Overexpression of Small GTP-binding Protein RhoA Promotes Invasion of Tumor Cells

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ABSTRACT

Adhesion of tumor cells to host cell layers and subsequent migration are pivotal steps in cancer invasion and metastasis. The small GTP-binding protein RhoA controls cell adhesion and motility through organization of the actin cytoskeleton and regulation of actomyosin contractility. Cultured rat MM1 hepatoma cells migrate through a mesothelial cell monolayer in vitro in a serum-dependent, RhoA-mediated manner (K. Yoshioka et al., J. Biol. Chem., 273: 5146–5154, 1998). Furthermore, the ROCK family of RhoA-associated serine-threonine protein kinases is involved in this migration, and an inhibitor for these kinases effectively inhibits the invasion of MM1 cells in vitro and in vivo (K. Itoh et al., Nat. Med., 5: 221–225, 1999). Although there have been no reports of genetic alterations directly affecting RhoA in human cancer, the expression level of RhoA in tumors has been several times higher than that of surrounding normal tissue; RhoA was especially highly expressed in the metastatic region. To determine whether RhoA is activated by its overexpression, we made stable transfectants of MM1 cells expressing various levels of wild-type human RhoA. These transfectants showed promoted invasive ability in vitro in the absence and presence of 1-oleoyl-lysophosphatidic acid, marked adherence to the plastic culture dish with scattered shape, elevated phosphorylation of Mr 20,000 myosin light chain, and translocation of RhoA protein from the cytosol to the membrane. All of these phenotypes were similar to those of active RhoA transfectants, correlated with the expression level of RhoA and reversed by the treatment of the cells with Clostridium botulinum exoenzyme C3 ADP-ribosyltransferase. In addition, overexpression of wild-type RhoA in MM1 cells also conferred invasive ability in vivo after the cells were transplanted into the syngeneic rats. Thus, high expression of RhoA in the cell facilitates the translocation of this protein to the membrane, where it is activated, resulting in the stimulation of the RhoA-ROCK-actomyosin system, leading to invasion.

INTRODUCTION

Transcellular migration of tumor cells through host cell layers such as endothelium of blood vessels and methothelium of the visceral cavity is crucial in cancer invasion and metastasis (1–4). We previously developed a quantitative in vitro invasion assay (5). In this assay, cultured rat hepatoma cells are seeded on a MCL from the syngeneic rat, and the invasive ability is evaluated as number of tumor cells that penetrate through the monolayer and form colonies underneath. The in vitro invasive potency measured by this method is well correlated with the in vivo ability of tumor cells to establish tumor nodules and dissemination after implantation in the peritoneal cavity of the syngeneic rat (6). In this in vitro system, rat hepatoma MM1 cells (a clone isolated from parental AH130 cells) could transmigrate through the MCL in the presence of serum (7), and LPA was a completely effective substitute for serum (8). The effect of LPA on the induction of transmigration was abolished by the pretreatment of MM1 cells with C3 (from Clostridium botulinum), which is known to specifically inhibit the function of small GTP binding protein RhoA (9, 10). Recently, we demonstrated that RhoA-actomyosin pathway plays a pivotal role in this transmigration (11) using the stable transfectants of active RhoA (VRhoA). Furthermore, we also reported that the ROCK family of RhoA-associated serine-threonine protein kinases is involved this migration and that an inhibitor for these kinases effectively inhibits the invasion of MM1 cells in vitro and in vivo (12).

Rho protein is a well-known member of the p21 Ras superfamily of small GTPases, which shuttles between an inactive GDP-bound state and an active GTP-bound state and exhibits intrinsic GTPase activity. RhoA regulates signal transduction from cell surface receptors to intracellular target molecules and is involved in a variety of biological processes, including cell morphology (13), motility (14), cytokinesis (15, 16), smooth muscle contraction (17, 18), and tumor progression (19, 20), and it acts as a molecular switch in the cells (for reviews see Refs. 21–23). Thus far, even after extensive screening of clinical cases, there is no evidence that RhoA is activated by mutation in human malignancies (24). In addition, the expression level of RhoA in tumors has been reported to be several times higher than in surrounding normal tissue, and the expression level of RhoA was positively correlated with the stage in colon cancer (25). Especially, RhoA (25) and RhoC (26), another Rho family protein, were expressed at a relatively higher level in the metastatic region. These findings have led us to examine a role for the overexpression of RhoA in the invasion of tumor cells. The present study is undertaken to address how overexpression of RhoA regulates actomyosin-based contractility, leading to transmigration of tumor cells, and promotes invasive ability in vitro and in vivo.

MATERIALS AND METHODS

Reagents. G418 (geneticin) and culture medium were obtained from Life Technologies, Inc. (Rockville, MD). Pwo DNA polymerase was purchased from Boehringer Mannheim (Mannheim, Germany). LPA was obtained from Sigma Chemical Co. (St. Louis, MO) and was first dissolved in 20% ethanol at a concentration of 10 mM and diluted with serum-free medium. GDP, GTP, GTPyS, ATP, Tween 20, Triton X-114, Triton X-100, and fatty acid-free BSA were obtained from Sigma. [γ-32P]NAD (1000 Ci/mol) and [α-32P]NAD (800 Ci/mol) were purchased from Daiichi Pure Chemicals (Tokyo, Japan).

Cell Culture. Mesothelial cells were isolated from Donryu rat (obtained from Japan SLC, Inc., Shizuoka, Japan) mesentery and cultured in the MEM containing 2-fold amino acid and vitamins supplemented with 10% FCS, as reported previously (5). MM1 cells, isolated from parental AH130 cells, were maintained as suspension in the MEM containing 2-fold amino acid and vitamins supplemented with 10% FCS and split at a 1:20 ratio every 3 days.

Construction of Mutants RhoA Expression Vectors and Transfection. The expression plasmids were designed to generate from pEXV-wtRhoA vector (13), provided by Dr. A. Hall (University College, London, United
Kingdom). A 0.63-kb EcoRI-NotI fragment, containing full-length human wtRhoA cDNA tagged at the NH₂ terminus with a 8-amino acid FLAG sequence (DYKDDDDK; Ref. 27), was generated by PCR using the forward primer 5'-CTTGAATTCATGACTAAAGGACGAGTACAAAGGCTGCCATCCGAAGAAACTGGTG-3' (60-mer) and the reverse primer 5'-TTTGGCGGCCTCTCATCAGAAGAAGCGCAACCC-3' (32-mer) with pEXV-wtRhoA as the template. PCR was carried out as follows: denaturation for 1 min at 94°C, annealing for 1 min at 56°C, and extension for 1 min at 72°C with 30 thermal cycles, followed by a 7-min extension using Pwo DNA polymerase. The generated cDNA was introduced into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) at the site of EcoRI and NotI. The expression vector for active VRhoA was prepared as reported previously (11). All plasmids were sequenced with dideoxynucleotide terminus method (26) using [α-35S]ATP and Sequi-Gen sequencing kit (Bio-Rad Labora- tories, Richmond, CA) to verify the correct substitutions. All plasmids were purified using plasmid Maxi kit (Qiagen K. K., Tokyo, Japan). The transfection procedure was described previously (11).

Cell Monolayer Invasion Assay. The assay procedure of in vitro invasive activity of tumor cells was described previously (5, 11, 12). Briefly, after mesothelial cells from rat mesentery had reached confluence in 35-mm dish, the culture medium was removed, and 2 × 10⁴ tumor cells were seeded onto the MCL in the MEM containing 2-fold amino acid and vitamins with LPA or FCS. The number of the penetrated single tumor cells and tumor cell colonies (invasion foci) was counted under a phase-contrast microscope (Olympus, Tokyo, Japan) in 16 different visual fields (0.59 mm² each). The in vitro invasion activity was calculated as infiltrated cells per 35-mm dish. In some experiments, tumor cells were pretreated with 50 μg/ml C3 for 24 h, followed by washing twice with MEM containing 2-fold amino acid and vitamins.

In Vivo Invasion Assay. MM1 (hepatoma) cells (2 × 10⁶ cells) stably expressing empty vector only (mock), wtRhoA, or VRhoA, were implanted into the peritoneal cavity of the syngeneic male Donryu rats (100-g body weight.). After 11 days, all rats were sacrificed and checked with the amount of ascites, tumor nodules, and the dissemination into the peritoneum (12). For fluorescence microscopy (IX-FLA and IX70; Olympus) attached with a camera. Cells were grown to subconfluency (60–70%) in MEM containing 2-fold amino acid and vitamins with 10% FCS, grown to confluency. Confluent cells were then washed twice with the medium without FCS and rendered quiescent by incubating in the medium for 16 h. Medium was removed, and cells were directly dissociated in Laemmli’s sample buffer (1 μl per 10⁶ cells, Ref. 30). Samples were then heated at 100°C for 3 min and subjected to 12% SDS-PAGE, transblotted to a nitrocellulose membrane (0.2 μm; Bio-Rad), and blotted with anti-PMLC20 polyclonal Abs (specific for phosphorylated 19Ser; Ref. 31). The blot membrane was scanned with GT-9500 flat scanner (Epson, Japan) and analyzed with NIH image software using a Power Macintosh computer (Apple, Tokyo, Japan). The phosphorylation level of ML20 in MM1 cells was estimated as reported previously (11, 12) and expressed as a percentage of total.

Immunoblotting. Cells were washed with PBS twice and extracted 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, 0.1% SDS, 1% 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, loaded onto SDS-12% PAGE, transblotted to a Fine trap NT-31 membrane (Nihon Eido, Tokyo, Japan), and blotted with anti-RhoA polyclonal Abs (diluted 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Rac monoclonal Ab (diluted 1:1000; Upstate Biotechnology, Inc., Lake Placid, NY), anti-Cdc42 polyclonal Abs (diluted 1:1000; Santa Cruz Biotechnology), anti-RhoGDI monoclonal Ab (diluted 1:250; Transduction Laboratories), anti-FLAG M5 monoclonal Abs (9 μg/ml; Eastman Kodak Co.), or antiactin monoclonal Ab (20 μg/ml; Boehringer Mannheim; 11). The blot membrane was scanned as described above.

Table 1 Augmentation of the peritoneal invasion of MM1 hepatoma cells in vivo by the expression of wtRhoA or active RhoA (VRhoA).

<table>
<thead>
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<th>Parameter</th>
<th>Transfectants</th>
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<tr>
<td></td>
<td>Mock</td>
<td>wtRhoA</td>
<td>VRhoA</td>
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<tr>
<td>Ascitesa</td>
<td>14/14 (100%)</td>
<td>14/14</td>
<td>13/14</td>
<td>93%</td>
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<td>Mean ascites volume (ml)b</td>
<td>55.6 ± 6.5</td>
<td>56.4 ± 6.1</td>
<td>56.4±5.1</td>
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<tr>
<td>Tumor nodulesc</td>
<td>3/14 (21%)</td>
<td>6/11 (55%)</td>
<td>13/14</td>
<td>93%</td>
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<tr>
<td>Tumor cell disseminationd</td>
<td>0/14 (0%)</td>
<td>3/11 (27%)</td>
<td>12/14</td>
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*Incidence (no. of animals positive for indicated parameter/total no. of animals tested); values in parentheses represent percentage of total.

RESULTS

Expression of wtRhoA Induced in Vitro Invasiveness and Morphological Change in M11 Cells. To examine the role of the expression level of RhoA protein in tumor cell motility and morphology, we expressed wtRhoA protein in rat hepatoma MM1 cells. Full-length cDNA of wtRhoA was constructed with NH₂-terminal FLAG tag sequence and introduced into the mammalian expression vector pcDNA3. These plasmids were transfected into MM1 cells, and stable transfectants were isolated by the selection using geneticin. We obtained a number of clonal stable transfectants and analyzed them with immunoblotting using anti-FLAG monoclonal Ab or anti-RhoA polyclonal Abs, as shown in Fig. 1A. The expression levels of FLAG-RhoA protein were 24.5 ± 4.8% (clone 1), 68.3 ± 18.6% (clone 2), to 139.1 ± 25.4% (clone 3) of those of endogenous RhoA in these transfectants. In contrast, the expression levels of RhoGDI and actin...
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Fig. 1. Augmentation of the MLC20 phosphorylation and in vitro invasiveness in MM1 cells by the expression of wtRhoA and VRhoA. A. Immunoblot analysis. Cell lysates prepared from each transfectant were separated on 12% SDS-PAGE followed by immunoblotting with anti-RhoA, anti-FLAG, anti-RhoGDI, and anti-actin Abs. The expression levels of wtRhoA and VRhoA were determined by scanning the intensity of each band on the blots and normalizing to the intensity of endogenous RhoA. Black and white arrowheads (top), positions of expressed and endogenous RhoA, respectively. To determine the phosphorylation level of MLC20, we deprived cells of serum for 24 h, and prepared lysates were separated on 12% SDS-PAGE followed by immunoblotting with anti-MLC20 polyclonal Abs. The pattern of MLC20 that appeared as a doublet in this cell line is indicated by double arrowheads (bottom). B, relative expression level of Rho protein was estimated as the total Rho protein content (endogenous plus expressed) in the cell lysates. The expression level of Rho protein in the mock transfectants was represented as 10. ■, phosphorylation level of MLC20 in each transfectant. C, in vitro invasion assay. Cells (2 × 10^5) were seeded onto a MCL. After incubation for 20 h, the penetrated tumor cells and the cell colonies were examined by phase-contrast microscopy. Invasion ability was calculated the number of the infiltrated cells per 35-mm dish. □, in vitro invasiveness in the absence of FCS; ■, invasiveness in the presence of FCS in the assay medium. Columns, means of three independent experiments; bars, SD (when error bars are not shown, it indicates that the range falls within the size of the bar). * P < 0.01 to mock transfectants obtained by Student’s t test.

Fig. 2. Morphological changes in RhoA overexpressing MM1 cells. A. Phase-contrast micrographs of empty vector-alone transfectants (mock, left), wtRhoA transfectants (clone 3 in Fig. 1, middle), and 50 μg/ml C3-treated (for 24 h at 37°C) wtRhoA transfectants (right). Scale bar, 50 μm. B. Rhodamine phalloidin staining of mock (left), and wtRhoA transfectants (clone 3 in Fig. 1, right) to visualize F-actin. Scale bar, 50 μm.

protein did not change in these transfectants. To ascertain whether the wtRhoA overexpressing MM1 cells stimulated the actomyosin system or not, we examined the phosphorylation level of MLC20 with immunoblotting using anti-MLC20 polyclonal Abs (specific for phosphorylated Ser; Ref. 31). The phosphorylation level of MLC20 in wtRhoA transfectants was increased 2.24 ± 0.39-fold (clone 1), 2.70–0.53-fold (clone 2), and 3.41 ± 0.41-fold (clone 3) compared to that in mock transfectants (10.0 ± 2.03% total phosphorylation of MLC20; Fig. 1B). Fig. 1C demonstrates that these transfectants showed 6.2-fold (clone 1), 14.0-fold (clone 2), and 18.7-fold (clone 3) higher in vitro invasive ability through the MCL in the absence of FCS in the assay medium than did mock transfectants. Notably, there was a positive correlation between the expression level of wtRhoA, the phosphorylation level of MLC20, and in vitro invasiveness among these transfectant clones (Fig. 1, B and C). On the other hand, active RhoA (V-RhoA) transfectants (expressed VRhoA level was only 11.8 ± 4.6% of endogenous RhoA) markedly increased the phosphorylation level of MLC20 (4.19 ± 0.44-fold compared to mock transfectants) and in vitro invasiveness (73.2-fold) through the MCL in the absence of FCS in assay medium than did those of wtRhoA transfectants. These results indicated that the RhoA signaling pathway and actomyosin system were linked in the transcellular migration of tumor cells through the host cell layer.

We next examined the shape of wtRhoA expressing cells in the presence of serum under phase-contrast microscope. In contrast to the mock transfectants, which grew only in suspension and failed to attach to the dish, the wtRhoA transfectants adhered to the plastic culture dish with scattered shape, developed pointed edges, and began to spread (Fig. 2A, left and middle) with lamellipodial protrusions and fine stress fibers stained with rhodamine phalloidin (Fig. 2B, right). Treatment with 50 μg/ml exoenzyme C3 for 24 h rendered wtRhoA-expressing cells completely round, and the cell shape reversed to that of parental MM1 cells (Fig. 2A, right).

Next, we tested the effect of C3 on the phosphorylation level of MLC20 and in vitro invasiveness of wtRhoA and VRhoA transfectants. As shown in Fig. 3, the phosphorylation levels of MLC20 (Fig. 3A) in the cells expressing wtRhoA (clone 3 in Fig. 1: 32.5 ± 9.5%) and VRhoA (39.2 ± 8.5%) were greatly enhanced compared to that of
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mock transfectants (20.0 ± 4.54%) in the presence of 10% FCS, as was the invasiveness of these transfectants [11,500 ± 940 cells/dish (mock), 39,460 ± 1,660 cells/dish (wtRhoA), and 32,830 ± 1,320 cells/dish (VRhoA); Fig. 3B]. All of these changes were markedly reduced by the treatment of the cells with 50 μg/ml C3 for 24 h in the presence of 10% FCS. Both endogenous RhoA and expressed RhoA were effectively ADP-ribosylated 68–98% by the treatment with 50 μg/ml C3 for 24 h (Fig. 3A, middle). These results strongly suggested that overexpression of Rho regulates the actomyosin system, resulting in enhanced adhesion, leading to invasion of tumor cells in vitro.

**wtRhoA Transfectants Promoted the Invasiveness in Vivo.** To examine the in vivo invasive ability of these transfected cells, we implanted 2 × 107 cells in the peritoneal cavity of the syngeneic rat. The wtRhoA-transfected cells (clone 3 in Fig. 1) invaded more extensively into the peritoneum and formed numerous tumor nodules compared to mock transfectants. The incidence of macroscopic tumor nodule present in the peritoneum of rats implanted with wtRhoA transfectants (6 of 11 or 55%; see Table 1) was significantly higher than that of rats implanted with mock transfectants (3 of 14, or 21%; P < 0.01) and significantly lower than that of rats implanted with VRhoA transfectants (13 of 14, or 93%; P < 0.01). The incidence of tumor cell dissemination in the peritoneal cavity of rats implanted with wtRhoA transfectants (3 of 11, or 27%) was also significantly higher than that of rats implanted with mock transfectants (0 of 14, or 0%; P < 0.01) and significantly lower than that of rats implanted with VRhoA transfectants (12 of 14, or 86%; P < 0.01). In terms of the appearance of ascites, there was no significant difference among these three groups, and the average amount of ascites was even higher in the rats receiving mock transfectants (55.5 ± 6.5 ml) than those receiving VRhoA transfectants (36.4 ± 5.1 ml; P < 0.01). These results suggest that the overexpression of wtRhoA activated the function of RhoA and promoted the tumor invasive ability in vivo as well as in vitro in the modest degree between mock and active RhoA. We could not find any macroscopic metastatic lesion (lung, liver, spleen, and stomach) in these rats.

**wtRhoA Enhanced and the Translocation of RhoA from the Cytosol to the Membrane.** Next, we checked whether the overexpression of RhoA enhances the translocation of RhoA protein from the cytosol to the membrane in the cell because evidence was accumulated that RhoA required to be targeted to the membrane for its activation (32, 33). We first examined whether GTP would affect the intracellular localization of Rho family small GTPase, Cdc42, Rac1, and RhoA in MM1 cells. Incubation of cell lysates from MM1 cells with 300 μM GTPγS stimulated the translocation of RhoA, Rac1, and Cdc42 from the cytosol to the particulate (membrane) fraction. In contrast, RhoGDI was always found in the cytosolic fraction. None of these proteins were detected in the detergent-insoluble fraction. Treatment with 1 mM GTP also stimulated the translocation of all three GTPases, with less degree. In contrast, the distribution of three GTPases was not affected by 1 mM GDP or 1 mM ATP (data not shown).

We next examined the effect of LPA in the translocation of RhoA from the cytosol to the membrane fraction in MM1 cells. In unstimulated MM1 cells, most (>95%) of the RhoA protein was found in the cytosolic fraction. Stimulation of MM1 cells with 0.1–25 μM LPA for 30 min at 37°C resulted in a significant increase in the amount of RhoA translocated from the cytosol to the membrane fraction in a dose-dependent fashion (4.7 ± 1.2% without LPA stimulation, 8.2 ± 2.1% (with 0.1 μM LPA), 12.1 ± 0.9% (0.25 μM; P < 0.01 compared to without LPA), 20.2 ± 0.9% (1 μM), 29.4 ± 3.3% (2.5 μM), 34.5 ± 4.6% (5 μM), 35.7 ± 4.8 (10 μM) and 35.8 ± 5.1 (25 μM)). Because the phosphorylation of mitogen-activated protein kinase (11) and shape changes, such as budding on the surface and filopodia formation, in MM1 cells induced by LPA stimulation was observed in a similar dose-dependent manner (data not shown), we used 5 μM LPA stimulation (a concentration designed to evoke the submaximal biological responses) for the following experiments. As shown in Fig. 4A, RhoA was translocated from the cytosol to the membrane in a time-dependent manner. Translocation of RhoA became evident at 10 min after stimulation of MM1 with 5 μM LPA, was maximal for 30 min and was sustained for 60 min with the amount of RhoA at 33.6 ± 4.9% from the cytosol to the membrane fraction. LPA also evoked the translocation of Cdc42 to a similar degree (33.8 ± 7.7% at 60 min) but not of Rac1 or RhoGDI, from the cytosol to the membrane in a time-dependent fashion (Fig. 4A). Pretreatment of MM1 cells with 50 μg/ml C3 for 24 h completely blocked both LPA-stimulated translocation of RhoA to the membrane and the increase in phosphorylation level of MLC20 but not the increase in...
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Fig. 4. Time course of translocation of Cdc42, Rac1, RhoA, and RhoGDI induced by LPA (A) or PDGF (B). MM1 cells were stimulated with 5 μM LPA (A) or 50 ng/ml PDGF (B) for the times indicated, extracted, and then fractionated as described in “Materials and Methods.” Cdc42, Rac1, RhoA, and RhoGDI were detected by immunoblotting. Data points, means of three or four independent experiments; bars, SD (when error bars are not shown, it indicates that the range falls within the size of the data symbol).

mitogen-activated protein kinase phosphorylation in MM1 cells. On the contrary, PDGF evoked the translocation of Rac1 but not RhoA and Cdc42 from the cytosol to the membrane (Fig. 4B) and did not show any evidence for MLCK phosphorylation (actomyosin-based contractility) resulting in the in vitro invasion (LPA: 14,020 ± 980 cells/dish versus PDGF: 710 ± 120 cells/dish).

Finally, we examined whether the overexpression of RhoA enhances the translocation of RhoA in the wtRhoA and the VRhoA expressing cells. As shown in Fig. 5, A and C (open columns), most (99.1%) of RhoA protein was found in the cytosolic fraction in the serum-starved mock transfectants. In contrast, in the serum-starved wtRhoA transfectants, 18.0 ± 5.2% of expressed FLAG-wtRhoA and 26.2 ± 4.4% of endogenous RhoA were present in the membrane fraction without any stimulation. In the serum-starved VRhoA transfectants, 78.1 ± 8.9% of the expressed FLAG-VRhoA and 50.7 ± 5.4% of endogenous RhoA were found in the membrane fraction without any stimulation. These results suggested that the expression of wtRhoA or active RhoA (VRhoA) facilitated the translocation of RhoA from the cytosol to the membrane and then activated both endogenous and expressed RhoA, thereby promoting their invasive ability, as shown in Fig. 1C. Fig. 5, B and C (filled columns), represent the situation after the cells were stimulated with 5 μM LPA for 60 min at 37°C. A portion of the total RhoA in the cell (11.3 ± 2.5%) was translocated from the cytosol to the membrane fraction in the mock transfectants. In the wtRhoA transfectants, 32.5 ± 6.0% of the expressed and 38.1 ± 6.5% of the endogenous RhoA were translocated from the cytosol to the membrane fraction after LPA stimulation. In the VRhoA transfectants, the magnitude of LPA-stimulated translocation of RhoA was much greater than unstimulated cells (95.4 ± 7.0% of the expressed VRhoA and 76.2 ± 10.8% of endogenous RhoA exist in the membrane fraction). These results indicated that the overexpression of RhoA itself stimulates the translocation of endogenous and expressed RhoA from the cytosol to the membrane, and LPA markedly enhanced this membranous localization of RhoA. Then, membrane-bound RhoA stimulated the Rho-ROCK-actomyosin system followed by tumor cell invasion.

DISCUSSION

In this study, we have prepared several MM1 hepatoma cell clones that stably express FLAG-wtRhoA. Although, several lines of evidence (19, 34, 35) suggested the involvement of Rho in cell proliferation, the wtRhoA-overexpressing MM1 cells demonstrated little changes in growth [estimated doubling times from the growth curve were 13.0 h for MM1 cells and 13.0 h for wtRhoA transfectants (clone 3 in Fig. 1)]. In contrast, the wtRhoA transfectants showed dramatic shape changes such as adherence to the culture dish and developed pointed edges with filopodial protrusion (Fig. 2). They also enhanced ability for migration through the MCL even in the absence of FCS or LPA (Fig. 1C) and increased ability of invasiveness in vivo (Table 1). It is interesting to note that control cells (mock transfectants) developed solid tumors after implantation into the peritoneal cavity, whereas the wtRhoA transfectants formed numerous small tumor nodules disseminated in the peritoneum. All of these characteristics were similar to those of active RhoA transfectants reported previously (11), but to a lesser degree. These results strongly suggest that expressed wtRhoA itself promoted the Rho-actomyosin-based motility in the cell.

To confirm the activation of RhoA in the cell, we next addressed the translocation of RhoA protein from the cytosol to the membrane in the wtRhoA and active RhoA (VRhoA) transfectants. First, we checked the effect of GTPγS, an analogue of GTP that is resistant to hydrolysis in the cell, on the distribution of Rho family small GTPase such as Rho, Rac, and Cdc42Hs. All three of these small G proteins were effectively translocated from the cytosol to the membrane fraction when the cell lysates were incubated with GTPγS. GTP did show the similar effect but a lesser degree, and GDP and ATP did not show such an effect. Similar results were already reported using other cells.

4 Unpublished observation.
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such as smooth muscle (32) or Swiss 3T3 fibroblasts (33). In contrast, using Swiss 3T3 fibroblasts, Fleming et al. (33) reported that bioactive phospholipid LPA induces a sustained, time-dependent translocation of RhoA and Cdc42 from the cytosol to the membrane fraction. They also reported that LPA had no effect on the distribution of Rac1 and RhoGDI. As in MM1 cells, LPA also effectively promoted the translocation of RhoA and Cdc42 but not Rac1 nor RhoGDI from the cytosol to the membrane fraction (Fig. 4A). On the other hand, PDGF evoked the translocation of Rac1 but not RhoA and Cdc42 from the cytosol to the membrane (Fig. 4B) and did not show any evidence for actomyosin-based contractility resulting in the cellular migration (see “Results”). Together, these results show that the LPA-Rho-actomyosin cascade was critical in the invasion of MM1 cells and that the PDGF-Rac pathway is less prominent in this invasion. This is different from the critical role of Tiam1-Rac cascade in the invasion of T-lymphoma cells reported by Collard and colleagues (36–38). These controversial results of the function of Rho and Rac in cellular invasion might be attributable to the different activators (guanine exchange proteins; GEP) for Rho-family GTPases (see below) in the different cell types. The role of activation of Cdc42 in the invasion should be addressed in the future.

Next, we further examined the translocation of RhoA from the cytosol to the membrane in the wtRhoA or active RhoA transfectants. In these transfectants, both endogenous and expressed RhoA proteins already existed in the membrane fraction, even in the absence of LPA stimulation (Fig. 5), and the translocation from the cytosol to the membrane was markedly enhanced after LPA stimulation. In addition, endogenous and wtRhoA were translocated in the same manner, whereas expressed active (GTPase activity-deficient) VRhoA was effectively targeted to the membrane, because this mutant protein mimics the GTP-binding form of RhoA. The translocation of RhoA was completely inhibited by the pretreatment of cells with C3 (data not shown). Taken together, these data show that overexpressed RhoA stimulates the translocation of RhoA from the cytosol to the membrane, activating RhoA protein on the membrane, followed by the stimulation of ML20 phosphorylation, leading to cell migration. On the other hand, other Rho-family GTPases, Rac1 and CDC42 did not show any translocation in these wtRhoA and active RhoA expressing cells, suggesting the less prominent role of these two GTPases in the invasion of MM1 cells. We previously hypothesized the positive feedback loop in the activation of RhoA in MM1 cells (11), because the expression of active RhoA in W1 cells, lower invasive counterpart of MM1 cells, rendered this cell phenotype from LPA resistant to LPA sensitive and demonstrated activation of endogenous RhoA by LPA stimulation. It might be implied that the expression level of RhoA in the cell is a critical regulator in determining the sensitivity of LPA receptor-RhoA cascade for the activation of RhoA. In other words, we might be able to postulate the threshold-level in the expression of RhoA in each cell, for the activation cascade of RhoA in the cell.

Recently, two functional transmembranous heterotrimeric G-protein coupled receptors, vzg-1/Edg2 (39) and Edg4 (40), were reported as functional receptors for LPA. In addition, G-protein G13, but not G12, was reported to be used for the signal propagation from the LPA receptor to Rho activation and to induce the rapid remodeling of the actin cytoskeleton (41). Furthermore, some of the Rho GDP/GTP exchange proteins (RhoGEPs) were reported as oncogenes, such as Dbl (42), Ost (43), and Vav (44), and most recently, Takahashi et al. (45) reported that Dbl protein competes with RhoGDI when binding to the NH2-terminal region of radixin on the plasma membrane, and it also activates RhoA protein on the membrane. Because Dbl protein has been reported as GEP for Rho and Cdc42 but not Rac1 (42), our present results that showed LPA-induced translocation of RhoA and Cdc42 but not Rac1 (42), our present results that showed LPA-induced translocation of RhoA and Cdc42 but not Rac1 to the plasma membrane in MM1 cells (see Fig. 4) might suggest the LPA-Dbl-Rho-family GTPase cascade on the membrane during the cellular invasion. Although the upstream of Rho signaling cascade including the activation of Rho in the cell rapidly progressed, the precise function of this dbl family proteins in the human malignancy should be focused on in more detail in the future.

In the clinical situation, human malignant tumor consists of heterogeneous cell population. Another family of small GTPase Ras was frequently mutated in these populations and resulted in the active form such as Val12 or Lys61 to enhance the cell proliferation and tumor progression (46). In contrast, thus far, there has been no report of the mutation of RhoA in the human malignancy, even in the extensive search (24). Furthermore, the expression levels of RhoA and RhoC were positively correlated with the stages of colon cancer (25) and pancreas adenocarcinoma (26), respectively. Likewise, some cells acquired overexpressing Rho protein and gained the ability of enhanced motility and adhesion to the extracellular matrix in the primary site; these cells might facilitate the metastasis of cancer. The next question that should be addressed is: What are the most important factors regulating the expression level of Rho in the tumor cells, and how can they be manipulated?
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