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Y-27632, an Inhibitor of Rho-associated Protein Kinase, Suppresses Tumor Cell Invasion via Regulation of Focal Adhesion and Focal Adhesion Kinase

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Migration of rat ascites hepatoma (MM1) cells, invasion and phagokinetic movement were induced by the combination of lysophosphatidic acid (LPA) and fibronectin (FN). Induction of migratory activity was tightly correlated with morphological change of MM1 cells from spherical or polygonal-shaped cells to fusiform-shaped ones with pseudopodia. MM1 cells were mobile in a fusiform shape, whereas those of a spherical or polygonal shape were not. A small GTPase Rho and one of its downstream effectors ROCK (Rho-associated coiled-coil forming protein kinase), play essential roles in these processes, as evidenced by suppression of migration and morphological change of MM1 cells by *Clostridium botulinum* C3 exoenzyme, an inhibitor of Rho, or by Y-27632, an inhibitor of ROCK. Y-27632 also suppressed the formation of fusiform-shaped pseudopodia-carrying MM1 cells that was induced by stimulation with the combination of LPA and FN. LPA and FN also evoked the formation of focal adhesions and actin bundles, and tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin. The inhibitory effect of Y-27632 on LPA-induced migration and morphological change of MM1 cells was considered to be mediated, at least in part, by impaired formation of focal adhesions and actin bundles. Y-27632 suppressed LPA-induced tyrosine phosphorylation of FAK and paxillin, suggesting that ROCK regulates these molecules and Y-27632 inhibits cellular migration and morphological change, at least in part, through this regulation.

Key words: Invasion — ROCK — Y-27632 — Focal adhesion — Focal adhesion kinase

Cellular morphology and motility tightly correlate with invasive and metastatic capacity of cancer cells. They are partly regulated by signaling through complex adhesive structures called focal adhesions. Focal adhesion kinase (FAK) and paxillin are key molecules controlling their formation and function.[1–3] The Rho family of small GTPases, such as Rho, Rac, and Cdc42, and their downstream effectors, including Rho-associated coiled-coil forming protein kinase (ROCK) or p21-activated kinase (Pak), are considered to be critically involved in controlling focal adhesions and the dynamics of actin fibers.[4–7]

Rat ascites hepatoma (MM1) cells invade a mesothelial cell monolayer *in vitro* (transcellular migration) and form tumor cell nests under the monolayer (invasion foci). Invasive capacity of MM1 cells can be quantified by counting the number of invasion foci formed.[8] Using this system, we showed that MM1 cells require stimulation with lysophosphatidic acid (LPA) to exhibit invasive capacity: MM1 cells do not invade a mesothelial cell monolayer in the absence of LPA, but show extensive invasion in its presence.[9] Requirement of LPA for MM1 cell migration was confirmed using a phagokinetic motility assay, in which MM1 cells exhibited phagokinetic movement in the presence of both LPA and fibronectin (FN), but not in the absence of either of the two.[10] Because anti-FN antibody or anti-β1 integrin antibody inhibited the invasion of MM1 cells through a mesothelial cell monolayer, it was concluded that both LPA and FN are commonly required for MM1 cell migration in the two different assay systems. The requirement of extracellular ligands and integrins for cellular migration is widely accepted in various types of cells.

The mechanism by which LPA and FN induce migratory ability is closely related to morphological change of MM1 cells from a spherical or polygonal shape to a fusiform shape with pseudopodia.[11] When MM1 cells were stimulated with LPA and FN, they became fusiform-shaped and exhibited migratory activity. Analysis of intracellular signaling revealed that Rho plays a central role in these processes. Briefly, *Clostridium botulinum* C3 exoenzyme (C3) inhibited LPA-induced morphological change of MM1 cells as well as the invasion, and conversely, the expression of a constitutively active mutant of Rho conferred LPA-independent invasiveness upon MM1 cells.[11, 12] Recently, several downstream effectors of Rho were identified.[6] We have revealed that the role of Rho in inducing MM1 cell invasiveness can be substituted for by one of its downstream effectors, ROCK.[13] Y-27632, an

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inhibitor of ROCK,[14] suppressed the invasion of MM1 cells; the expression of a constitutively active mutant of ROCK induced the invasion of MM1 cells in the absence of LPA in a similar fashion to that of the constitutively active mutant of Rho. Critical participation of LPA and the Rho/ROCK-signaling pathway in tumor invasion was recently confirmed using a different assay system.[15] ROCK is known to activate LIM-kinase, and the activated LIM-kinase phosphorylates cofilin, resulting in reorganization of the actin cytoskeleton.[16–18] The activation of this pathway also generates contractile force of actin fibers by increasing myosin phosphorylation.[19]

We have also shown that LPA evokes tyrosine phosphorylation of FAK and paxillin, and this phosphorylation is involved in MM1 cell invasion.[20] FAK and paxillin are regulators of focal adhesions and actin structures. In the present manuscript, using Y-27632, we examined the role of ROCK in the regulation of focal adhesions and actin bundles of MM1 cells with special reference to their migratory ability.

MATERIALS AND METHODS

Chemicals Bovine serum albumin (BSA, fraction V), 1-oleoyl-sn-LPA, FN, and anti-vinculin antibody VIN-11-5 were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). LPA was dissolved in phosphate-buffered saline (PBS) supplemented with 0.1% BSA. Anti-FAK antibody, anti-paxillin antibody, and anti-phosphotyrosine antibody (4G10) were purchased from Transduction Laboratory (Lexington, KY), Zymed Laboratories (San Francisco, CA), and Upstate Biotechnology Inc. (Lake Placid, NY), respectively. Rhodamine-labeled phalloidin was purchased from Molecular Probes Co., Ltd. (Eugene, OR). A chemiluminescence detection kit (ECL) was from Amersham Co., Ltd. (Buckinghamshire, England). C3 exoenzyme was a kind gift from Dr. R. Komagome (Hokkaido University). Y-27632 was a gift from Yoshitomi Pharmaceutical Industries, Ltd. (Osaka), and was dissolved in sterile water.

Cells and cell culture MM1 cells were cultured in suspension in minimum essential medium (MEM) containing 2-fold amino acids and vitamins (modified MEM, M-MEM) (Nissui, Tokyo) supplemented with 10% fetal calf serum (FCS) (Bioserum, Wako, Osaka). Rat mesothelial cells were isolated from the mesentery of Donryu rats (Japan SLC, Shizuoka) and cultured in M-MEM supplemented with 10% FCS as reported previously.[8] The mesothelial cells and MM1 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. When mesothelial cells became confluent in a 35-mm dish, the monolayer was used for the assay of invasion. When necessary, MM1 cells were pretreated with 10 μg/ml C3 for 24 h. Y-27632 was added 15 min before the addition of LPA. Y-27632 at the final concentration of 30 μM was used for the analysis of cellular morphology and phagokinetic movement, whereas 10 μM was adopted for the invasion assay because mesothelial cells were more vulnerable to Y-27632.

Measurement of invasive capacity The assay procedure of *in vitro* invasive capacity of MM1 cells was essentially the same as described in our previous report.[8] MM1 cells (2×10^5 cells) were seeded over a mesothelial cell monolayer and cultured. Twenty hours later, the supernatant was removed and the mesothelial cell monolayer was fixed with 10% formalin together with invaded MM1 cells. The number of penetrated single tumor cells and tumor cell colonies (collectively called invasion foci) was counted under a phase contrast microscope. Invasive capacity was expressed as the number of invasion foci per square centimeter.

Phagokinetic track motility assay The assay procedure of phagokinetic motility of MM1 cells was as follows. Uniform carpets of colloidal particles were prepared on slide glasses with a slight modification of the method previously described by Albecht-Buehler and Goldman.[21] The slide glasses were further coated with FN, when necessary. The glasses were placed in 35-mm tissue culture dishes containing 2 ml of culture medium, and 1.2×10^5 MM1 cells that had been washed once with M-MEM were added to each dish and incubated at 37°C for 7 h in a CO_2 incubator. The area swept out by MM1 cells was measured with an image analyzer (Olympus XL-20, Tokyo). The cell motility was expressed as total area of gold particles swept out by 100 cells in 7 h.

Observation of MM1 cell morphology A 35-mm dish was coated with 20 μg/ml FN at room temperature for 3 h. The FN-coated dish was blocked with modified M-MEM supplemented with 1.5% BSA, and then the morphology of MM1 cells was observed in it in the absence or presence of 25 μM LPA.

Staining of actin fibers and focal adhesions After incubation under appropriate conditions, MM1 cells were fixed with 3.7% formaldehyde in PBS and then permeabilized with 1% Triton X-100 in PBS. After blocking with 1% BSA in T-TBS (Tris-HCl-buffered saline supplemented with 0.5% Tween 20), filamentous actin (F-actin) was stained using rhodamine-labeled phalloidin. Focal adhesions were stained by incubating the cells with VIN-11-5, an antibody to vinculin, at room temperature for 2 h, followed by detection using rhodamine-conjugated goat anti-mouse IgG (Tago Biosource International, Camerill, CA) under a BX-50 fluorescence microscopy (Olympus).

Western blot analysis Total cell lysates were prepared by mixing the cell suspension with an equal volume of two-fold concentrated sodium dodecylsulfate (SDS) sample buffer (SDS, dithiothreitol, Tris-HCl, pH 7.2). The mixture was then heated at 100°C for 5 min and applied to a SDS-

polyacrylamide gel. For immunoprecipitation analysis, MM1 cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.2, 1 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* sodium orthovanadate, 50 m*M* sodium fluoride). The lysate was clarified by centrifugation at 16 000*g* at 4°C for 10 min. The protein to be analyzed was immunoprecipitated with the antibody against it that was conjugated with protein A and G agarose in RIPA buffer. Western blot analysis of the total cell lysate or immunoprecipitate was performed by essentially the same method as described.[20] Briefly, proteins separated in SDS-polyacrylamide gel were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 1% BSA solution in T-TBS, and incubated with a primary antibody in T-TBS for 120 min or overnight. After washing with T-TBS, the membrane was reacted with a suitable secondary antibody conjugated with horseradish peroxidase (HRP). Signals were detected using a chemiluminescence detection kit (ECL) after intensive washing.

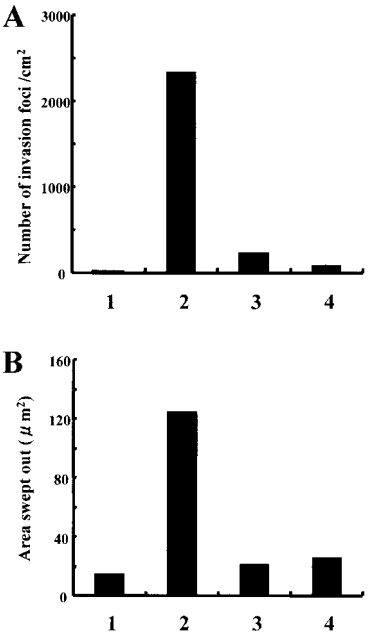

Fig. 1. The effect of C3 or Y-27632 on the *in vitro* invasion (A) and phagokinetic movement (B) of MM1 cells. The effect of C3 on the invasive capacity of MM1 was tested by pretreating them with 10 μg/ml C3 for 24 h. The effect of Y-27632 was tested by adding it to the invasion assay system 15 min before the addition of LPA to a final concentration of 10 μM. The invasive capacity was expressed as the mean of at least three determinations. Phagokinetic movement was assayed in the presence of FN. 1, LPA-free; 2, 25 μM LPA; 3, 25 μM LPA with the pretreatment of MM1 cells with C3; 4, 25 μM LPA in the presence of Y-27632 (10 μM in the invasion assay and 30 μM in the assay of phagokinetic movement).

RESULTS

Inhibition of invasion and phagokinetic movement of MM1 cells by Y-27632 MM1 cells exhibited invasion through a mesothelial cell monolayer in response to LPA. This transcellular migration was inhibited by the pretreatment of MM1 cells with C3 or by the addition of Y-27632 to the invasion assay medium. A representative result is shown in Fig. 1A. We also tested the effects of C3 and Y-27632 on phagokinetic movement of MM1 cells. C3 and Y-27632 again remarkably suppressed phagokinetic movement of MM1 cells (Fig. 1B). These results indicate that C3 and Y-27632 suppress the migration of MM1 cells independently of assay systems. In addition, a contribution of the Rho/ROCK pathway to MM1 cell migration is strongly indicated.

Inhibition of LPA-induced morphological changes of MM1 cells by Y-27632 We have already clarified the correlation between motility and morphology of MM1

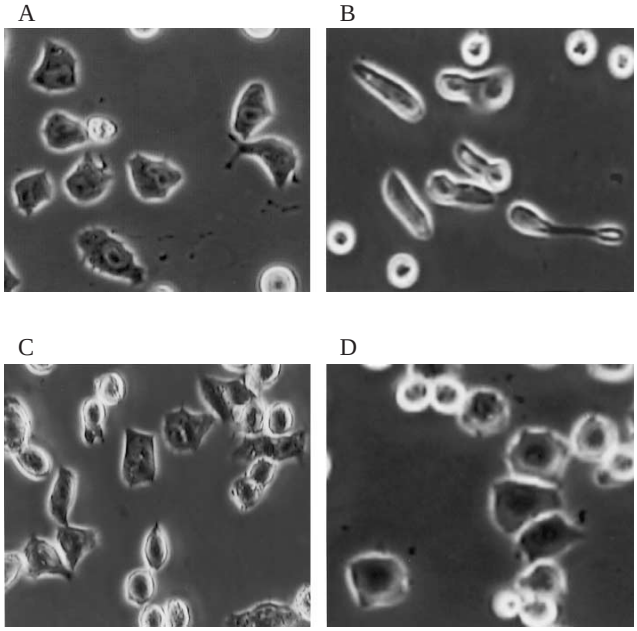

Fig. 2. Morphological response of MM1 cells to LPA, and suppression of it by 30 μM Y-27632. MM1 cells on an FN-coated dish were polygonal-shaped in the absence of the stimulation (A). When stimulated with 25 μM LPA for 30 min, they became fusiform-shaped with pseudopodia formation (B). This morphological response of MM1 cells to LPA was abolished by the pretreatment of MM1 cells with C3 (C), or by the addition of 30 μM Y-27632 (D).

cells. Briefly, an appreciable ratio of mobile MM1 cells exhibited a fusiform shape with pseudopodia, whereas immobile MM1 cells were spherical or polygonal without pseudopodia. For example, serum-starved MM1 cells were spherical or polygonal as shown in Fig. 2A, whereas fusiform-shaped MM1 cells with pseudopodia appeared after stimulation with the combination of LPA and FN (Fig. 2B). As described above, motility of MM1 cells is induced by this combination. Y-27632 suppressed this morphological response of MM1 cells to LPA and FN, and maintained MM1 cells in spherical or polygonal shape even in the presence of LPA (Fig. 2D). Note that MM1 cells that were treated with C3 showed a similar morphology to serum-starved MM1 cells (Fig. 2C).

Focal adhesions and actin fibers in Y-27632-treated MM1 cells Prominent differences between polygonal-shaped MM1 cells and fusiform-shaped ones were present in their focal adhesions and actin fibers. No focal adhesions or actin bundles were observed in polygonal-shaped MM1 cells stained with anti-vinculin antibody or rhodamine-labeled phalloidin, respectively (Fig. 3, A and B). In contrast, MM1 cells that became fusiform-shaped after stimulation with LPA on FN possessed focal adhesions and actin bundles (Fig. 3, C and D). When MM1 cells were stimulated with the combination of LPA and FN in the presence of Y-27632, no focal adhesions or actin bundles were observed (Fig. 3, E and F), indicating that the formation of these structures is regulated by ROCK. The expression of vinculin in MM1 cells was not influenced by treatment with Y-27632, when tested by western blot analysis (data not shown).

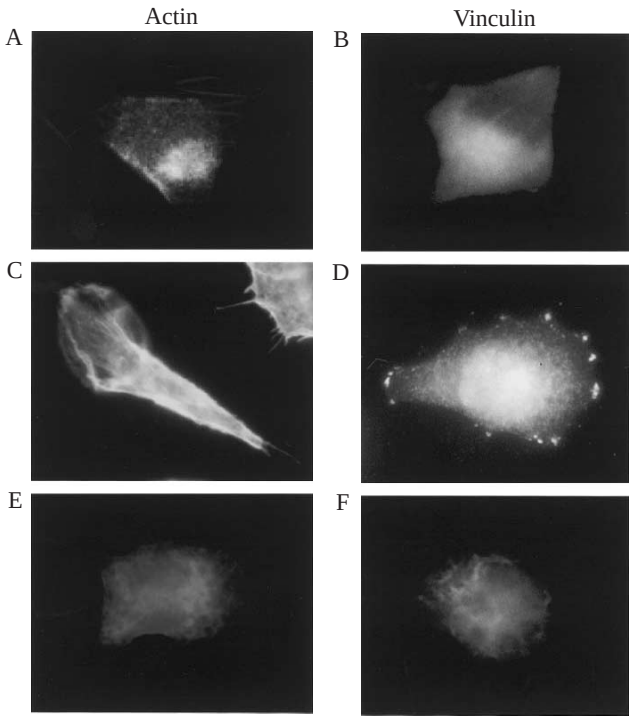

Fig. 3. Focal adhesions and actin fibers in MM1 cells. Focal adhesions were visualized by staining vinculin with anti-vinculin antibody VIN-11-5. Actin staining was performed with rhodamine-labeled phalloidin. No focal adhesions or actin bundles were observed in polygonal-shaped MM1 cells (A and B). In contrast, when stimulated with 25 μM LPA on FN, MM1 cells became fusiform-shaped and possessed actin bundles and focal adhesions (C and D). Y-27632 at the concentration of 30 μM suppressed these responses of MM1 cells to the combination of LPA and FN (E and F).

Suppression of LPA-induced tyrosine phosphorylation of FAK and paxillin by Y-27632 FAK and paxillin are among the most critical molecules in regulating focal adhesions and actin dynamism.[2, 3] We have already shown that tyrosine phosphorylation occurred mainly in three proteins of MM1 cells, FAK, paxillin, and p41/42 MAP kinase (MAPK). Our previous studies revealed that tyrosine phosphorylation of FAK and paxillin, in cooperation with other signaling pathways, was involved in cellular migration. Fig. 4 shows the results of western blot analysis of whole cellular lysate with anti-phosphotyrosine antibody. LPA-induced tyrosine phosphorylation of FAK and paxillin was suppressed by Y-27632, whereas that of MAPK was not. By immunoprecipitation, we confirmed increased tyrosine phosphorylation of FAK in response to

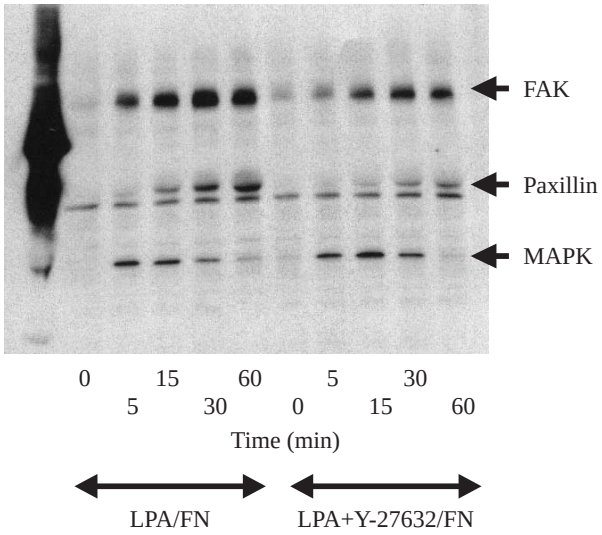

Fig. 4. Suppression by Y-27632 of LPA-induced tyrosine phosphorylation of FAK and paxillin. MM1 cells were stimulated with 25 μM LPA for 5 min. Y-27632 was added 15 min before the addition of LPA to the final concentration of 30 μM. Western blot analysis using whole cellular lysate of MM1 cells is shown.

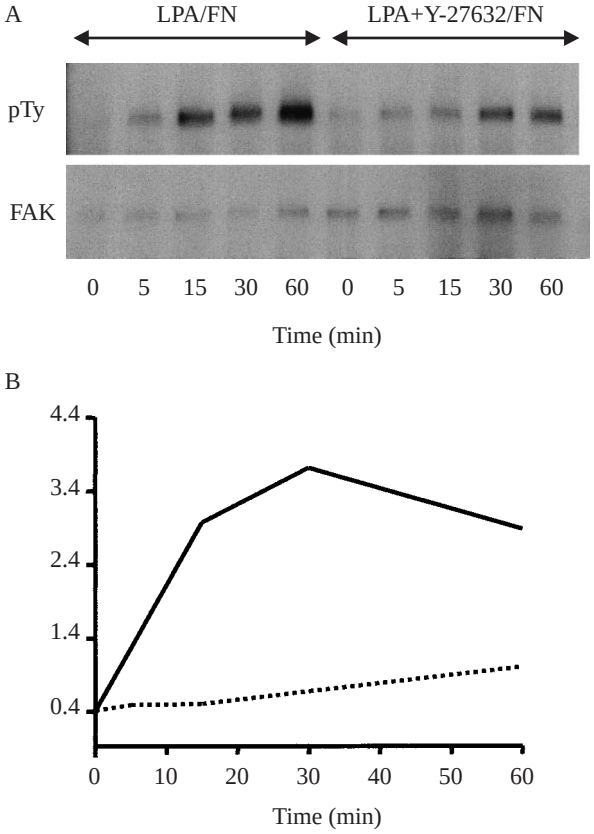

Fig. 5. Suppression by Y-27632 of LPA-induced tyrosine phosphorylation of FAK. MM1 cells were stimulated with 25 μM LPA for 5 min. Y-27632 was added 15 min before the addition of LPA to the final concentration of 30 μM. Western blot analysis using the immunoprecipitate of MM1 cells by anti-FAK is shown. ── Y-27632 (−), ---- Y-27632 (+).

LPA and the suppression of this response by Y-27632 (Fig. 5).

DISCUSSION

Cellular movement including invasion of cancer cells is regulated by various intracellular signals. The Rho/ROCK pathway plays central roles in the invasion and phagokinetic movement of MM1 cells in cooperation with integrins. In the present study, we showed that an inhibitor of ROCK, Y-27632, suppressed MM1 cell migration as evidenced by suppressed invasiveness and decreased phagokinetic movement in the presence of the inhibitor. ROCK is involved in Rho-mediated induction of migration of MM1 cells. The inhibitory effect of Y-27632 on MM1 cell migration had a tight correlation with its effect on the morphology of MM1 cells. Y-27632 suppressed the formation of fusiform-shaped cells with pseudopodia, which represent mobile MM1 cells.

Decreased motility of MM1 cells by Y-27632 seems to be ascribed to the impaired formation of intracellular structures required for cell movement, typically focal adhesions and actin bundles. ROCK is known to contribute to the formation of focal adhesions and stress fibers of N1E-115 neuroblastoma cells.[22] The involvement of ROCK in focal adhesion formation of HeLa cells was also shown using a mutant ROCK cDNA.[23] Our results are on the same lines as these studies. Na-H exchange acts downstream of RhoA and is mediated by ROCK.[24] The inhibition of Na-H exchange activity is reported to suppress cell spreading and to impair the accumulation of integrin, paxillin, and vinculin at focal adhesions.[25] The inhibition of focal adhesion formation of MM1 cells by Y-27632 may be ascribed to the suppression of the Na-H exchanger. ROCK also activates LIM-kinase, resulting in cofilin phosphorylation, which induces the reorganization of actin fibers. LIM-kinase may be essential for LPA-induced actin bundle formation of MM1 cells. In accordance with this observation, LPA-induced tyrosine phosphorylation of FAK and paxillin was remarkably suppressed by Y-27632. In contrast, LPA-induced tyrosine phosphorylation of MAPK was not affected by Y-27632. PD-98059, an inhibitor of MAPK, did not suppress the invasion of MM1 cells (data not shown).

In summary, the formation of focal adhesions and actin bundles is regulated by ROCK. Since FAK and paxillin regulate the function of focal adhesions, at least in part, via their tyrosine phosphorylation, ROCK also controls the function of focal adhesions by regulating the phosphorylation of FAK and paxillin. Our results suggest that Y-27632 suppresses migration of MM1 cells by inhibiting this function of ROCK.

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