Small GTP-binding Protein Rho Stimulates the Actomyosin System, Leading to Invasion of Tumor Cells*

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We have shown previously that Rho plays a pivotal role in 1-oleoyl-lysophosphatidic acid (LPA)-dependent invasion of rat hepatoma cells (MM1). Herein we made stable transfectants of MM1 expressing active and Botulinum exoenzyme C3 (C3)-sensitive (Val14), or active and C3-insensitive (Val14/Ile41) forms of human RhoA. Both transfectants showed greatly promoted invasive ability in vitro in the absence of LPA as well as in vivo, adherence to the dish with scattered shape, and enhanced phosphorylation level of 20-kDa myosin light chain (MLC20). A specific MLC kinase inhibitor (KT5926) could inhibit their invasion and the phosphorylation level of MLC20. Stable active RhoA transfectants of W1 cells (low invasive counterpart of MM1) also demonstrated promoted invasive ability in vitro and in vivo, and enhanced phosphorylation level of MLC20. C3 treatment inhibited the invasiveness of the Val14 RhoA transfectant but not that of the Val14/Ile41 RhoA transfectant. LPA enhanced the invasiveness of both transfectants, and this enhancement was abolished by the C3 treatment. These results suggested that 1) the Rho signaling pathway and actomyosin system were linked in the transmigration of tumor cells, and 2) expressed active RhoA enhanced LPA-induced tumor cell invasion via the activation of endogenous RhoA pathway, indicating a positive feedback mechanism in the activation of RhoA.

Transcellular migration of tumor cells through host cell layer is one of the most crucial steps in cancer invasion and metastasis (1–4). We have previously developed a cell monolayer invasion assay, in which rat hepatoma cells (AH130) invade through a cultured mesothelial cell monolayer (MCL)5

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§ The abbreviations used are: MCL, mesothelial cell monolayer; Ab, antibody; C3, C3 ADP-ribose transferase; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PCR, polymerase chain reaction; GTPγS, guanosine 5′-3-O-(thiotriphosphate); LPA, 1-oleoyl-lysophosphatidic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum; V14RhoA and V14/I41RhoA, Val14 and Val14/Ile41 forms of RhoA, respectively; MLCK, myosin light chain kinase; MLC, myosin light chain; PMLC, phosphorylated myosin light chain; GST, glutathione S-transferase.

EXPERIMENTAL PROCEDURES

Materials

G418 (Geneticin) and all culture medium were obtained from Life Technologies, Inc. Pico DNA polymerase was purchased from Boehringer Mannheim (Mannheim, Germany). LPA and KT5926 were from Sigma. LPA was dissolved in PBS containing 0.1% BSA. KT5926 was dissolved in Me2SO. [α-32P]dCTP (3000 Ci/mol), [α-32P]NAD (800 Ci/μmol), and all other reagents were purchased from Amersham.
Mesothelial cells were isolated from Donryu rat mesentery and cul-
tured in minimum essential medium containing 2-fold concentrations of
amino acid and vitamins (M-MEM) supplemented with 10% fetal calf
serum (FCS), as reported previously (5). MM1 cells (a highly invasive
clonal) (5) and W1 cells (a very low invasive clone) (6), isolated from the
same parental AH130 cells, were maintained in a suspension in the
M-MEM supplemented with 10% FCS and split at a 1:20 ratio every 3
days.

Construction of Mutant RhoA Expression Vectors

The expression plasmids were designed to generate from pEXV-
active (Val14) RhoA vector, provided by Dr. A. Hall (University college,
London). A 0.63-kilobase pair
insertion was carried out as follows: denatu-
rating for 1 min at 94 °C, annealing for 1 min at 56 °C, extension for 1
min at 72 °C with 30 thermal cycles, following 7-min extension using
Pvo DNA polymerase. This fragment contained the FLAG tag sequence
at the N terminus of full-length human RhoA cDNA with an EcoRI site
engineered before the start ATG codon and a NotI site after the 3’-end
of the open reading frame. The generated mutant cDNA was introduced
into the mammalian expression vector pcDNA3 (Invitrogen, San Diego,
CA) at the site of EcoRI and NotI. Mutation of active RhoA (C3-sensit-
ive) to constitutively active RhoA (Val14, codon 41 Asn (AAC) to Ile
(ATA)) was generated as follows. First PCR used two sets of primers: first set forward primer, 5’-CTTTGAGATCTATGTGGCAGAT-3’ (20-mer); sec-
tend set forward primer, 5’-TGTGATCCTGGACCAATGAT-3’ (20-mer); first set reverse primer, 5’-TCACAAGAAGACACAG3’ (15-mer) with pcDNA3-FLAG-Val14Rho as the template. 30 cycles of PCR were
utilized as follows: denaturation for 1 min at 94 °C, annealing for 1
min at 60 °C, extension for 1 min at 72 °C following a 7-min extension.
The PCR products were separated by agarose gel electrophoresis and
were extended to generate double-stranded DNA in the presence of
Pvo DNA polymerase and dNTPs for 5 min at 72 °C. The products were gel-pu-
rifed and then served as the template for the second series of PCR
using the forward primer 5’-CTGGAGAAGACATG3’ (15-mer) and the reverse
primer 5’-TCACAAGAAGACACAG3’ (15-mer). The mutant cDNAs were then
inserted into the expression vector pcDNA3 at the sites of EcoRI and NotI. All plasmids were sequenced with the dideoxy
termination method (30) using (α-35S)ATP and the Sequi-Gen sequence
apparatus (Bio-Rad) to verify the correct substitutions.

Transfection

Cells (MM1 or W1) in log phase growth were centrifuged and resus-
pended in Ca2+- and Mg2+-free PBS at a concentration of 1 × 106
cells/ml. 5 μg of plasmid was added to 80 μl of cell suspension, and
electroporation was performed using a Bio-Rad Gene Pulser (capaci-
tance, 25 microfarads; field strength, 2.5 kV/cm) as described previously
(27). After the transfection, the cells were grown in the M-MEM with
10% FCS for 48 h, following the selection with G418 (200–300 μg/ml).
Clonal transfectants were isolated with limiting dilution method.

Cell Monolayer Invasion Assay

The assay procedure of in vitro invasive ability of tumor cells was
described previously (5). Briefly, after mesothelial cells from rat mesen-
tery had reached confluency in a 35-mm dish, the culture medium
was changed to M-MEM containing LPA or FCS. The number of
penetrated single tumor cells and tumor cell colonies (invasion foci)
was counted under a phase contrast microscope (Olympus, Japan) in 16
different visual fields (5 mm2 each). The in vitro invasion ability was
quantitatively calculated as the percentage of infiltrated cells out of
total seeded cells. In some experiments, tumor cells were pretreated with 50 μg/ml C3 for 24 h or with 15 μM KT5926 for 30 min, followed by
washing twice with M-MEM. Statistical comparisons were made using
Student’s t test.
Expression of Active RhoA (Val and Val/Ile) Induced in Vivo—Invasiveness and Morphological Change in MM1 Cells—To examine the role of RhoA protein in tumor cell motility and morphology, we expressed RhoA protein mutants in MM1 cells (a highly invasive cell line from parental AH130 cells). Full-length CDNA of active and C3-sensitive Val (V14), or active and C3-insensitive (A31), the catalytic site of C3 to Ile, Val/Ile (V14/I41) form of human RhoA was constructed with N-terminal FLAG tag sequence and introduced into the mammalian expression vector pcDNA3 (see Fig. 1; compare the determined by the previously reported urea-PAGE immunoblotting method using anti-MLC20 Abs (36).

Detection of FLAG-RhoA and Endogenous RhoA—Cells were washed with PBS twice and lysed in a lysis buffer (10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM DTT, 1% Triton X-100, 1% SDS, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, and 10 μg/ml aprotinin) for 30 min in ice. The lysates were centrifuged to remove insoluble materials, normalized according to their protein content, and loaded onto SDS-12% PAGE. The gel was transferred to a Finetrap NT-31 membrane (Nippon Eido, Tokyo, Japan) with a semidry blotting apparatus and blocked for 1 h at room temperature with 3% BSA (Promega, Madison, WI) in TPBS. Primary antibodies were 1 μg/ml anti-Rho polyclonal Abs (SC-179, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which could detect human and rat RhoA equally. Secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) used at a 1:50,000 dilution, and final signal was detected by ECL (Amersham). Next, FLAG-RhoA proteins were detected on the same blots. Membrane was stripped with a solution containing 2% SDS, 100 mM 2-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50 °C, washed with TPBS, and blocked with 3% BSA in TPBS for 16 h at 4 °C. Membrane was then reprobed with anti-FLAG M5 monoclonal Abs (Eastman Kodak Co.) (9 μg/ml) for 1 h at room temperature, washed with TPBS, and incubated with a 1:20,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham) for 1 h at room temperature following detection by ECL.

Estimation of MLC20 Phosphorylation in Situ—Cells were starved in the M-MEM without FCS for 16 h and subsequently treated with LPA at 37 °C for various times. The cell lysate was prepared as described under “Estimation of MLC20 Phosphorylation in Situ.” The lysates were separated by SDS-8% PAGE. Proteins were transferred to a nitrocellulose membrane with the semidy blotting method and blocked with 3% BSA in TPBS for 1 h at room temperature. Primary antibodies were a 1:1000 dilution of anti-phosphospecific MAPK polyclonal Abs (New England Biolabs, Beverly, MA), and secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase used at a 1:7500 dilution and developed with the same L-phenyl-2-thiouracil and 5-bromo-4-chloro-3-indolyl-phosphate (Promega) as substrate. The blot membrane was scanned with a GS-9500 gel scanner (Epson, Japan) and analyzed with NIH Image software. Western blot analysis of human RhoA protein mutants in MM1 cells (a highly invasive cell line from parental AH130 cells). Full-length CDNA of active and C3-sensitive Val (V14), or active and C3-insensitive (A31), the catalytic site of C3 to Ile, Val/Ile (V14/I41) form of human RhoA was constructed with N-terminal FLAG tag sequence and introduced into the mammalian expression vector pcDNA3 (see Fig. 1; compare the
size of transcripts of endogenous and expressed RhoAs). These plasmids were transfected into MM1 cells, and stably expressed transfectants were isolated by the selection using G418. We obtained a number of clones of stable transfectants and analyzed them with RNA blotting using a RhoA probe as shown in Fig. 2A. The expression levels of active RhoA transcript varied from 10 to 80% of those of endogenous RhoA in these transfectants. Immunoblotting analysis using anti-FLAG monoclonal Abs or anti-RhoA polyclonal Abs (Fig. 2B) revealed that the protein expression level of FLAG-RhoA was 5–10% of that of endogenous RhoA in these cells (Fig. 2, compare A and B), suggesting posttranscriptional regulation in the expression of RhoA. The mobility difference between V14RhoA and V14/I41RhoA on SDS-PAGE (Fig. 2B) was likely to be the conformational difference in these RhoA mutants, which has already been reported previously (12). Fig. 2C demonstrates that these transfectants showed 25–100 times higher in vitro invasiveness through the MCL in the absence of FCS in the assay medium than did mock transfectants. It is of note that there was a positive correlation between the expression level of active RhoA and in vitro invasiveness among these transfectant clones (Fig. 2C).

We next examined the morphology of these cells in the presence of serum. In contrast to mock transfectants, which grew in suspension, the V14RhoA and V14/I41RhoA transfectants adhered to the plastic culture dish with scattered shape (Fig. 3, A, B, and D). Treatment with 50 μg/ml exoenzyme C3 for 24 h made V14RhoA cells completely round up, and the cell morphology was reversed to that of parental MM1 cells (Fig. 3C); however, V14/I41RhoA cells were resistant to the C3 treatment and remained adhered to the culture dish (Fig. 3E).

**Active RhoA Promoted Invasiveness in Vivo**—To examine the in vivo invasive ability of these transfected cells, 2 × 10⁵ cells were implanted in the peritoneal cavity of the syngenic rat. The active RhoA-transfected cells invaded more extensively into the peritoneum and formed many more tumor nodules as compared with mock transfectants (data not shown). The incidence of macroscopic tumor nodule present in the peritoneum of rats implanted with active RhoA transfectants (6 of 10 for V14RhoA and 5 of 7 for V14/I41RhoA) was higher than that of rats implanted with mock transfectants (2 of 8) (see Table I). These results suggested that the activation of RhoA greatly promoted the tumor invasive ability in vivo as well as in vitro. We could not find any macroscopic metastatic legion (lung, liver, spleen, stomach) in these rats.

**Active RhoA Enhanced MLC20 Phosphorylation**—MM1 cells require the presence of FCS to exert their transcellular migration in vitro, and it has been shown that LPA can be substituted for serum (8). Recently, a series of reports (26, 37, 38) indicated that stimulation of fibroblasts with LPA activated Rho-induced contractility and stress fiber formation. These processes seem to be linked to the activation of the actomyosin system (26). To ascertain whether the active RhoA-transfected MM1 cells stimulate the actomyosin system, the phosphorylation level of MLC20 in the MCL was examined with immunoblotting using anti-phosphorylated MLC20 polyclonal Abs (specific for phosphorylated Ser¹⁹) (39). The phosphorylation level of MLC20 was increased 2–5-fold in both V14RhoA and V14/I41RhoA transfectants compared with mock transfectants (Fig. 4, A, upper panel, and B, left two columns), but it was completely abolished by the treatment with 15 μM KT5926 for 30 min (a specific inhibitor for MLC kinase (40); Fig. 4B, column 3). However, this effect of KT5926 was temporal and declined in a time-dependent fashion (Fig. 4, A bottom) and B). After the removal of 15 μM

**FIG. 2.** Induction of in vitro invasiveness in transfected MM1 cells with human active RhoA. A, RNA blot analysis. 4 μg of total RNA isolated from several clones (clones 1–3) of transfectants, MM1 cells, and transfec tant of vector alone were separated in a 1% agarose gel. Filter was prepared and hybridized with the radioactive probe indicated on the left. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, immunoblot analysis. Cell lysates prepared from each cells were separated on SDS-12% PAGE followed by immunoblotting with anti-RhoA Abs. Blots were stripped and reprobed with anti-FLAG Abs. The expression levels of V14RhoA and V14/I41RhoA were determined by scanning the intensity of each band on the blots and averaging. C, expression level of V14RhoA and V14/I41RhoA transfectants showed 25–100 times higher in vitro invasion ability was calculated as the percentage of the infiltrated cells out of the total seeded cells. Dotted bars represent in vitro invasiveness, and closed bars represent the level of expression of active RhoA estimated from panel B. Error bars indicate the S.E. of three different experiments for the in vitro invasion assay and duplicate scanning of the blot for the expression level.
TABLE I
Augmentation of invasiveness in MM1 and W1 cells by active human RhoAs in vivo

Rats were sacrificed 10 days after implanted each 2 × 10^7 cells in the peritoneal cavity. A solitary tumor nodule was formed in the peritoneum of the rats that received control pDNA3 alone transfectants (data not shown). Numerous tumor nodules formed in the peritoneum of rats that received V14RhoA-2 transfectants or V14/I41RhoA-1 transfectants (data not shown).

| Cells           | Tumor incidencea |
|-----------------|------------------|
| MM1             | 2/8              |
| W1              | 2/5              |

| Vector alone transfectants | V14RhoA-2 transfectants | V14/I41RhoA-1 transfectants |
|---------------------------|-------------------------|-----------------------------|
| 6/10                      | 3/5                     | 5/7                         |

a Values for tumor incidence are number of animals showing invasive tumor nodules/number of animals implanted.

KT5926 and wash out of this inhibitor, the phosphorylation level of MLC20 in the V14/I41RhoA-1 cells gradually elevated to 32.2% (4 h), 37.5% (8 h), 54.2% (12 h), and 84.9% (20 h) of that of the untreated transfectants (percentage of MLC20 phosphorylation was 47.9%). The impaired in vitro invasive ability was also restored in a similar manner (Fig. 4C), suggesting that the Rho signaling pathway and actomyosin system were linked in the transcellular migration of tumor cells through the host cell layer.

Active RhoA Induced Invasiveness in W1 Cells in Vitro and in Vivo—We have previously isolated a number of AH cell sublines showing different in vitro invasive ability from the same parental AH130 cells (6). A clone named W1 showed very low in vitro invasive even in the presence of serum or LPA, whereas MM1 demonstrated high invasiveness. Thus, we compared the response to LPA between MM1 and W1 cells. The treatment with 25 µg of LPA increased the phosphorylation level of MAPK in a time-dependent manner peaking at 5 min in both cells (Fig. 5, A and B, top). However, W1 cells showed little change in the phosphorylation level of MLC20 by LPA stimulation (Fig. 5B, bottom), while MM1 cells showed increased MLC20 phosphorylation peaking at 30 min after stimulation with LPA (percentage of MLC20 phosphorylation was 11.6% at time 0 and 33.5% at 30 min after LPA stimulation; Fig. 5A, bottom). Moreover, the treatment of MM1 cells with 50 µg/ml of C3 for 24 h completely abolished the change in phosphorylation level of MLC20 with LPA stimulation (data not shown). These observations suggested two notions: 1) both cells expressed the putative LPA receptors, and 2) W1 cells lacked Rho-actomyosin stimulation induced by LPA, which resulted in little invasiveness of these cells in vitro.

To try to focus on the second question, we introduced active human RhoA (V14RhoA and V14/I41RhoA) into W1 cells. We obtained several stable transfectants expressing 5–10% of active RhoA protein compared with the endogenous RhoA (Fig. 6A, top and middle). These transfectants also showed an increased phosphorylation level of MLC20 (Fig. 6A, bottom) as well as in vitro invasive ability in the absence of serum (Fig. 6B). Notably, the increased invasiveness of these transfectants was enhanced 3–4-fold by adding the serum into the assay medium, while mock transfectants represented little effect of serum stimulation (Fig. 6B). Furthermore, these active RhoA transfectants showed enhanced invasive ability after implantation into the peritoneal cavity similar to the MM1 transfectants (Table I). The active RhoA-transfected cells invaded more extensively into the peritoneum and formed more tumor nodules compared with the mock transfectants (data not shown). These results suggested that active RhoA expression with coincident MLC20 phosphorylation was sufficient to significantly enhance the invasiveness in vivo as well as in vitro in both cell types.

We also examined the effect of C3 on the morphology of these cells. Both V14RhoA and V14/I41RhoA W1 transfectants adhered to the culture dish with scattered shape. Treatment with C3 made V14RhoA transfectants round up; however, V14/I41RhoA transfectants were resistant to the C3 treatment.
tively ADP-ribosylated 68–96% by the treatment with 50 and expressed C3-sensitive active RhoA (V14RhoA) were effec-

shown in Figs. 7

in vitro

invasiveness of both MM1 and W1 transfectants. As

Next, we tested the effect of C3 on

enous RhoA Activation—

(data not shown).

Constitutively Active RhoA Stimulated LPA-induced Endog-

enous RhoA Activation—Next, we tested the effect of C3 on in vitro invasiveness of both MM1 and W1 transfectants. As shown in Figs. 7A and 8A, both endogenous RhoA in the cell and expressed C3-sensitive active RhoA (V14RhoA) were effectively ADP-ribosylated 68–96% by the treatment with 50 μg/ml C3 for 24 h, whereas C3-insensitive RhoA (V14/I41RhoA) was not ADP-ribosylated, although it was present in the cell lysates as detected in the immunoblot (Figs. 7B and 8B, lane 3). In the absence of LPA, the C3 treatment greatly reduced the invasiveness of V14RhoA clone (p < 0.001 for both MM1 and W1), and the invasiveness of the V14/I41RhoA clone was resistant to the C3 treatment (p = 0.04 for MM1; p = 0.11 for W1) as expected (Figs. 7C and 8C, pairs of columns 3 and 5). The invasiveness of both transfectants was enhanced 2–4-fold in the presence of LPA (Figs. 7C and 8C, pairs of columns 4 and 6). Moreover, C3 inhibited the LPA-induced invasiveness in not only V14RhoA (p < 0.001 for both MM1 and W1) but also V14/I41RhoA clones (p = 0.01 for MM1 and p = 0.004 for W1), suggesting that V14/I41RhoA enhanced the invasiveness of both MM1 and W1 cells in two ways: 1) direct activation of the RhoA downstream cascade including MLC20 phosphorylation (C3-insensitive), and 2) promotion of LPA-induced endogenous RhoA activation (C3-sensitive), for which we postulate a positive feedback mechanism in RhoA activation.

DISCUSSION

In the present study, we have prepared the MM1 cells (a highly invasive cell line from parental AH130 cells) stably expressing FLAG-V14RhoA or FLAG-V14/I41RhoA. Although several lines of evidence suggested the involvement of Rho in cell proliferation, the active RhoA-transfected MM1 (V14RhoA and V14/I41RhoA) showed little change in growth (estimated doubling times from growth curve were 14.0 h for MM1 cells, 15.4 h for V14RhoA transfectants, and 15.4 h for V14/I41RhoA transfectants, respectively). By using rat fibroblasts (Rat-1) and mouse fibroblasts (NIH 3T3), human RhoA transfectants were found to show an increase in growth and saturation density (41), and the tumorigenicity of human RhoA-transfected Rat-1 cells was correlated with amplification and expression of RhoA (42). In addition, NIH 3T3 cell transfectants overexpressing normal or active RhoA proteins from Aplysia were also reported to have increased cell growth, and tumors were induced when inoculated into nude mice (16). Yamamoto et al. (43) indicated that the treatment with exoenzyme C3 caused inhibition of cell growth and accumulated in the G1 phase of the cell cycle in Swiss 3T3 cells. Likewise, C3 also stopped the growth and differentiation of PC12 cells (44). In contrast, C3 treatment hardly affected the growth of both active RhoA transfected cells and mock transfectants during the period of in vitro invasion assay in the present study (data not shown). We suggest that the data, taken together, indicate that activation...

Fig. 5. Alteration of phosphorylation levels of MAPK and MLC20 in MM1 (A) and W1 (B) cells stimulated with LPA. Cells were deprived of serum for 24 h and stimulated with 25 μM LPA during the indicated period. Cell lysates were separated on SDS-8% PAGE followed by immunoblotting with anti-phosphospecific MAPK poly-
clonal Abs (top). Blots were stripped and reprobed with anti-p44/42 MAPK polyclonal Abs to ensure equal protein loading (middle). At the bottom, cell lysates were separated on SDS-12% PAGE followed by immunoblotting with anti-PMLC20 polyclonal Abs. The horizontal axis of each graph represents the phosphorylation level of MAPK (arbitrary units) using NIH Image software, and the phosphorylation level of MLC20 was normalized using cultured endothelial cells as an internal control (for percentage of phosphorylation, see “Experimental Procedures”). A typical result from three independent experiments is shown.

Fig. 6. Induction of invasiveness in transfected W1 cells with active human RhoA. A, immunoblot analysis. Cell lysates from four different clones (two of V14RhoA and two of V14/I41RhoA) were sepa-
rated on SDS-12% PAGE followed by immunoblotting with RhoA Abs. Blots were stripped and reprobed with anti-FLAG Abs. The level of MLC20 phosphorylation was semiquantitated using anti-PMLC20 polyclo-

nal Abs. B, in vitro invasion assay. Cells (2 × 10⁴ cells/dish) were seeded on MCL in the absence or presence of FCS in the assay medium. In vitro invasion ability was calculated in the manner described for Fig. 2C. Results were representative of three independent experiments.
of RhoA largely affects the stimulation of motility but not growth in AH cells. These controversial results of the function of Rho might be attributable to properties of the different cell types.

Using both MM1 and W1 cells (a low invasive counterpart of MM1 cells), we have shown here that the active RhoA transfectants show morphological changes such as adherence to the culture dishes, the ability for migration through MCL even in the absence of FCS or LPA, and the ability of invasiveness in vivo. It is interesting to note that control cells (mock transfectants) developed solid tumors after implantation into the peri toneal cavity, while active RhoA transfectants formed many more small tumor nodules disseminated in the peritoneum using both MM1 and W1 cells. These results strongly suggest that expressed active RhoA plays a pivotal role in the invasion of AH cells. This was further confirmed by the fact that the invasiveness of V14/141RhoA transfectants showed little response to the exoenzyme C3, indicating that C3-insensitive expressed active V14/141RhoA could drive the ability of invasiveness.

Next, we focused on how Rho regulates transmigration of AH cells in vitro and hypothesized that the stimulation of the actomyosin system induced by active RhoA leads to the subsequent transmigration of AH cells. The above speculation was supported by the following three findings. 1) Active RhoA transfectants enhanced MLC20 phosphorylation. 2) KT5926, a specific inhibitor of MLCK, greatly suppressed MLC20 phosphorylation as well as the transmigration of active RhoA transfectants. 3) The time-dependent decline of the effect of KT5926 restored the transmigration ability in these cells.

A series of reports support this hypothesis using different cell systems. Jalink et al. (25) reported that LPA-induced neurite retraction of NIE-115 and PC12 cells was abolished by the treatment with C3. They proposed that RhoA regulated LPA induced actin-myosin contractility through the balance between MLCK and MLC20 phosphatase activities employing KT5926 in these cells. Chrzanowska-Wodnicka and Burridge (26) reported that KT5926 blocked contraction, formation of stress fibers and focal adhesions, and tyrosine phosphorylation in LPA-stimulated fibroblasts. They also found that 2,3-butanedione-2-monoxime, an inhibitor of actin-myosin interactions, blocked active RhoA-induced formation of stress fibers, suggesting that activation of Rho results in change of cytoskeletal structures, promoted by MLC20 phosphorylation. In permeabilized pig aortic smooth muscle cells, GTPγS-induced Ca2+ stimulation of MLC20 phosphorylation has been reported to involve Rho (22). Gong et al. (23, 24) reported that GTPγS-induced Ca2+ stimulation of MLC20 phosphorylation has been reported to involve Rho (22). Gong et al. (23, 24) reported that GTPγS-induced translocation of RhoA associated with Ca2+ sensitization of rabbit portal vein smooth muscle. Using a cell-free system, Amano et al. (45) reported that GTPγS-RhoA can activate Rho-kinase, and then activated Rho-kinase directly phosphorylates MLC20 at the site of Ser19 in vitro. It is important to...
Among them, the multiple domains in Rho-associated kinase activity or decreased MLC20 phosphatase activity. A number of studies have shown that activation of active Rho was attributable to either increased MLCK or decreased MLC20 phosphatase activity. Therefore, complete suppression of active Rho-induced MLC20 phosphorylation by KT5926 could inhibit MLCK; however, this inhibitor had little effect on other kinases. Therefore, complete suppression of active Rho-induced MLC20 phosphorylation by KT5926 was due to the inhibition of MLCK rather than Rhokinase, and the elevation of MLC20 phosphorylation induced by active Rho was attributable to either increased MLCK activity or decreased MLC20 phosphatase activity. A number of putative Rho target proteins have been identified (46–53). Among them, the multiple domains in Rho-associated kinase which could interact with reorganization of the cytoskeleton (54, 55). Most recently, we made MM1 cells stably expressing the constitutively active form of Rhokinase. These cells showed a striking phenotype (including spreading and adhering on the plastic dish), increased in vitro invasiveness without LPA stimulation, and enhanced the phosphorylation level of MLC20.4 LPA was a crucial regulator in the transmigration of MM1 cells through MCL (6). Conversely, W1 cells did not show transmigration upon serum (LPA) stimulation (6). We compared the responses to LPA, such as the phosphorylation levels of MAPK and MLC20 between MM1 and W1 cells. Both cells responded to LPA in respect to MAPK activation, suggesting the absence of LPA. We also detected significant elevation of the phosphorylation level of MLC20 in MM1 cells; however, W1 cells had little response to LPA concerning the phosphorylation level of MLC20. This implies lack of Rho-actomyosin stimulation induced by LPA in W1 cells, resulting in very low invasiveness of these cells. To clarify this possibility, we introduced active RhoA (V14RhoA or V14/I41RhoA) into W1 cells. Consequently, these transfectants showed enhanced MLC20 phosphorylation and a significant increase in invasiveness in the absence of LPA. Furthermore, the invasiveness of V14/I41RhoA transfectants in the absence of LPA showed little response to C3, suggesting that the invasiveness was largely mediated by C3 insensitive expressed active RhoA.

It is interesting to note that active RhoA W1 transfectants responded to LPA, showing enhanced extensive invasiveness, while parental W1 cells represented little invasiveness even in the presence of LPA. We speculate that expressed active RhoA enhanced LPA-induced invasiveness via the activation of endogenous RhoA pathway. This hypothesis is supported by the following findings. In V14RhoA W1 transfectants, LPA-enhanced transmigration was C3 sensitive, indicating both endogenous and expressed RhoA activation (Fig. 8, pairs of columns 3 and 4). Conversely, in V14/I41RhoA W1 transfectants, transmigration was C3-insensitive in the absence of LPA, representing expressed RhoA-induced invasion (Fig. 8, pair of columns 5). LPA enhanced the transmigration of these cells and this enhancement was C3-sensitive (p = 0.004, Fig. 8, pair of columns 6), suggesting that C3-insensitive active RhoA (V14/I41RhoA) stimulates endogenous RhoA activation, indicating a positive feedback mechanism. The major findings in this study were summarized as the proposed model in Fig. 9.

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