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Low Substratum Rigidity of Collagen Gel Promotes ERK Phosphorylation Via Lipid Raft to Augment Cell Migration

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Abstract Previous study demonstrated that low substratum rigidity down-regulates focal adhesion proteins. In this study we found that cells cultured on collagen gel exhibited higher migration capacity than those cultured on collagen gel-coated dishes. Low rigidity of collagen gel induced delayed but persistent phosphorylation of ERK1/2. Inhibition of collagen gel-induced ERK1/2 phosphorylation by MEK inhibitors and ERK2 kinase mutant induced a rounding up of the cells and prevented collagen gel-induced cell migration. Interestingly, phosphorylated ERK1/2 induced by low rigidity was present in focal adhesion sites and the lipid raft. MβCD (Methyl-β-cyclodextrin), a lipid raft inhibitor, inhibited collagen gel-induced ERK1/2 phosphorylation, and cell migration. Overexpression of FAK C-terminal fragment (FRNK) in MDCK cells triggered ERK phosphorylation. Meanwhile, low substratum rigidity induced degradation of FAK into a 35 kDa C-terminal fragment. A calpain inhibitor that partially rescued FAK degradation also prevented low rigidity-induced ERK phosphorylation. However, MβCD did not prevent low rigidity-induced FAK degradation. Taken together, we demonstrate that the degradation product of FAK induced by collagen gel triggers activation of ERK1/2, which in turn facilitates cell spreading and migration through the lipid raft. J. Cell. Biochem. 103: 1111–1124, 2008.

Key words: substratum rigidity; ERK1/2; migration; spreading; lipid raft; FAK

The interaction between cell and extracellular matrix (ECM) is the pivotal event for cell behavior and physiology [Hynes, 2002]. Physical properties have been shown to play an important role in regulating cycle progression, apoptosis, differentiation as well as migration [Pelham and Wang, 1997; Wang et al., 2000; Wozniak et al., 2003]. Cell movement and focal adhesions can be regulated by physical interactions at the cell-substrate interface. Recent studies suggested that focal adhesion or stretch-activated channels served as mechanosensors regulating the downstream signal transduction in response to the physical property of environment [Geiger and Bershadsky, 2002]. We previously demonstrated that low substratum rigidity down-regulated focal adhesion complex proteins via α2β1 integrin [Wang et al., 2003]. Among several focal adhesion complex proteins, focal adhesion kinase (FAK) is one of the most important components in the regulation of cell motility. Several studies show

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that FAK controls the formation and disassembly of focal contact that is required for cell migration. Overexpression of FAK enhances cell migration [Cary et al., 1996], whereas FAK-deficient cells exhibit an increased number of prominent focal adhesions and decreased motility [Ilic et al., 1995]. In addition, overexpression of FRNK (FAK-related non kinase), the dominant negative FAK, inhibits the rate of cell migration [Taylor et al., 2001]. Reconstitution of FAK-deficient cells with wild type FAK restores cell migration, whereas reintroduction of FAK mutants lacking kinase activity (Y397F mutant) fails to do so [Sieg et al., 1999]. Thus, it is likely that low substratum rigidity would hamper cell migration, unless it may trigger alternative signal pathways leading to cell migration.

In addition to FAK, there are several other signal pathways that serve to regulate cell migration. Recent data showed that mitogen-activated protein kinases (MAPK), including ERK1/2, Jun N-terminus kinase (JNK) and p38, also play crucial roles in cell migration [Huang et al., 2004]. Among these signaling pathways, activation of ERK1/2 is the most important pathway involved in cell spreading and migration under certain conditions [Nguyen et al., 1999; Fincham et al., 2000; Ku and Meier, 2000; Carragher et al., 2003; Subauste et al., 2004]. In this study, we explored whether low substratum rigidity of collagen gel could trigger ERK phosphorylation to regulate cell migration.

Lipid raft/caveolae is the special microdomain in cell membrane comprised of glycerophospholipids and enriched in cholesterol and sphingolipids [Simons and Toomre, 2000]. Caveolae are specialized lipid rafts which contain caveolin proteins to form cave-like structure. Functions of lipid raft/caveolae are quite diverse, including cholesterol transport, endocytosis, protocytosis, and signal transduction [Sowa et al., 2001]. We were particularly interested in whether low rigidity triggered signal pathways other than FAK and the role of lipid raft/caveolae in mediating these pathways.

The study reported here shows that low substratum rigidity augments cell migration by promoting ERK phosphorylation instead of canonical FAK phosphorylation. Low rigidity-induced phosphorylation of ERK was localized at focal adhesion and thus was associated with cell spreading and migration. Furthermore, lipid rafts were involved in low substratum rigidity-induced ERK phosphorylation. This study provides a new concept that low substratum rigidity triggers cell migration by shifting the controlling machinery from FAK to ERK pathways.

MATERIALS AND METHODS

Materials and Reagents

MDCK 3B5 cells harboring FAK and FRNK, FAKY397F and FAK Y925F were the gifts from Dr. Hong-Chen Chen. Rabbit anti-phospho-ERK and rabbit anti-ERK1/2 were purchased from Cell Signaling Technology. Rabbit anti-FAKY397P was purchased from Biosource. Rabbit anti-FAK residues 903–1052 and HA were purchased from Santa Cruz. Mouse anti-paxillin and anti-FAK were purchased from BD Transduction Laboratories. Mouse anti-β-actin was purchased from Amersham. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. Fluorescence (Alex 488)-conjugated goat anti-rabbit IgG, fluorescence (Alex 594)-conjugated goat anti-mouse IgG, phalloidin-TRITC and DAPI were purchased from Molecular Probes. U0126, Calpeptin, and Calpastatin were purchased from Calbiochem. LY294002, SP600125, and SB203580 were purchased from TOCRIS.

Cell Culture

MDCK, NIH3T3, LLCPK1, BSC-1, U373MG, and HeLa cells were cultured in DMEM (high or low glucose, Invitrogen) supplemented with 5% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C of in air contained 5% CO_2.

Extraction of Collagen and Preparation of Collagen Gel and Collagen Gel-Coated Condition

The type I collagen was prepared from rat-tail tendons according to the established procedure described previously [Jiang et al., 2000, 2001; Wang et al., 2005, 2006]. For the preparation of 0.3% collagen gel, 3 volumes of acid collagen solution were mixed with 5.7× DMEM (1 volume), 2.5% NaHCO3 (0.5 volume), 0.1 M HEPES (1 volume), 0.17 M $CaCl_2$ (0.1 volume), 1 N NaOH (0.1 volume), and 4.3 volume of 1× culture medium (DMEM plus 10% fetal calf serum). In serum free condition, the culture medium was exchanged by DMEM only. The mixture were dispensed in the culture dish (2 ml/60-mm dish) and placed in an incubator

(5% CO_2 in air, 37°C) to allow for gelation [Wang et al., 2001]. After gelation, each culture was supplemented with 1.5 ml of culture medium containing DMEM and 10% FCS. To prepare the collagen gel-coated dish, chilled 0.3% collagen solution was added into 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 washed twice by normal culture medium prior to the use.

Western Blot Analysis

Cells were harvested by using RIPA lysis buffer contained 50 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidt P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride. The homogenates were stored at −80°C prior to analysis.

For Western blot, 30 μg of cell homogenate from specific samples was resolved by 10% SDS–PAGE and electrophoretically blotted onto nitrocellulose paper. The nitrocellulose paper was incubated with specific antibody and then immunocomplexes were detected with horseradish peroxidase-conjugated IgG, and finally the immunocomplexes were made visible by fluorography with enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia International PLC, UK).

In some study, the MDCK cells were seeded on culture dishes (10^6/60-mm dish) for 24 h and then the serum-containing medium was removed. After serum starvation for 12 h, the cells were pre-treated with indicated inhibitors for 1 h at 37°C. After treatment, the cells were replated on dish, collagen gel-coated dish or collagen gel and the cell lysates were harvested and stored at −80°C of for further study.

Cell Transfection and Immuno-Fluorescence Study

For transient transfection, MDCK cells were plated at a density of 3×10^5 cells/30 mm dish for 24 h before transfection with lipofectamine plus reagent according to the manufacturer's instructions (Life Technologies, Inc.). The final amount of the transfected DNA for a 30 mm dish was adjusted to 4 μg plasmid plus 20 μl lipofectamine. The plasmid of ERK2 kinase mutant was a gift from Dr. Nishida [Matsubayashi et al., 2004]. After transfection for 6 h, the serum-containing medium was added for 24 h and the cells were re-plated on collagen gel-coated dish or collagen gel.

MDCK cells were cultured on collagen gel-coated dish or collagen gel for 1 h or 4 h were washed three times with ice phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde prepared in PBS (pH 7.4) for 20 min at room temperature. Cells were then washed several times in cold PBS and permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature and then incubated with first Ab (anti-phospho-ERK and anti-paxillin) at 4°C overnight. Cells were then washed and incubated with Alexa Fluor 488-conjugated goat anti-rabbit Ab, Alex Fluor 594-conjugated goat anti-mouse Ab for 1 h. The immunofluorescent images were taken by confocal microscopy (Leica, TCS-SP2).

Time-Lapse Microscopy

Cells were trypsinized and plated on collagen gel coated dish or collagen. After 30 min, the culture dish was transfer to the chamber of time-lapse microscopy (Leica, AS MDW) contained sterilized water and 5% CO_2 in 37°C. Images of time-lapse microscopy were taken every 5 min for 24 h. To measure the distance of cell movement, Image Tool (from UTHSCSA) was employed.

In some experiments, cell were cultured in serum-free medium for 12 h and then treated with the inhibitors for 1 h. After treatment, the cells were plated on collagen gel-coated dish or collagen gel. The culture dish was transferred to the chamber of time-lapse microscopy containing sterilized water and 5% CO_2 in 37°C and the images were taken as described above for 24 h.

Cell Spreading Assay

MDCK cells received serum starvation for 12 h and then treated with inhibitors for 1 h. Cells were trypsinized and replated on collagen gel-coated dish or collagen gel. At indicated time, the cells were fixed with 4% paraformaldehyde and the immunofluoscent protocol was employed as described above. Phalloidin-TRITC was used to stain the actin filament to reveal the cell shape. After staining, five random fields were photographed and counted in each condition. The cell area was measured by the software of Image Tool (from UTHSCSA). For each experiment, minimally 15 cells were measured.

Statistical Analysis

The results of experiments were expressed as mean ± SEM (standard error of mean) of three independent experiments. One-way ANOVA was used to test for statistical differences. $P < 0.05$ was taken to be statistically significant.

RESULTS

Low Substratum Rigidity Triggers Cell Migration in All Cells Examined

To evaluate the effect of low substratum rigidity on cell migration, we employed various lines of cells cultured on collagen gel-coated dishes or collagen gel, including MDCK, LLC-PK1, NIH3T3, BS-C-1, HeLa, and U373-MG. After serum starvation for 12 h, different cell lines were cultured on collagen gel-coated dish or collagen gel. Cell migration was observed under time-lapse microscopy. The migratory images of the cells were taken every 5 min in a 24 h time course. The results of cell migration tracking revealed that cell migration ability was significant higher on collagen gel than collagen gel-coated dishes in all cell lines examined (Fig. 1). These data indicate that low substratum rigidity increases cell migration ability, which is a general phenomenon for different cell lines.

Low Substratum Rigidity Triggers ERK Activation

To elucidate if low rigidity affects MAPK signal pathways, we analyzed the phosphorylation pattern of ERK, P38, and JNK in MDCK cells cultured on dish, collagen gel-coated dish or collagen gel in the presence of fetal calf serum. Low substratum rigidity did not affect phosphorylation of P38 or JNK (data not shown). The phosphorylated ERK1/2 level was increased at 1 h and the activation of ERK1/2 was persistent for at least 8 h in MDCK cells cultured on collagen gel as compared with those on dish and collagen-gel coated dish (Fig. 2A,E). To rule out the possibility that the ERK1/2 phosphorylation is triggered by serum, MDCK cells were serum starved for 12 h and then cultured on dish, collagen gel-coated dish or collagen gel without serum. We found that low substratum rigidity also induced ERK phosphorylation, which was persistently activated up to at least 8 h in MDCK cells in the absence of serum (Fig. 2B,F). To examine whether collagen gel-induced ERK phosphorylation results from integrin signaling, we employed Western blot to assess β1 integrin activity by using specific antibodies against LIBS (ligand-induced binding site) of β1 integrin. Our data showed that low rigidity down-regulated β1 integrin activation within 4 h (data not shown). Thus, low rigidity-induced ERK phosphorylation is not dependent on α2β1 integrin. We also employed HeLa and NIH 3T3 cell line. Low rigidity induced an increase in ERK1/2 phosphorylation within 1 h, which was sustained for at least 24 h in HeLa cells (Fig. 2C,F). The low rigidity-induced phosphorylation of ERK1/2 in NIH 3T3 cells reached its peak at 4 h, which gradually decreased and returned to control levels at 24 h (Fig. 2D,F). These data indicate that low rigidity-induced a delayed but persistent phosphorylation of ERK.

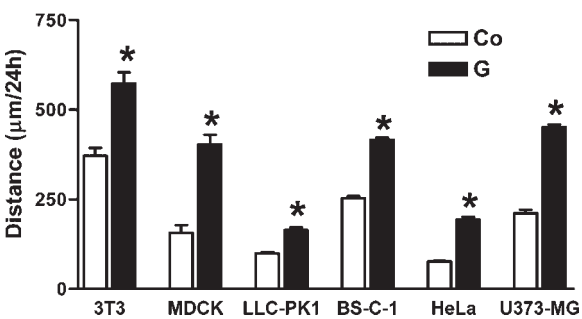

Fig. 1. Collagen gel augments the cell migration ability. Different cell lines were cultured on collagen gel-coated dish (Co) and collagen gel (G). After 24 h, the pictures were obtained by time-lapse microscopy and the migratory distance of each cell was assessed by Image Tool software. Each bar indicates the mean ± SE of three independent experiments with tracing of at least 30 cells in each condition. $^{*}P < 0.05$ versus cells cultured on collagen gel-coated dish.

Cell Spreading on Collagen Gel is Mediated by Activation of ERK

To explore the function of low rigidity-induced ERK phosphorylation, the MEK inhibitor, U0126, was employed. MDCK cells were pre-treated with U0126 for 1 h and then cultured on collagen gel-coated dish or collagen gel in the presence or absence of U0126. U0126 did not affect the ability of cell spreading on collagen gel-coated dish. However, it inhibited spreading of MDCK cells cultured on collagen gel (Fig. 3A). Different chemical inhibitors for phosphorylation of JNK, P38, and PI3K were also used to test whether these signal pathways affected the cell spreading on collagen gel. None of these inhibitors affected cell spreading on collagen gel or collagen gel-coated dish (Fig. 3A).

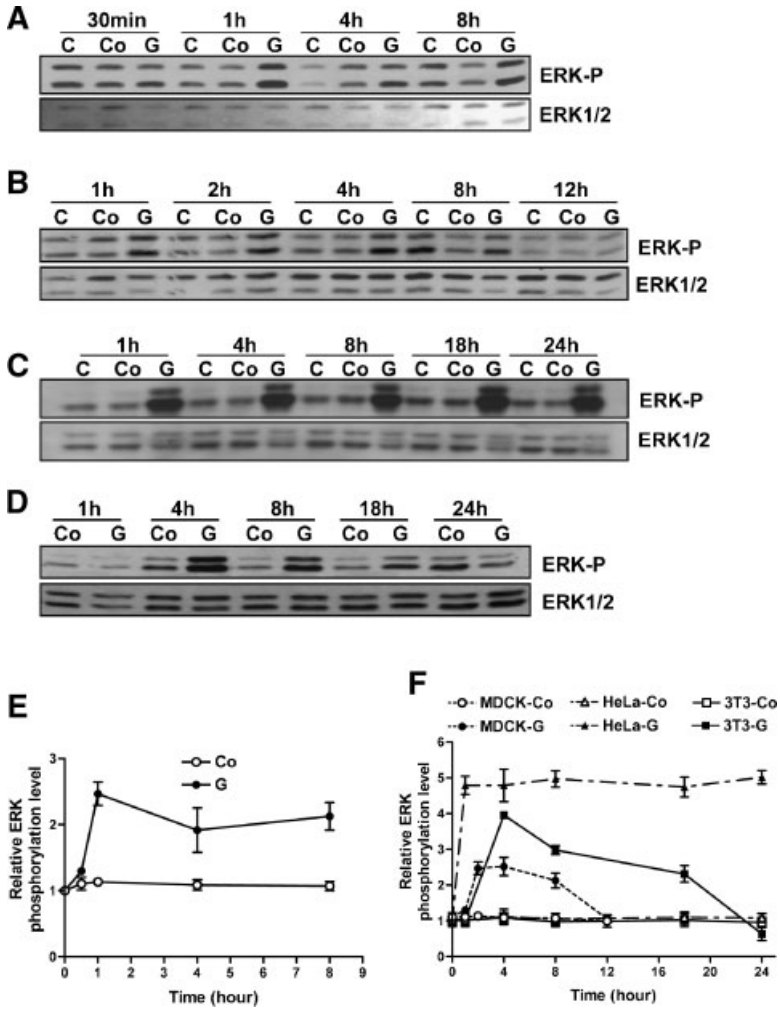

Fig. 2. Low substratum rigidity induces phosphorylation of ERK1/2 in different cell lines. **A**: MDCK cells were cultured on dish (**C**), collagen gel-coated dish (Co) or collagen gel (G) with 10% fetal calf serum for the indicated time. **E**: Quantification of relative ERK phosphorylation levels in cells cultured on collagen gel-coated dish (Co) or collagen gel (**G**) for the indicated time. **B**: MDCK, (C) HeLa and (**D**) NIH 3T3 cells were serum starved for 12 h and then cultured on dish (C), collagen gel-coated dish (Co) or collagen gel (G) with serum-free medium for the indicated time. Cells were harvested and the protein expression was analyzed by Western blot using antibodies recognizing phosphorylated ERK or total ERK. **F**: Quantification of relative ERK phosphorylation level in MDCK, HeLa, 3T3 cells cultured on collagen gel-coated dish (Co) or collagen gel (G) for the indicated time. Each point indicates the mean ± SE of three independent experiments.

Quantification of cell spreading was performed by measuring cell area on collagen gel or collagen gel-coated dish in the presence or absence of different inhibitors. We found that cells cultured on collagen gel displayed a smaller area than cells cultured on collagen gel-coated dish. However, U0126 markedly inhibited cell spreading while cell were cultured on collagen gel (Fig. 3B). Similar results were obtained when U0126 treatment was extended to 4 h. U0126 decreased cell spreading only in those cells cultured on collagen gel (Fig. 3C). Furthermore, we applied ERK2 kinase mutant conjugated with GFP to confirm this observation. After transfection, control cells expressing GFP only spread out on collagen gel-coated dish or collagen gel as expected, but cells expressing the ERK2 kinase mutant displayed a roundish morphology (Fig. 3D). We further measured the cell area in MDCK cells expressing ERK2 kinase mutant cultured on collagen gel. The results showed that inhibition of ERK2 kinase markedly lowered low rigidity-triggered cell spreading (Fig. 3E), indicating that activation of ERK is required for cell spreading on collagen gel.

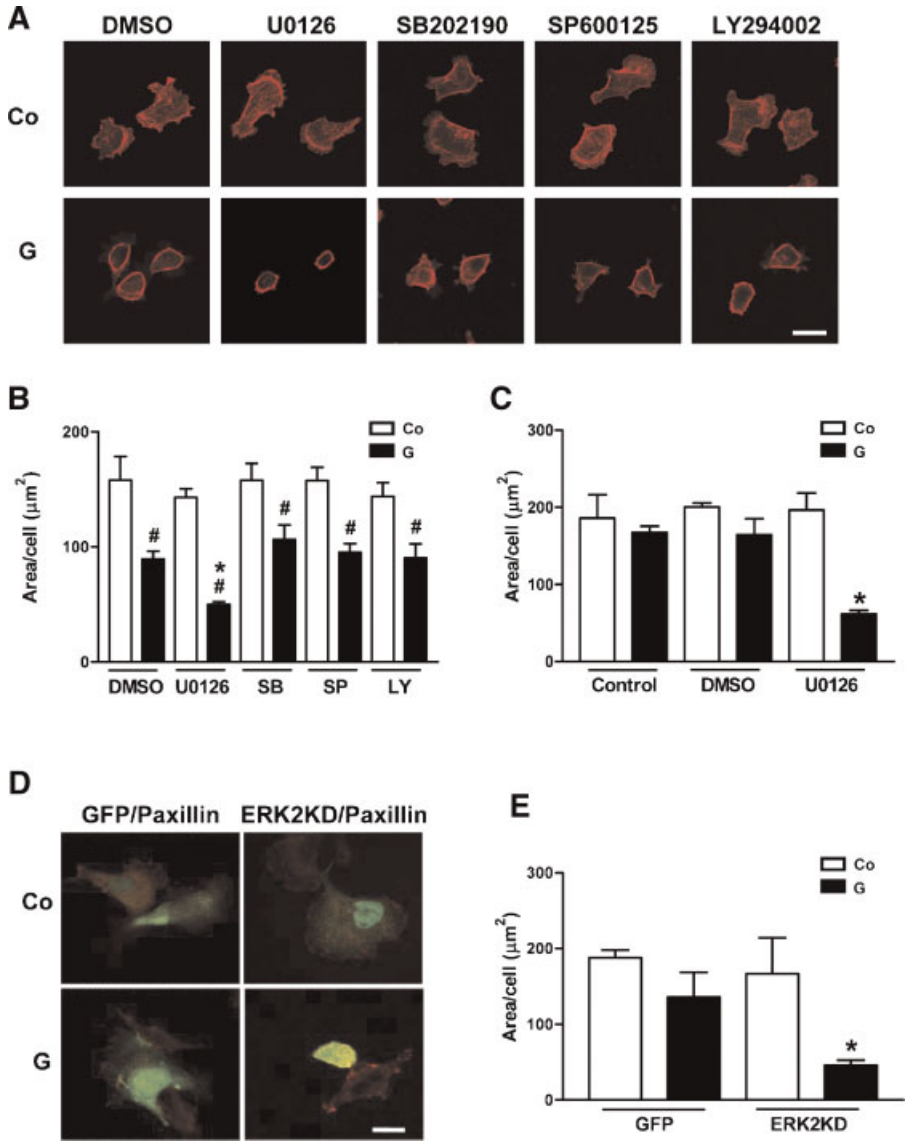

Fig. 3. Low rigidity-induced phosphorylation of ERK1/2 is associated with cell spreading. **A**: Immunofluorescence study to examine cell size of MDCK cells cultured on collagen gel-coated dish (Co) or collagen gel (G) for 1 h in the presence of inhibitors, including LY294002 (20 μM), U0126 (20 μM), SP600125 (50 μM), or SB203580 (20 μM). The actin filament was visualized through staining with phalloidin-TRITC. Bar = 10 μm. The areas of cells seeded on collagen gel-coated dish (Co) or collagen gel (G) for 1 h (**B**) or 4 h (**C**) were assessed by Image Tool software. About 150 cells in each condition were evaluated. Results are the mean ± SE of three independent experiments. $^{*}P < 0.05$ versus cells cultured on collagen gel with treatment of DMSO. $^{\#}P < 0.05$ versus cells cultured on collagen gel-coated dish. **D**: Immunofluorescence image of cells transiently transfected with the plasmid of GFP and ERK2 kinase mutant (ERK2KD) were cultured on collagen gel-coated dish (Co) or collagen gel (G) for 4 h. Bar = 10 μm (D) Areas of transfected cells were measured by Image Tool software. About 100 cells in each condition were evaluated. The result shows the mean ± SE of three independent experiments. $^{*}P < 0.05$ versus cells transfected with ERK2KD were cultured on collagen gel. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Low Rigidity Induces Translocation of Phosphorylated ERK to Focal Adhesion

Under normal culture conditions, activated ERK is translocated into the nucleus to activate transcription factors. To examine the localization of active ERK in cells cultured on collagen gel, we employed immune-fluorescence studies. Within 1 h in culture, activated ERK was observed in the nuclei of MDCK cells cultured on collagen gel-coated dish. In contrast, activated ERK was expressed in both nuclei and cell membrane in cells cultured on collagen gel (Fig. 4A). The total immunofluo-

rescence of active ERK in cells cultured on collagen gel was markedly higher than that observed on collagen gel-coated dish. This observation is consistent with that assessed? by Western blot analysis (Fig. 2A,B). Furthermore, activated ERK could be observed as dot form near the cell edge and was co-localized with paxillin (Fig. 4A). We also quantified cells that exhibited co-localization of phosphorylated ERK and paxillin in MDCK cells cultured on collagen-gel coated dish or collagen gel for 1 h. For the quantification, cells were immunostained with paxillin (green), active ERK1/2 (red) or nucleus (blue) and the yellow immunofluorescence indicates co-localization of phosphorylated ERK and paxillin. As shown in Figure 4B,

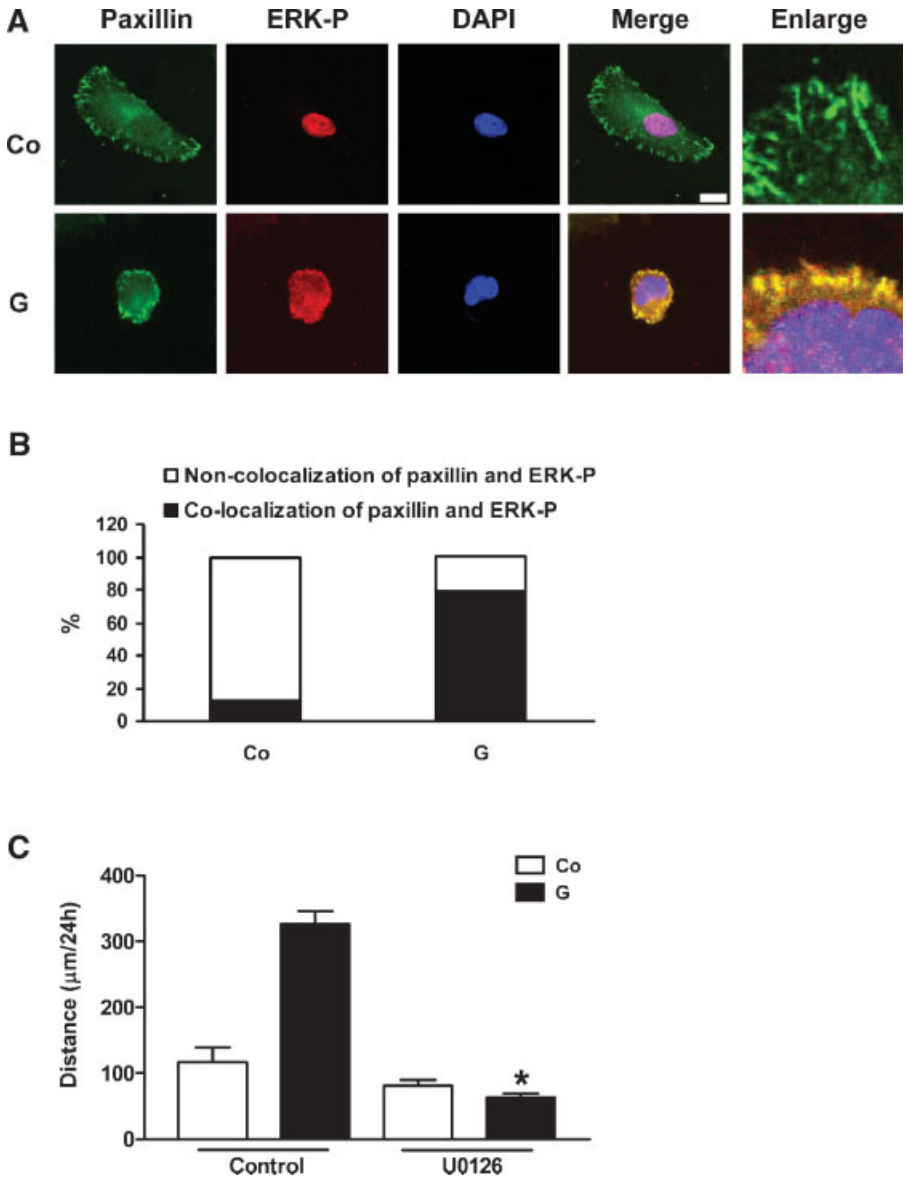

Fig. 4. Collagen gel-induced activation of ERK1/2 is associated with focal adhesions. **A**: MDCK cells were cultured on collagen gel-coated dish (Co) or collagen gel (G) for 1 h and immunofluorescence study was carried out with images taken by confocal microscopy. Cells were immunostained and show paxillin (green), active ERK1/2 (red) or nucleus (blue). The yellow immunofluorescence indicates co-localization of phosphorylated ERK and paxillin. Bar = 10 μm. **B**: Quantification of the cell that exhibited co-localization of phosphorylated ERK and paxillin was made in MDCK cells cultured on collagen-gel coated dish (Co) or collagen gel (G) for 1 h. About 100 cells in each condition were evaluated. **C**: Time lapse microscopic tracing of cell migration distance in MDCK cells cultured on collagen gel-coated dish (Co) and collagen gel (G) in the presence or absence of U0126 (10 μM). Each bar indicates the mean ± SE of three independent experiments with tracing of at least 15 cells in each experiment. *$P < 0.05$ versus cells cultured on collagen gel.

collagen gel markedly increased the percentage of cells exhibiting co-localization of phosphorylated ERK and paxillin. Low rigidity-induced translocation of activated ERK to cell periphery, particularly in focal adhesions, suggests its association with cell migration.

Inhibition of ERK Phosphorylation Suppresses Low Rigidity-Induced Cell Migration

Because inhibition of collagen gel-induced phosphorylation of ERK by MEK inhibitor suppressed cell spreading on collagen gel, we further examined whether MEK inhibitor suppressed collagen gel-induced cell migration. MDCK cells were pretreated with 10 μM U0126 and then cultured on collagen gel in the presence or absence of U0126 and the cell migration was assessed by time-lapse microscopy. U0126 completely abolished low rigidity-induced ERK phosphorylation (data not shown). U0126 did not affect the cell migration on collagen-coated dish, but it inhibited collagen gel-induced cell migration (Fig. 4C). Taken together, collagen gel-induced phosphorylation of ERK is associated with augmentation of cell spreading as well as cell migration.

Low Rigidity-Induced ERK Phosphorylation and Cell Migration is Mediated by Raft/Caveolae

Lipid rafts are specialized membrane structures controlling several cell signaling. In order to examine whether low rigidity-induced phosphorylated ERK is localized in lipid rafts, we employed sucrose gradients to separate raft and non-raft fractions. Caveolin-1 is a marker for raft fractions and β subunit of Na^+/K^+ pump for non-raft fractions. When cells were cultured on collagen gel-coated dish, phosphorylated ERK was present in non-raft fractions. However, when cells were cultured on collagen gel, there was a general increase in phosphorylation level of ERK and a proportion of phosphorylated ERK was present in raft fractions (Fig. 5A). These results confirm that under low rigidity conditions phosphorylated ERK is translocated to lipid rafts. To examine whether lipid rafts

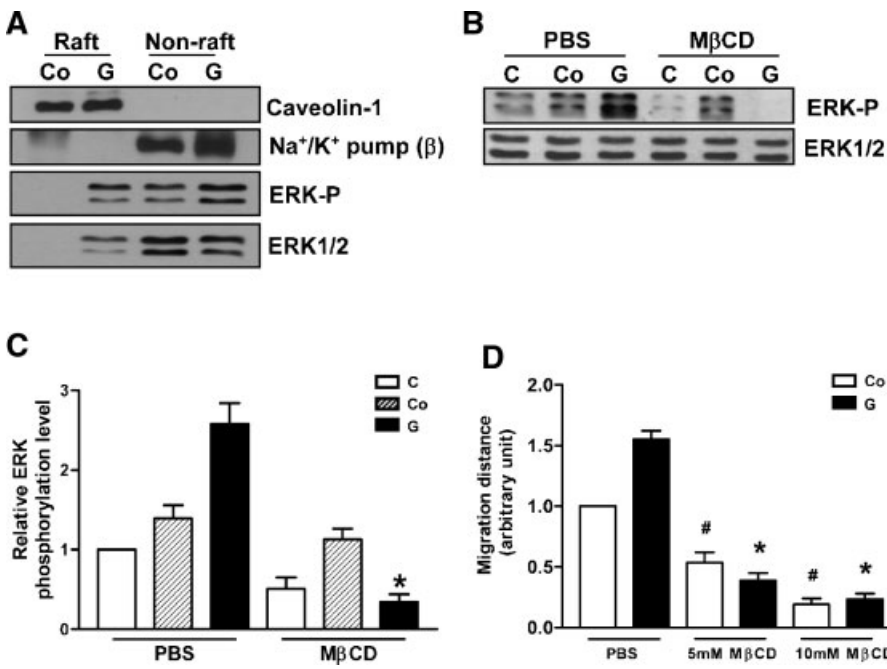

Fig. 5. Lipid rafts are involved in low substratum rigidity-induced ERK phosphorylation and cell migration. **A**: MDCK cells were cultured on collagen gel-coated dish (Co) or collagen gel (G) for 1 h. Cell lysates were fractionated by OptiPrep and analyzed by Western blot. Antibodies specific for caveolin-1, β subunit of Na^+/K^+ pump and ERK-P were used. **B**: MDCK cells were cultured on dish (C), collagen gel-coated dish (Co) or collagen gel (G) for 1 h in the absence or presence of 10 mM MβCD. Cell lysates were analyzed by Western blot using specific antibodies to detect phospho-ERK1/2 and ERK1/2. **C**: Quantification of relative ERK phosphorylation level in cells cultured on dish (C), collagen gel-coated dish (Co) or collagen gel (G) with or without 10mM MβCD for 1 h from three independent experiments. $^*P < 0.05$ versus cells cultured on collagen gel treated with PBS. **D**: Time lapse microscopic tracing of cell migration distance in MDCK cells cultured on collagen gel-coated dish (Co) and collagen gel (G) in the absence or presence of 5 mM and 10 mM MβCD. The migration distance was analyzed by Image Tool software. Results are the mean ± SE of three independent experiments. $^*P < 0.05$ versus cells cultured on collagen gel. $^\#P < 0.05$ versus cells cultured on collagen gel-coated dish.

were involved in collagen gel-induced activation of ERK, MDCK cells were pretreated with MβCD and cultured on dish, collagen gel-coated dish or collagen gel. As shown in Figure 5B and C, MβCD did not affect ERK phosphorylation in cells cultured on dish or collagen gel-coated dish but completely alleviated collagen gel-induced phosphorylation of ERK. Moreover, we assessed the effects of MβCD on cell migration ability under the time-lapse microscope. The results showed that MβCD markedly suppressed cell migration ability in cells cultured on collagen gel or collagen-gel coated dish. These data indicate the significance of lipid raft integrity in cell migration (Fig. 5D). Because the integrin-mediated signal plays an important role in cell migration, we rationed that MβCD suppressed cell migration on collagen gel-coated dish via inhibition of β1 integrin activation. In order to test this possibility, we cultured MDCK cells on collagen gel-coated dish with or without MβCD. Our data showed that MβCD down-regulated β1 integrin activation (data not shown). Thus, MβCD-induced reduction of migration on collagen gel-coated dish may result from inhibition of β1 integrin activity. Taken together, low rigidity-triggered cell migration could be mediated by activation of ERK through lipid rafts.

Low Rigidity-Induced ERK Phosphorylation Could be Triggered by FRNK or C-Terminal Degraded Product of FAK

In order to delineate whether low substratum rigidity-induced ERK1/2 activation could be mediated by down-regulation of FAK phosphorylation or FAK degradation, we employed various MDCK stable transfectants overexpressing wild type FAK, FAKY397F, FAKY 925F, or FRNK, which is a C-terminal fragment of FAK. These transfectants were cultured on dish and treated with or without MEK inhibitor U0126 for 1 h afterwards their ERK phosphorylation levels were examined. As shown in Figure 6A, cells overexpressing FAK, FAKY 397F or FAKY925F exhibited similarly low levels of ERK phosphorylation. Only cells overexpressing FRNK exhibited significantly enhanced ERK1/2 phosphorylation levels. U0126 completely abolished ERK phosphorylation in all cells examined. It is possible that low rigidity-induced ERK phosphorylation is correlated with FAK activity. In order to test this possibility, we employed cells overexpressing FAKY397F which is an inactive FAK. However, as shown in Figure 6A, cells expressing FAKY397F did not show altered ERK phosphorylation level. On the other hand, it has been established that activation of FAK leads to down stream elevation of ERK activity. These data taken together indicate that low rigidity-induced ERK phosphorylation is not caused by FAK inactivation. Since overexpression of FRNK triggered ERK phosphorylation, we hypothesized that low rigidity-induced ERK phosphorylation might be mediated through the FAK C-terminal fragment. To examine whether low substratum rigidity might trigger formation of such a similar fragment, antibodies, specifically detecting FAK residues 903–1052, were used. As shown in Figure 6B, a reduction of FAK combined with the generation of a C-terminal fragment of FAK (35 kDa) was observed only when cells were cultured on collagen gel but not on dish or collagen gel-coated dish. We also showed that with the reduction of intact 125 kDa FAK, both FAK 35 kDa fragment and phosphorylated ERK increased (Fig. 6B,C). Our previous study showed that low substratum rigidity of collagen gel triggered degradation of FAK, which could be partially reversed by the pan-calpain inhibitor, calpeptin [Wang et al., 2003]. It has been shown that FAK is the substrate for calpain which cleaves FAK into two products: 90 (N-terminal) and 35 kDa (C-terminal) [Carragher et al., 1999]. The 35 kDa product may function as FRNK. In order to delineate whether prevention of FAK degradation could revert low rigidity-induced ERK phosphorylation, we employed calpain inhibitors. As shown in Figure 6D, calpastatin did not affect low-rigidity-induced FAK degradation and ERK phosphorylation. However, calpeptin partially reversed low rigidity-induced FAK degradation, which is consistent with our previous report. Interestingly, calpeptin completely inhibited low substratum rigidity-induced FAK degradation into a C-terminal 35 kDa fragment and ERK phosphorylation. These results taken together suggest that low substratum rigidity induces degradation of FAK into a C-terminal FRNK-like fragment which may subsequently triggers ERK1/2 activation.

FRNK-Induced ERK Phosphorylation is Associated With Cell Spreading

To verify whether low rigidity-induced ERK phosphorylation is mediated by FRNK-like

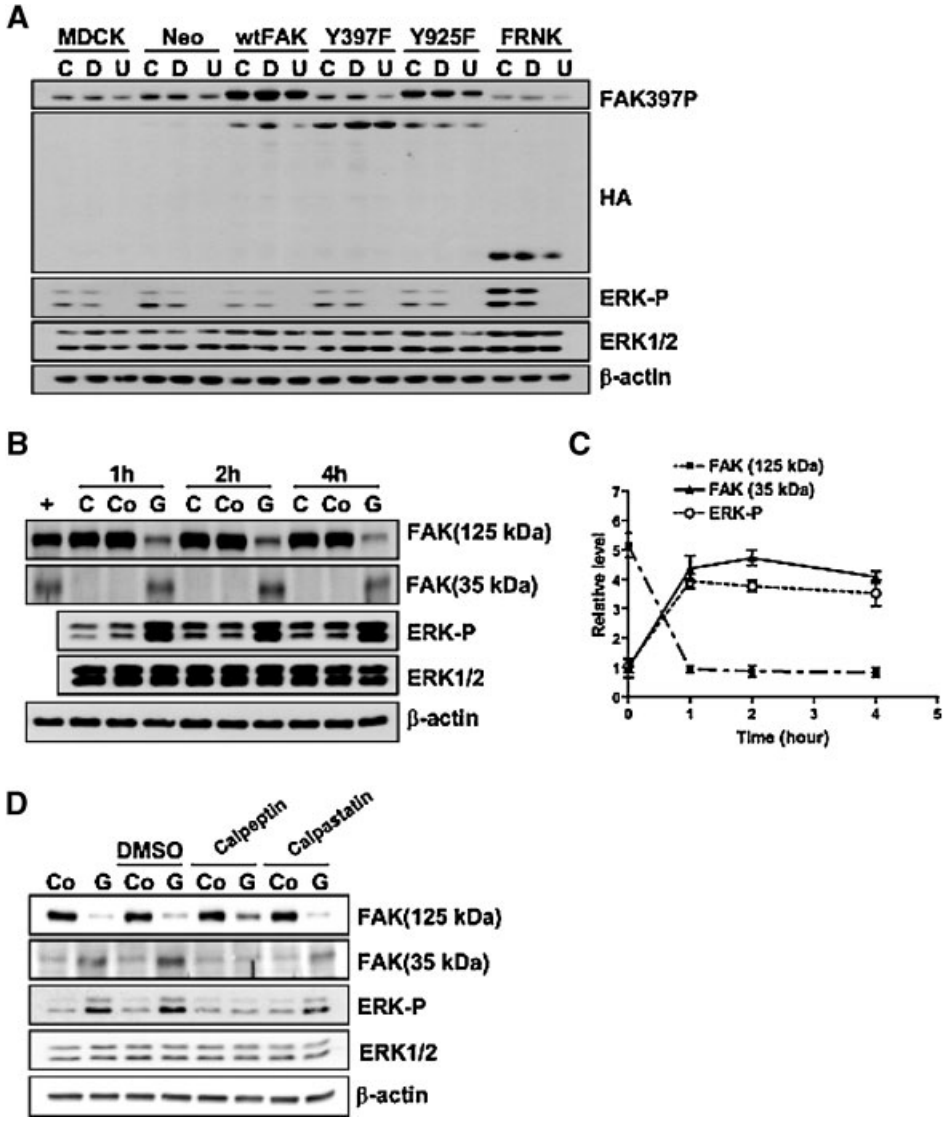

Fig. 6. FRNK is involved in low rigidity-induced ERK1/2 phosphorylation. **A**: Cultured MDCK transfectants harboring HA-tagged FAK, FAKY397F, FAKY925F, or FRNK were treated in the absence (C) or presence of DMSO (D) or 20 μM U0126 (U) for 1 h. Cell lysates were harvested and analyzed by Western blot using specific antibodies to detect FAKY397P, HA, phospho-ERK1/2, and ERK1/2. **B**: MDCK cells were cultured on dish (C), collagen gel-coated dish (Co), or collagen gel (G) for the indicated time and the cell lysates were harvested and analyzed by Western blot using specific antibodies to detect FAK residues 903–1052, phosphor-ERK1/2, and ERK1/2. Lysates of MDCK cells treated with 250μg/ml degraded collagen for 30 min were used as a positive control for the presence of FAK 35kDa fragments. **C**: Quantification of relative level of intact FAK (125 kDa), FAK 35 kDa fragments, or phosphor-ERK1/2 in cells cultured on collagen gel within 4 h. Each point indicates mean ± SE of three independent experiments. **D**: MDCK cells were cultured on collagen gel-coated dish (Co) and collagen gel (G) with the treatment of calpeptin (200 μM) or calpastatin (100 nM) for 4 h. Cell lysates were harvested and analyzed by Western blot using specific antibodies to detect FAK, phosphorylated ERK 1/2, and ERK 1/2.

fragments, cells harboring control vectors or FRNK were cultured on dish, collagen gel-coated dish, or collagen gel. As shown in Figure 7A, FRNK-induced ERK phosphorylation remained high regardless of the culture conditions. Since lipid rafts were involved in low rigidity-induced ERK phosphorylation, we tried to determine whether lipid rafts were also involved in low rigidity-induced FAK degradation. Although MβCD significantly blocked FRNK-induced ERK phosphorylation, it had no effect on low rigidity-induced FAK degradation (Fig. 7A). In order to further confirm whether FRNK-induced ERK phosphorylation was associated with cell spreading, MDCK cells harboring control vectors or FRNK were

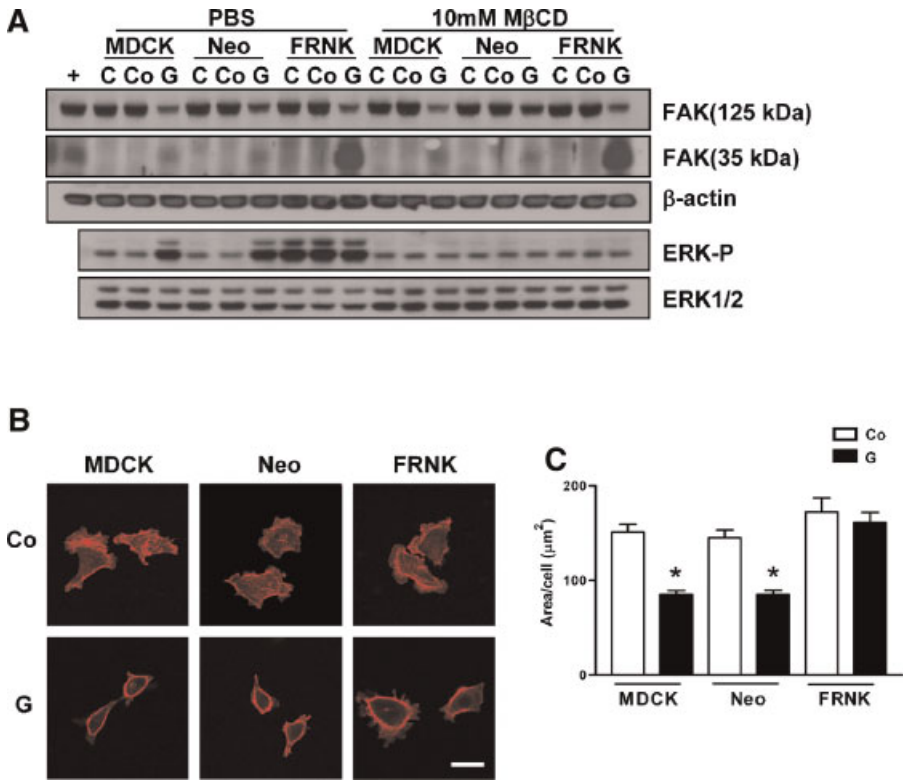

Fig. 7. Overexpression of FRNK induces ERK phosphorylation and cell spreading. **A**: MDCK cells harboring control vector (Neo) or FRNK were cultured on dish (C), collagen-gel coated dish (Co), or collagen gel (G) in the presence or absence of 10 mM MβCD for 1 h. Cell lysates were collected and analyzed by Western blot using antibodies against FAK, phosphorylated ERK or total ERK. β-Actin was used as an internal control. Lysates of MDCK cells treated with 250 μg/ml degraded collagen for 30 min were used as a positive control for the presence of FAK 35kDa fragment. **B**,**C**: MDCK cells harboring control vector (Neo) or FRNK were cultured on collagen-coated dish (Co) or collagen gel (G) for 1 h. B: The results of the immunofluorescence study. Actin filament was visualized by staining with phalloidin-TRITC. Bar = 10 μm. Cell morphology was observed by confocal microscopy. C: Quantitative results of cell areas were assessed by Image Tool software. About 100 cells in each condition were evaluated. $^{*}P < 0.05$ versus cells cultured on collagen gel-coated dish. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cultured on collagen gel-coated dish or collagen gel for 1 h and cell areas were measured. Overexpression of FRNK did not alter spreading morphology in cells cultured on collagen gel-coated dish. However, cells overexpressing FRNK cultured on collagen gel regained their ability to spread out in comparison to the control groups (Fig. 7B,C). Since FRNK and the degradation product of FAK contribute in a similar way to low rigidity-induced ERK phosphorylation and cell spreading, these data indicate that low rigidity-induced ERK phosphorylation may be mediated by the 35 kDa C-terminal fragment of FAK.

DISCUSSION

Previous studies showed that low substratum rigidity induced a down-regulation of focal adhesion complex proteins [Wang et al., 2003]. Low substratum rigidity also down-regulated FAKY397 phosphorylation (data not shown), which is consistent with the finding of Paszek et al. [2005]. Under physiological conditions, cell motility is regulated by canonical FAK signaling. It is anticipated? that low rigidity would suppress cell motility since it not only down-regulates FAK expression, but also inhibits FAK Y397 phosphorylation. However, here we demonstrate for the first time that low substratum rigidity augments cell motility. In this case, low rigidity must trigger other signal pathways than FAK. Our data show that low rigidity induces persistent phosphorylation of ERK1/2, which is subsequently translocated to cell adhesions to mediate cell spreading and cell migration. Low substratum rigidity-regulated phosphorylation of FAK and ERK was observed in various cell lines. These results provide very important physiological implication, since most of the cells in vivo are surrounded by soft tissue

with rather low rigidity. Cukierman et al. [2001] showed that cells cultured under 3D cell/tissue-derived matrix display lowered FAKY397 phosphorylation and elevated ERK phosphorylated levels. It is likely that the low rigidity of 3D matrix may be the cause.

We also observed a novel distribution of phosphorylated ERK under low substratum rigidity. When cells are cultured on collagen gel, low rigidity triggers activation of ERK, which is present in focal adhesion and participates in regulation of cell spreading and migration. Speculations on how activated ERK promotes cell motility have been proposed. Activated ERK may be directly associated with some focal adhesion proteins (e.g., paxillin, myosin light chain kinase or calpain) to facilitate cell spreading and migration [Nguyen et al., 1999; Fincham et al., 2000; Ku and Meier, 2000; Carragher et al., 2003]. Alternatively, activated ERK might stimulate expression of specific proteins related to cell migration through transcriptional regulation [Liang and Chen, 2001; Howe et al., 2002]. Since we found that activated ERK was present in focal adhesion, the former theory is more likely.

Lipid rafts are membrane micro-domains enriched in cholesterol, sphingolipids, and saturated phospholipids and are considered as platforms for cellular signaling. Retardation of lipid raft/caveolae formation by MβCD attenuated low rigidity-induced phosphorylation of ERK, cell spreading, and migration, suggesting the role of lipid rafts in low rigidity-triggered cell migration. Recent studies showed that lipid rafts are involved in activation of Src family proteins Fyn [Young et al., 2003]. On the other hand, Src kinase may promote the activity of receptor kinase EGFR when recruited to lipid rafts [Nanjundan et al., 2003]. Whether Src kinase is involved in low rigidity-induced ERK phosphorylation is therefore very intriguing. Small GTPase, Ras, is the upstream of ERK signal pathway. It also has been reported that Ras can translocate to lipid rafts and transduce its downstream signals [White and Anderson, 2001; Niv et al., 2002]. The other small GTPase, Rac, also triggers the phosphorylation of PAK in lipid rafts [del Pozo et al., 2004]. PAK activates the phosphorylation of Raf and indirectly increases the phosphorylation of ERK [Eblen et al., 2002]. According to these results, it is also likely that low substratum rigidity might induce the accumulation of Ras or Rac in lipid rafts, which in turn accelerates ERK phosphorylation and results in cell spreading and migration on collagen gel.

The study reported here showed that low substratum rigidity-induced down-regulation of FAK could be partially prevented by pan-calpain inhibitor, calpeptin, but not calpastatin, indicating that low rigidity induces activation of calpain II. In addition to the stimulation by elevated calcium concentration, calpain can be activated by the binding of phospholipids through its domain III [Arthur and Crawford, 1996; Tompa et al., 2001]. Recent data showed that calpain could be co-localized with lipid rafts or caveolae [Morford et al., 2002; Kifor et al., 2003]. However, our data shows that MβCD blocked low-rigidity induced ERK phosphorylation but not FAK degradation (Fig. 7A). Thus, low rigidity-induced calpain activation is not mediated through lipid rafts. On the other hand, previous studies showed that degraded collagen also activates calpain, which leads to FAK degradation [Carragher et al., 1999; von Wnuck Lipinski et al., 2006]. We cannot rule out the possibility that low rigidity triggers activation of metalloproteinases (MMPs), for example MMP2 or MMP9, which in turn accelerates degradation of collagen fibrils and subsequently results in calpain activation. We show that overexpression of FRNK promotes ERK phosphorylation, which is consistent with the reported by Segarra et al. [2005]. They demonstrate that FRNK cooperates with Src tyrosine kinase to mediate p130CAS downstream signaling events in regulating ERK phosphorylation. Taken together, we conclude that low rigidity induces calpain activation to trigger FAK degradation into a FRNK-like product, which may subsequently cause elevation of ERK phosphorylation.

Biophysical properties of environments are critical for cell physiology. In this paper, we demonstrate that the substratum rigidity regulates the cell spreading and migration through phosphorylation of ERK, mediated by lipid rafts, and degradation of FAK. Lipid rafts play an important role in mediating the biophysical stimuli of the environment (Fig. 8). The characterization of low rigidity-induced lipid raft formation awaits further investigation. In addition, how lipid raft transmits signals in response to biophysical stimulations to regulate cell behaviors is also an interesting issue to be investigated in the future.

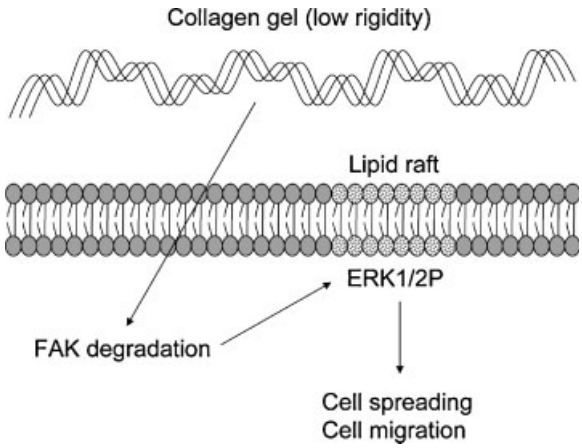

Fig. 8. A schematic figure to depict a signaling mechanism by low rigidity induced ERK phosphorylation. Low substratum rigidity induces FAK degradation, which triggers ERK1/2 phosphorylation to regulate cell spreading and migration via lipid rafts.

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