits information from integrin to the various signaling pathways, including those that regulate cellular events such as cell shape, migration, growth, differentiation, and apoptosis (22, 23, 44, 52). FAK is a 125-kDa cytoplasmic tyrosine kinase and is autophosphorylated at Tyr397 immediately after integrin clustering. This creates a binding site for the src homology 2 (SH2) domain of Src (46, 58). The recruited, active Src phosphorylates several other tyrosine residues in FAK and creates phosphotyrosine binding sites for other signaling molecules, such as Grb2, p130cas, and phosphatidylinositol 3-kinase (6, 24). There are five major sites phosphorylated by Src: Y407, Y576/577, Y616, and Y925. Phosphorylation at Y576/577 and Y861 enhances FAK kinase activity (3), and Y925 is a binding site for the SH2 domain protein Grb2 (47).

Current understanding of how ECM affects cell behaviors has been taken primarily from in vitro studies in which cells were cultured on rigid dishes coated with a thin layer of ECM. However, recent studies showed that sensing mechanical stimuli is also crucial for cells to detect several useful signals from the external environment during development and tumorigenesis (14, 15, 27, 42, 51). The three-dimensional collagen gel culture system has been used as a model to study ECM signaling because it provides not only biochemical signals but also biophysical properties (52, 53). The elastic modulus of soft tissue is in the few hundreds to several thousands of pascals range that can be made by collagen gel (37, 46, 57). Previous studies showed that low substratum rigidity downregulates focal adhesion complex protein expression and phosphorylation (13, 54). However, the mechanosensing machinery as well as its mode of action are unclear.

In this study, we attempted to examine whether and how collagen gel affects ECM signaling via its biophysical property. Our data indicate that cells sense external mechanical stimulation by using a set of mechanical sensing machinery that is composed of lipid rafts, β₁-integrin, FAK, and the actin cytoskeleton. We also delineated the mode of action of this mechanical sensing machinery.

MATERIALS AND METHODS

Cells and cell culture. Madin-Darby canine kidney (MDCK) and HEK293T transfectants were maintained in DMEM (from GIBCO) supplemented with 10% fetal bovine serum under 5% CO₂ at 37°C

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(51). These cells were seeded on different substrata at a density of 10^6 cells per 100-mm dish. In some experiments, cells were pretreated with different inhibitors such as cytochalasin D, colcemide (from Biosource), and methyl-β-cyclohexyliden (MβCD; from Sigma).

Preparation of collagen gel and polyacrylamide gel. For preparation of collagen gel, type I collagen was prepared according to the established procedure described previously (29) from tendons of rat tails provided by the National Cheng Kung University Medical College animal center. To prepare 0.3% collagen gel, collagen stock was mixed with 5.7× DMEM, 2.5% NaHCO₃, 0.1 M HEPS, 0.17 M CaCl₂, 1 N NaOH, and culture medium. The mixtures were dispensed in the culture dish (4 ml/100 mm dish) and allowed to gelate. To prepare the collagen gel-coated dish, 0.3% collagen gel solution was added to culture dishes to cover the surface. The culture dish was then tilted, and the excess amount of collagen gel was aspirated. The collagen gel-coated dishes were air-dried and washed twice with normal culture medium before use. Polyacrylamide gel was prepared according to a previously described method (55) with the following modifications. Gels were prepared with 5% acrylamide and bisacrylamide ranging from 0.02% to 0.085%. The elastic modulus of the gels was detected by rheometer (Thermo, HAAK Rheometer). The polyacrylamide gel was coated with 200 μg/ml type I collagen (from BD) by using a photocactivatable sulfonated cross-linker, sulfo-SANPAH (from Pierce), before addition of cells.

Collection of cell lysate and Western blot. Cell lysates were harvested in modified RIPA buffer (150 mM NaCl, 1 mM EGTA, 50 mM Tris pH 7.4, 10% glycerol, 1% Triton X-100, 1% sodium orthovanadate, and protease inhibitor cocktail). The lysates were analyzed by Western blot with antibodies against FAK

RESULTS

Low substratum rigidity downregulates FAKY397 phosphorylation and β₁-integrin activation. To investigate whether low substratum rigidity affects focal adhesion signals, we examined FAK phosphorylation levels of MDCK cells cultured on dish, collagen gel-coated dish, or collagen gel. By rheometer measurement, the rigidity of the collagen gel-coated dish and the collagen gel are 10⁷ Pa and 20 Pa, respectively (57). When cells were seeded and allowed to spread on collagen gel for 30 min, only the level of FAKY397 phosphorylation was suppressed, while phosphorylation of FAK at other tyrosine sites (407, 577, 861, and 925) remained elevated similar to those of collagen gel-coated dish, they exhibited little FAKY397 phosphorylation and up-regulation of β₁-integrin, activated β₁-integrin (clone B44) (from Chemicon), Myc, hemagglutinin, discoi din domain receptor (DDR)-1 (from Santa Cruz Biotechnology), and β-actin (from Amersham) and an enhanced chemiluminescence (ECL) system (from Amersham-Pharmacia).

Immunofluorescence and confocal study. Cells cultured on collagen gel-coated dishes or collagen gel for 30 min were washed three times with ice-cold phosphate-buffered saline (PBS) and fixed and permeabilized with 4% paraformaldehyde and 0.5% Triton X-100 prepared in PBS for 15 min at room temperature. Fixed cells were incubated with anti-FAK or anti-phosphorylated FAKY397 antibody overnight at 4°C. Cells were then washed and incubated with the proper Alexa Fluor 488-conjugated secondary antibody (from Molecular Probes) and phalloidin-tetramethylrhodamine isothiocyanate (from Sigma) for 1 h at room temperature. The immunofluorescence images were taken by confocal microscopy (Olympus, FV-1000).

Raf7 fractionation and staining. Cells were cultured under different substratum rigidities for 30 min and were lysed in ice-cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl) containing 1% Triton X-100, 1 mM sodium orthovanadate, and protease inhibitor cocktail. Equal amounts of these cell lysates were brought to a volume of 2 ml with 40% OptiPrep and then overlaid with 4 ml of 30% OptiPrep and 2 ml of sucrose 5% OptiPrep. Lysates were then ultracentrifuged in a SW40.1Ti rotor (Beckman, Palo Alto, CA) at 40,000 rpm for 16 h to result in a gradient. The resulting pellets of each fraction were dissolved in lysis buffer and stored at −80°C before being tested.

Fluorescence resonance energy transfer measurement. Fluorescence resonance energy transfer (FRET) efficiency was measured as described previously (34). In brief, 293T cells were transiently transfected with wild-type, constitutively activated, or autoclustering β₁-integrin for 48 h and were suspended or cultured on different substratum rigidities for 30 min. Cells exhibiting relatively similar intensity levels of monomeric cyan fluorescent protein (mCFP) and yellow fluorescent protein (mYFP) under confocal microscope (Olympus, FV-1000) were selected for experiments. Ten to fifteen regions of interest (ROIs) at adhesion points of the mCFP image were randomly chosen and analyzed. Each data set contains ~100 individual ROIs from 10–15 individual cells in at least 3 separate experiments. ROIs exhibiting saturation in either the CFP or YFP channel were eliminated from the analysis. FRET efficiency (E, %) was calculated as E = [1 − (FmCFP/Pre/FmCFP/Pos1)] × 100, where FmCFP/Pre and FmCFP/Pos are intensity of mCFP emission before and after mYFP photobleaching, respectively. Data were fit to Lineweaver-Burk plots (1/E = 1/Eₘₐₓ + K/Eₘₐₓ × 1/F) with Prism software. K was calculated from the slope of Lineweaver-Burk plots (K/Eₘₐₓ) and 1/Eₘₐₓ at the y-intercept. The curves shown in Figs. 5 and 6 were drawn by using the K and Eₘₐₓ values with Prism software.

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with FAKY397 phosphorylation and β1-integrin activation, cells were seeded and allowed to spread on collagen-coated polyacrylamide gel with different moduli of rigidity for 30 min. The levels of FAKY397 phosphorylation as well as β1-integrin activation depended on substratum rigidity, particularly at rigidities lower than 386 Pa, where they react in a dose-responsive manner (Fig. 2C). The responses of β1-integrin activation and FAKY397 phosphorylation fit a simple hyperbola in elastic modulus. The \( K_D \) (half-saturation) values for β1-integrin and FAKY397 are 346.3 Pa and 277.1 Pa, respectively (Fig. 2D). These data indicate that low substratum rigidity fails to fully activate FAKY397 and β1-integrin.

Lipid raft plays crucial role in activating β1-integrin. Since β1-integrin activation is the upstream signal of FAKY397 phosphorylation, we addressed how low substratum rigidity regulates β1-integrin activation. Previous studies showed that FAK regulates the “inside-out” signal of integrin activation (16). However, neither FAK nor another collagen receptor, DDR-1, which inhibits \( \alpha_\beta_1 \)-integrin-induced signal (51), was involved in low-rigidity-regulated β1-integrin inactivation (data not shown). Recent studies suggested that the membrane microdomain, lipid raft, participated in regulation of integrin function (36). We employed live cell imaging to observe the distribution of lipid rafts in cells seeded and allowed to spread on a collagen-coated dish and collagen gel within 4 h. Cells were stained with Alexa Fluor 488-conjugated cholera toxin (CTX) B as a lipid raft marker. We found that low rigidity delayed the formation of lipid rafts (Fig. 3, A and B). A disperse distribution of lipid raft on the cell membrane was observed when cells were seeded and allowed to spread on collagen gel-coated dishes for 30 min. In contrast, little rafts were seen when cells were seeded on collagen gels for the same time. An immunofluorescence study was performed in order to delineate the localization of β1-integrins and rafts in the cell membrane. We found that active β1-integrins were present and colocalized with lipid rafts when cells were cultured on the collagen gel-coated dish but not the collagen gel for 30 min (Fig. 3C). Furthermore, we employed a sucrose gradient to separate raft and nonraft fractions of cells. β1-Integrin was not present in raft fractions when cells were cultured on collagen gel (Fig. 3D). Thus our data show that the activated β1-integrin is associated with markers of lipid rafts. To further confirm this, cells were cultured on a collagen gel-coated dish with treatment with MβCD, an inhibitor of lipid raft formation by depletion of membrane cholesterol. We found that MβCD inhibited β1-integrin activation as well as...
Fig. 2. Regulation of \( \beta_1 \)-integrin activation by substratum rigidity. A, after being cultured for 30 min, living cells were incubated with \( \beta_1 \)-integrin antibody and the membrane-bound \( \beta_1 \)-integrin was detected by FACScan analysis. B, suspended MDCK cells were treated with soluble collagen (200 \( \mu \)g/ml) or denatured collagen gelatin (200 \( \mu \)g/ml) for 30 min. Cell lysates were harvested and analyzed by Western blot using antibodies to detect activated \( \beta_1 \)-integrin (LIBS) and \( \beta_1 \)-integrin. C, MDCK cells were cultured on polyacrylamide gel with different moduli of rigidity for 30 min. Cell lysates were analyzed by Western blot using antibodies to detect \( \beta_1 \)-integrin (LIBS), \( \beta_1 \)-integrin, Y397-phosphorylated FAK, and FAK protein. D, quantitative densitometry of relative \( \beta_1 \)-integrin and FAKY397 activation level. Fitting curves were calculated by Prism software. Solid and dashed lines represent the curve fit for \( \beta_1 \)-integrin activation and FAKY397 phosphorylation, respectively.

FAKY397P, while repletion of membrane cholesterol by exposing cells to \( \Phi \)BCD saturated with cholesterol (39) reversed both \( \beta_1 \)-integrin activation and FAKY397 phosphorylation (Fig. 3E). These results indicate that under rigid substratum the activation of \( \beta_1 \)-integrin requires lipid rafts.

**\( \beta_1 \)-Integrin clustering is essential for FAKY397 phosphorylation.** To delineate whether low substratum rigidity-induced downregulation of FAKY397 phosphorylation is mediated by reduction of \( \beta_1 \)-integrin activation, MDCK cells stably overexpressing wild-type, constitutively active (G429N) \( \beta_1 \)-integrin (40) were employed. The results showed that constitutively active \( \beta_1 \)-integrin failed to rescue FAKY397 phosphorylation when cells were cultured under low substratum rigidity (Fig. 4A). To specify whether \( \beta_1 \)-integrin clustering is involved in regulating FAKY397 phosphorylation, green fluorescent protein (GFP)-conjugated wild-type, constitutively active, or autoclustered (V737N) \( \beta_1 \)-integrin (46) was transiently transfected into HEK293T cells. Autoclustered \( \beta_1 \)-integrin formed aggregations under low substratum rigidity (Fig. 4B). We found that autoclustered but not wild-type or constitutively active \( \beta_1 \)-integrin rescued FAKY397 phosphorylation when cells were cultured under low rigidity (Fig. 4C). To further investigate whether autoclustered \( \beta_1 \)-integrin regulates focal adhesion formation, actin organization, and cell spreading, GFP-conjugated wild-type, constitutively active, or autoclustered \( \beta_1 \)-integrin was transiently transfected into HEK293T cells. These \( \beta_1 \)-integrin transfecants were seeded and allowed to spread on a collagen gel-coated dish or collagen for 30 min and were immunostained with FAK (marker of focal adhesion) or actin. The focal adhesion sizes and numbers were then measured from the FAK-positive area with Image Pro Plus software under confocal microscopy (Fig. 4, D and E). In addition, the staining of actin filament that revealed the cell shape and the cell area was measured with the same software. The results are shown in Fig. 4, F and G. Autoclustered but not wild-type or constitutively active \( \beta_1 \)-integrin increased the sizes and number of focal adhesion formation, actin organization, and cell spreading even in cells cultured under low rigidity. These data indicate that \( \beta_1 \)-integrin clustering but not activation is prerequisite for FAKY397 phosphorylation, regardless of substratum stiffness.

**Low substratum rigidity downregulates level of \( \beta_1 \)-integrin clustering.** To delineate how substratum rigidity regulates \( \beta_1 \)-integrin clustering, we employed FRET. HEK293T cells transiently transfected with wild-type, constitutively active, or autoclustered \( \beta_1 \)-integrin-conjugated mCFP or mYFP were established for the measurement of FRET. Cells were suspended or seeded and allowed to spread either on a collagen gel-coated dish or collagen gel for 30 min and subjected to FRET examination. The FRET efficiency was assessed based on methods previously described (34, 60). Results showed that when cells were suspended \( \beta_1 \)-integrin was not clustered at all. Clustering of \( \beta_1 \)-integrin was markedly increased when cells were cultured on the collagen gel-coated dish. However, \( \beta_1 \)-integrin clustering remained at a low level when cells were cultured on collagen gel (Fig. 5, A and B). To delineate the correlation between substratum rigidity and \( \beta_1 \)-integrin clustering, HEK293T cells transiently cotransfected with wild-type \( \beta_1 \)-integrin-conjugated mCFP or mYFP were cultured on collagen-coated polyacrylamide gel with different moduli of rigidity. At a rigidity of >386 Pa, \( \beta_1 \)-integrin was highly clustered. The level of \( \beta_1 \)-integrin clustering was highly correlated with substratum rigidity in the range between 58 and 386 Pa. \( \beta_1 \)-Integrin clustering was diminished under substratum rigidity of 58 Pa (Fig. 5, C and D). The responses of value of FRET constant \( K \) fit a simple hyperbola in elastic modulus.
Fig. 3. Lipid raft is required for $\beta_1$-integrin activation and FAKY397 phosphorylation. A: MDCK cells were cultured on collagen gel-coated or collagen gel for indicated times. Living cells were stained with cholera toxin (CTX) B-conjugated Alexa Fluor 488. Immunofluorescence images were taken by confocal microscopy (Olympus, FV-1000). B: quantification of CTX B-positive area on the cell membrane with Image Pro Plus software. C: MDCK cells were culture on collagen gel-coated dish or collagen gel for 30 min. Cells were fixed but not permeabilized and then stained with mouse anti-active $\beta_1$-integrin (Huts-4) or anti-$\beta_1$-integrin antibodies followed by Alexa Fluor 594-conjugated secondary goat IgG (red) and Alexa Fluor 488-labeled CTX B (green). Immunofluorescence images were taken by confocal microscopy (Olympus, FV-1000). Bar, 10 $\mu$m. D: MDCK cells were cultured on collagen gel-coated dish or collagen gel for 30 min. Lysates were fractionated by OptiPrep and analyzed by Western blot using antibodies to detect $\beta_1$-integrin, caveolin-1, Na$/K^+$ pump. E: MDCK cells were pretreated with methyl-$\beta$-cyclodextrin (M$\beta$CD, 20 mM) or repletion of cholesterol for 1 h, followed by culture on collagen gel-coated dish for 30 min. Cell lysates were harvested and analyzed by Western blot using antibodies to detect activated $\beta_1$-integrin (LIBS), $\beta_1$-integrin, Y397-phosphorylated FAK, and FAK.
The $K_d$ (half-saturation) was 251.2 Pa (Fig. 5E). These data show that $\beta_1$-integrin clustering depends on an external force provided by substratum rigidity.

**Actin cytoskeleton contributes to $\beta_1$-integrin clustering.** We also explored whether internal forces provided by the cytoskeleton are also involved in regulating $\beta_1$-integrin clustering. Cells were cultured on a collagen gel-coated dish and then treated with cytochalasin D, myosin light chain kinase (MLCK) inhibitor ML-7, Rho kinase (ROCK) inhibitor Y27632, or colcemide to disrupt actin filaments or microtubules. Cells still spread out when microtubules were disrupted but spread very little after the disruption of actin filaments (Fig. 6A). Although $\beta_1$-integrin activation was not affected by disruption of the actin filaments or microtubules, $\beta_1$-integrin clustering and FAKY397 phosphorylation level were downregulated by cytochalasin D, ROCK inhibitor Y27632, and MLCK inhibitor ML-7 but not by colcemide (Fig. 6, B–D). These data indicate that $\beta_1$-integrin clustering but not activation depends on internal force provided by the actin cytoskeleton.

**Augmentation of $\beta_1$-integrin clustering enhances interaction of $\beta_1$-integrin, FAK, and talin.** We further investigated the molecular mechanism by which $\beta_1$-integrin clustering leads to FAKY397 phosphorylation. Recent evidence revealed that FAK activity is regulated through the interaction of its FERM-like NH$_2$-terminal domain with the cytoplasmic domain of $\beta_1$-integrin to release an autoinhibitory interaction of FERM domain with its kinase domain (11, 12). Other evidence also showed that talin is important in regulating integrin signaling (22). Therefore, whether $\beta_1$-integrin clustering affects the in-
The interaction between β₁-integrin, FAK, and talin was determined. HEK293T cells transiently transfected with wild-type, constitutively active (G429N), or autoclustered (V737N) β₁-integrin were cultured on polyacrylamide gel with different substratum rigidities, and the interactions between exogenous β₁-integrin, FAK, and talin were examined. With the substratum rigidity increase, the interaction between exogenous β₁-integrin, FAK, and talin increases (Fig. 7A). The response of the amount of β₁-integrin associate protein fits a simple hyperbola in elastic modulus (Fig. 7B). Transient transfection of equal amounts of GFP-conjugated wild-type, constitutively active (G429N), or autoclustered (V737N) β₁-integrin into HEK293T cells was performed (Fig. 7C). Cells were seeded and allowed to spread on a collagen gel-coated dish, collagen gel, or polyacrylamide gel with different moduli of rigidity for 30 min. β₁-Integrin clustering was assessed by fluorescence resonance energy transfer (FRET) under confocal microscopy (Olympus, FV-1000). C and D: HEK293T cells were transfected with wild-type β₁-integrin-conjugated mCFP and mYFP. Cells were cultured on polyacrylamide gel with different moduli of rigidity for 30 min. β₁-Integrin clustering was assessed by FRET under confocal microscopy (Olympus, FV-1000). C: representative image of FRET. D: quantitative results of FRET.

Fig. 5. Substratum rigidity controls β₁-integrin clustering. A and B: HEK293T cells transfected with wild-type, constitutively active (G429N), or autoclustered (V737N) β₁-integrin-conjugated monomeric cyan fluorescent protein (mCFP) and yellow fluorescent protein (mYFP) were suspended or cultured on collagen gel-coated dish or collagen gel for 30 min. β₁-Integrin clustering was assessed by fluorescence resonance energy transfer (FRET) under confocal microscopy (Olympus, FV-1000). A: representative image of FRET. B: quantitative results of FRET. C and D: HEK293T cells transfected with wild-type β₁-integrin-conjugated mCFP and mYFP. Cells were cultured on polyacrylamide gel with different moduli of rigidity for 30 min. β₁-Integrin clustering was assessed by FRET under confocal microscopy (Olympus, FV-1000). C: representative image of FRET. D: quantitative results of FRET.

E: plot of substratum rigidity vs. FRET constant K.
Fig. 6. Substratum rigidity-controlled β1-integrin clustering is regulated by actin cytoskeleton. HEK293T cells transfected with wild-type β1-integrin-conjugated mCFP and mYFP were cultured on a collagen gel-coated dish for 4 h and treated with cytochalasin D (10 μg/ml), ML-7 (50 μg/ml), Y27632 (10 μg/ml), or colcemide (10 μg/ml) for 30 min. A: cells were fixed and stained for actin (red), Y397-phosphorylated FAK (green), and nucleus (blue). Images were taken by confocal microscopy (Olympus, FV-1000). Bar, 10 μm. B and C: β1-integrin clustering was assessed by FRET under confocal microscopy (Olympus, FV-1000). B: representative image of FRET. C: quantitative results of FRET. D: cell lysates were analyzed by Western blot using antibodies to detect activated β1-integrin (LIBS), β1-integrin, Y397-phosphorylated FAK, and FAK.
Fig. 7. Autoclustered β1-integrin increases the interaction between β1-integrin, FAK, and talin in cells cultured under low substratum rigidity. A and B: transient transfection of equal amount of wild-type (WT), constitutively active (G429N), or autoclustered (V737N) β1-integrin into HEK293T cells was performed. A: cells were cultured on dish for 24 h, and lysates were analyzed by Western blot (IB) with antibodies against talin, activated β1-integrin (LIBS), β1-integrin, Y397-phosphorylated FAK, or FAK protein. IP, immunoprecipitation. B: cells were cultured on collagen gel-coated dish or collagen gel for 30 min. Cell lysates were immunoprecipitated with control bead (–), anti-GFP antibody and then immunoblotted with anti-talin, -Y397 phosphorylated FAK, -FAK, or -GFP antibodies. C: HEK293T cells transiently transfected with wild-type β1-integrin were cultured on polyacrylamide gel with different moduli of rigidity for 30 min. Cell lysates were immunoprecipitated with control bead (–), anti-GFP antibody and then immunoblotted with anti-talin, -Y397-phosphorylated FAK, -FAK, or -GFP antibodies. D: quantitative densitometry of relative β1-integrin associate protein level. Fitting curves were calculated with Prism software.

Fig. 8. Schematic figure depicting the mechanosensing machinery and the mode of action of cells in response to low rigidity. Substratum rigidity-induced β1-integrin activation is mediated by lipid rafts. Substratum rigidity also regulates β1-integrin clustering and FAKY397 phosphorylation through actin organization.
zation abolished FAKY397 phosphorylation. Cells overexpressing autophosphorylation site mutant FAK (FAK(Y397F)) retain well-constructed actin filaments when they are cultured on collagen gel (unpublished data). Thus we deduce that the feedback control loop leads to downstream FAKY397 phosphorylation.

We showed that lipid raft externalization to the membrane is associated with cell spreading. A recent study showed that adhesion-induced lipid raft externalization depends on microtubules (2). To test whether the cytoskeleton is also involved in regulating raft transport in our model, HEK293T cells were cultured on a collagen gel-coated dish for 4 h and treated with or without drugs to disrupt actin filaments or microtubules. However, our data showed that disruption of actin filament or microtubules does not affect the raft transport (data not shown). Although the cell spreading process is impeded by low substratum rigidity, we found that disruption of lipid rafts by MI6C reduced β1-integrin activation, FAK phosphorylation (Fig. 3E), the level of β1-integrin clustering, and cell spreading as well (data not shown). Thus we deduced that raft formation is prior to β1-integrin signaling. Recent work also suggested that line tension, the interfacial energy at the raft domain edge, is a key parameter determining the distribution of rafts (17). Applying lateral tension on the membrane increases raft formation through increasing line tension (1). Thus it is also possible that substratum rigidity regulates raft formation by controlling the physical property of the cell membrane. The details of this mechanism will be investigated in the future.

Talin is a cytoplasmic protein with a globular head domain and an elongated rod domain that acts as an essential linkage between integrins and the actin cytoskeleton. Previous studies showed that talin is also crucial in regulating integrin signaling. The NH2-terminal globular head domain of talin is responsible for promoting integrin activation and clustering (4, 10, 44, 56). Since force-induced exposure of vinculin-binding sites on for promoting integrin activation and clustering (4, 10, 44, 56).

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