Suppression of the Invasive Capacity of Rat Ascites Hepatoma Cells by Knockdown of Slingshot or LIM Kinase*§

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Actin cytoskeletal reorganization is essential for tumor cell migration, adhesion, and invasion. Cofilin and actin-depolymerizing factor (ADF) act as key regulators of actin cytoskeletal dynamics by stimulating depolymerization and severing of actin filaments. Cofilin/ADF are inactivated by phosphorylation of Ser-3 by LIM kinase-1 (LIMK1) and reactivated by dephosphorylation by Slingshot-1 (SSH1) and -2 (SSH2) protein phosphatases. In this study, we examined the roles of cofilin/ADF, LIMK1, and SSH1/SSH2 in tumor cell invasion, using an in vitro transcellular migration assay. In this assay, rat ascites hepatoma (MM1) cells were overlaid on a primary-cultured rat mesothelial cell monolayer and the number of cells that transmigrated under the monolayer in the presence of lyso phosphatidic acid (LPA) was counted. The knockdown of cofilin/ADF, LIMK1, or SSH1/SSH2 expression by small interfering RNAs (siRNAs) significantly decreased the LPA-induced transcellular migration of MM1 cells and their motility in two-dimensional culture. Knockdown of LIMK1 also suppressed fibronectin-mediated cell attachment and focal adhesion formation. Our results suggest that both LIMK1-mediated phosphorylation and SSH1/SSH2-mediated dephosphorylation of cofilin/ADF are critical for the migration and invasion of tumor cells and that LIMK1 is involved in the transcellular migration of tumor cells by enhancing both adhesion and motility of the cells.

The most prominent features of malignant tumor cells are their invasive and metastatic capacities. Tumor metastasis proceeds by several sequential steps, including detachment from a primary tumor, invasion into surrounding tissues, intravasation, extravasation, and proliferation in distant organs (1). The transcellular migration of tumor cells through the host cell layers, such as the vascular endothelium and the mesothelium of the visceral cavity, is one of the critical steps for tumor cell invasion and metastasis, because these cell layers can act as a barrier against tumor cell invasion, intravasation, and extravasation. However, the mechanism underlying transcellular migration is not well understood. Because the regulation of actin cytoskeletal reorganization is essential for cell adhesion, motility, and morphological change, actin-modulating proteins and their upstream signaling molecules appear to play crucial roles in a variety of processes during tumor cell invasion and metastasis, including transcellular migration.

Cofilin and actin-depolymerizing factor (ADF)2 are closely related actin-binding proteins that play a key role in controlling actin filament dynamics and reorganization by severing and depolymerizing actin filaments (2, 3). The activities of cofilin/ADF are inhibited by LIM kinase (LIMK)-mediated phosphorylation at Ser-3 (4, 5). The inactive Ser-3-phosphorylated cofilin (P-cofilin) and Ser-3-phosphorylated ADF (P-ADF) are reactivated by dephosphorylation, catalyzed by Slingshot (SSH) family protein phosphatases, composed of SSH1, SSH2, and SSH3 (6, 7). LIMKs are activated by the Rho family small GTPases, Rho, Rac, and Cdc42, via their downstream protein kinases, such as ROCK and PAK (8–10). SSH1 is activated by its association with actin filaments and is negatively regulated by 14-3-3 proteins that protect SSH1 from F-actin-mediated activation (11). Several lines of evidence suggest that cofilin/ADF and their upstream regulators, LIMKs and SSHs, play crucial roles in cell migration (12–17). Ectopic expression of LIMK1 increases the motility and invasiveness of breast and prostate cancer cells, and suppression of LIMK1 activity causes the decreased motility and invasiveness of these cells (18, 19). Similarly, knockdown of LIMK1 suppresses the motility and chemotaxis of Jurkat T lymphoma cells (15). These results suggest that LIMK1 promotes tumor cell migration and invasion. However, other groups have suggested that the overexpression of LIMK1 suppresses the motility and invasiveness of Ras-transformed fibroblasts and mammary tumor cells (20–22). Thus, it has remained unclear whether LIMK1 plays a positive or negative role in tumor cell migration and invasion. The functional role of SSHs in tumor cell invasion has not yet been investigated.

To examine the mechanisms of tumor cell invasion, an in vitro transcellular migration assay system has been developed

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§ The abbreviations used are: ADF, actin-depolymerizing factor; BSA, bovine serum albumin; CFP, cyan fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; hLIMK1, human LIM kinase-1; hSSH1, human slingshot-1; LIMK, LIM kinase; LPA, lyso phosphatidic acid; mSSH2, mouse slingshot-2; P-ADF, Ser-3-phosphorylated ADF; P-cofilin, Ser-3-phosphorylated cofilin; siRNA, small interfering RNA; SSH, slingshot; WT, wild type.

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In this assay, rat ascites hepatoma (AH130) cells are overlaid on a primary-cultured rat mesothelial cell monolayer and the number of AH130 cell colonies that penetrate the monolayer is counted. The invasive activity of AH130 cells determined by this in vitro system correlates well with the in vivo invasive capacity of the cells, as measured by implantation of the cells into the rat peritoneal cavity (24). Using this in vitro assay, previous studies have demonstrated that the transcellular migration of MM1 cells (a highly invasive clone derived from AH130 cells) through the mesothelial cell layer requires the presence of lysophosphatidic acid (LPA) and the activation of a Rho-ROCK signaling pathway (25–29).

In this study, we have assessed the roles of cofilin/ADF, SSH1/SSH2, and LIMK1 in tumor cell invasion, using the in vitro transcellular migration assay. We show that suppression of cofilin/ADF, SSH1/SSH2, or LIMK1 expression by small interfering RNAs (siRNAs) significantly reduces the transcellular migration of MM1 cells through the mesothelial cell monolayer as well as their motility in two-dimensional culture. We also show that knockdown of LIMK1, but not cofilin/ADF or SSH1/SSH2, inhibits the fibronectin-mediated cell adhesion of MM1 cells. These findings suggest that the proper regulation of cofilin/ADF activities by both LIMK1 and SSH1/SSH2 are critical for tumor cell invasion, and that LIMK1 is involved in the transcellular migration of MM1 cells by regulating both the adhesion and migration of tumor cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Rabbit anti-LIMK1 antibody (C10) was raised against the C-terminal peptide of LIMK1, as described (30). Anti-cofilin antibody was raised against recombinant cofilin-(His)$_6$ (31). Rabbit polyclonal antibodies were raised against the C-terminal peptides of mouse ADF, rat SSH1L, and mouse SSH2L. Anti-β-actin and anti-vinculin monoclonal antibodies were purchased from Sigma Aldrich.

**Plasmid Construction**—The plasmids for retroviral expression were constructed by subcloning the cDNAs encoding cofilin/ADF (CFP), or CFP-tagged human SSH1L, mouse SSH2L (nSSH2-CFP), human wild-type (WT) LIMK1 (hLIMK1(WT)-CFP), or human kinase-dead LIMK1 (hLIMK1(D460A)-CFP) (30), or Myc-tagged chick cofilin (ch-cofilin) or ADF (ch-ADF), into the pLNCX-neo retroviral vector (Clontech, Palo Alto, CA). The siRNA-targeting constructs were generated using the pSUPER.retro.purovector (OligoEngine, Seattle, WA), as described previously (32). The 19-base targeting sequences used in this study were as follows: 5’-GAAGAGAGGGAAGAAATGAG-3’ (rat SSH1), 5’-TCTGCTGAAACCTTGGGACGAGAC-3’ (rat SSH2), 5’-CTTGGGAACTATTGGGCTAGAA-3’ (rat LIMK1), 5’-CCGACCAAGGTGACTTGGGCTCTAGAA-3’ (rat cofilin), and 5’-CCAGCTGGATTCTCCGTTGATGAGGAA-3’ (rat ADF). As a control, we used a non-targeting sequence, 5’-TCTTCCCTCCCAAGAGAAATA-3’, which does not exist in the rat genome.

**Cells and Cell Culture**—MM1 cells, a highly invasive clone of rat ascites hepatoma AH130 cells (23), were cultured in suspension in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Rat mesothelial cells were isolated from the mesentery of Donryu rats and cultured in DMEM supplemented with 10% fetal calf serum, as described (23). The mesothelial cells and MM1 cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ in air. A monolayer of confluent mesothelial cells in a 35-mm dish was used for the transcellular migration assay. Plat-E cells were provided by Dr. T. Kitamura (University of Tokyo) and maintained in DMEM supplemented with 10% fetal calf serum, 10 μg/ml blasticidin (ICN Biochemicals, Irvine, CA), and 1 μg/ml puromycin (Sigma Aldrich) (33).

**Transfection and Retrovirus Infection**—To generate retroviral supernatants, Plat-E retrovirus packaging cells (33) were transfected with pLNCX-neo or pSUPER.retro.puro plasmids using FuGENE6 (Roche Applied Science, Mannheim, Germany). At 48 h after transfection, the culture medium was centrifuged, and the viral supernatant was used for infection after the addition of 8 μg/ml polybrene. Infected MM1 cells were cultured for 24 h, washed, and selected by culturing for 48 h with 2 μg/ml puromycin for cells infected with pSUPER.retro.puro retrovirus or for 2 weeks with 250 μg/ml G418 (Nacalai Tesque, Kyoto, Japan) for cells infected with pLNCX retrovirus.

**Transcellular Migration Assay (In Vitro Invasion Assay)**—The transcellular migration assay was performed, as described previously (23). Briefly, MM1 cells (2 × 10$^5$ cells) were seeded over a rat mesothelial cell monolayer and cultured in DMEM with or without 25 μM LPA (Sigma Aldrich). After incubation for 20 h, the supernatant was removed, and the resultant mesothelial cell monolayer and infiltrated MM1 cells were fixed with 4% formaldehyde. The number of penetrated single tumor cells and tumor cell colonies (collectively called infiltrated cell foci) was counted with a phase-contrast microscope in 10 different visual fields (0.95-mm$^2$ each). The in vitro transcellular migration activity was quantitatively determined as the number of infiltrated cell foci/dish.

**Immunoprecipitation and Immunoblot Analyses**—Cell lysates were prepared and subjected to SDS-PAGE and immunoblotting, as described previously (12). To analyze endogenous LIMK1, the cell lysates were immunoprecipitated with anti-LIMK1 antibody before immunoblot analysis (30).

**Cell Adhesion Assay**—MM1 cells (2 × 10$^5$ cells) cultured in suspension were washed twice with DMEM, suspended in 500 μl of DMEM containing 2.4% bovine serum albumin (BSA), and seeded onto 24-well uncoated dishes or dishes precoated with 10 μg/ml fibronectin (Sigma Aldrich). After incubation at 37 °C for 30 min in the presence of 25 μM LPA, the medium was carefully removed and the wells were washed three times with phosphate-buffered saline. The cells attached to the dishes were fixed with 4% formaldehyde and stained with 0.2% crystal violet in 1% ethanol. After the wells were rinsed three times with water, the dye was extracted with 0.1 M sodium citrate in 50% ethanol, and the absorbance at 550 nm was measured by a microplate spectrophotometer (Bio-Rad).

**Focal Adhesion Formation Assay**—MM1 cells were plated on coverslips precoated with 10 μg/ml fibronectin, cultured for 30 min, and then stimulated with 25 μM LPA for 1 h. Cells were fixed with 4% formaldehyde for 10 min, permeabilized in 0.2% Triton X-100 in PBS for 5 min, blocked with 5 mg/ml BSA for 1 h, and stained with anti-vinculin monoclonal antibody. Fluorescein isothiocyanate-conjugated anti-mouse IgG was used as a secondary antibody and visualized with a diaminobenzidine tetrahydrochloride substrate.
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Knockdown of Cofilin/ADF Suppresses Tumor Cell Invasion—To examine the role of cofilin and ADF in tumor cell invasion, we suppressed the expression of endogenous cofilin and ADF in MM1 rat ascites hepatoma cells with retrovirus-assisted siRNAs and analyzed the effects of their knockdown on the in vitro transcellular migration activity of MM1 cells. MM1 cells were infected with retrovirus coding for the siRNA sequences targeting rat cofilin or ADF, and the infected cells were selected by puromycin. Infection with cofilin and ADF siRNA retrovirus substantially reduced the expression of endogenous cofilin and ADF, respectively, in MM1 cells (Fig. 1A). The invasive capacity of MM1 cells was analyzed by an in vitro transcellular migration assay, in which MM1 cells were loaded on the cultured rat mesothelial cell monolayer and incubated for 20 h in the presence or absence of LPA, and the number of MM1 cell foci infiltrated into the monolayer was counted. Fig. 1B shows the phase-contrast image of control MM1 cells penetrated into the monolayer in the presence of LPA. Fig. 1C summarizes the quantitative data of the transcellular migration assays. As described previously (25), LPA was required for the transcellular migration of MM1 cells (Fig. 1C). Whereas knockdown of cofilin or ADF individually had no apparent effect on the invasive activity of MM1 cells, knockdown of cofilin and ADF together significantly reduced the number of infiltrated cells, as compared with the cells infected with the control siRNA (Fig. 1C). These results indicate that cofilin/ADF proteins are critical for the invasive activity of MM1 cells. Expression of chick cofilin or ADF, whose expression was not affected by siRNAs targeting rat cofilin or ADF, partially blocked the inhibitory effect of cofilin/ADF double knockdown on the invasive activity of MM1 cells (Fig. 1D), indicating that the effects of cofilin/ADF siRNAs are due to the suppression of cofilin/ADF expression.

Knockdown of SSH1/SSH2 Suppresses Tumor Cell Invasion—To investigate the role of SSH1/SSH2-mediated cofilin/ADF dephosphorylation in tumor cell invasion, we analyzed the effects of SSH1/SSH2 knockdown on the transcellular migration of MM1 cells. Infection with retrovirus coding for SSH1 and SSH2 siRNAs reduced the expression of endogenous SSH1 and SSH2, respectively (Fig. 2A). The P-cofilin/P-ADF levels increased in cells expressing SSH1/SSH2 siRNAs, compared with cells expressing control siRNA (supplemental Fig. S1), which suggests that SSH1/SSH2 are involved in cofilin/ADF
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A

FIGURE 2. Knockdown of SSH1/SSH2 suppresses tumor cell invasion. A, suppression of SSH1/SSH2 expression by siRNA. MM1 cells infected with retrovirus coding for the indicated siRNAs were selected by puromycin. Expression of endogenous SSH1, SSH2, and β-actin was analyzed by immunoblotting with antibodies specific for each protein. Relative levels of SSH1/SSH2 expression are shown as the means ± S.E. of triplicate experiments. B, quantitative analysis of the effects of SSH1/SSH2 knockdown on the transmigration activity of MM1 cells. MM1 cells were infected with retrovirus coding for the indicated siRNAs. In the right panel, MM1 cells stably expressing CFP, CFP-hSSH1, or mSSH2-CFP were infected with retrovirus coding for rat SSH1/SSH2 siRNAs. Cells were selected with puromycin and subjected to the transcellular migration assay. The number of MM1 cell foci infiltrated into the monolayer was counted. Data represent the means ± S.E. of three independent experiments. *, p < 0.005; **, p < 0.001.

B

C

FIGURE 3. LIMK1 is critical for the invasive activity of MM1 cells. A, suppression of LIMK1 expression by siRNA. MM1 cells infected with retrovirus coding for control or LIMK1 siRNA were selected by puromycin. Cell lysates were immunoprecipitated with anti-LIMK1 antibody and subjected to immunoblot analysis. Relative levels of LIMK1 expression are shown as the means ± S.E. of triplicate experiments. B, quantitative analysis of the effect of LIMK1 siRNA on the transmigration activity of MM1 cells. MM1 cells were infected with retrovirus coding for the control or LIMK1 siRNAs. Cells were selected with puromycin and subjected to the transcellular migration assay. The number of MM1 cell foci infiltrated into the monolayer was counted. Data represent the means ± S.E. of three independent experiments. **, p < 0.001. C, effects of S3 peptide on the invasive activity of MM1 cells. MM1 cells were subjected to the transcellular migration assay in the presence or absence of 10 μg/ml S3 or RV peptides. The number of MM1 cell foci infiltrated into the monolayer was counted. Data represent the means ± S.E. of three independent experiments. *, p < 0.05.

dephosphorylation in MM1 cells. Whereas knockdown of SSH1 had no apparent effect on the transcellular migration of MM1 cells, knockdown of SSH2 slightly suppressed transmigration and double knockdown of SSH1 and SSH2 more evi-}

stantly suppressed the transmigration (Fig. 2B). In MM1 cell lines stably expressing CFP-hSSH1 or mSSH2-CFP, whose expression was not affected by rat SSH1/SSH2 siRNA, double knockdown of rat SSH1/SSH2 did not affect the invasive activity of MM1 cells (Fig. 2B). Thus, the siRNA-resistant hSSH1 and mSSH2 have the potential to restore the invasive activity of SSH1/SSH2 siRNA cells, indicating that the effects of SSH1/SSH2 siRNAs are due to the suppression of endogenous SSH1/SSH2 expression. These findings suggest that SSH1 and SSH2 are required for the invasive activity of MM1 cells and that they can function redundantly in the invasion of MM1 cells.

LIMK1 Is Critical for Tumor Cell Invasion—To examine the role of LIMK1 in the invasive activity of MM1 cells, we analyzed the effect of LIMK1 knockdown on the transcellular migration of MM1 cells. Infection with retrovirus coding for LIMK1 siRNA suppressed the expression of endogenous LIMK1 in MM1 cells (Fig. 3A). The transcellular migration assay revealed that knockdown of LIMK1 markedly suppressed the invasive activity of MM1 cells (Fig. 3B). The LIMK1 siRNA-induced inhibition of the invasive activity was significantly attenuated in MM1 cells stably expressing hLIMK1(WT)-CFP, which was unaffected by rat LIMK1 siRNA (Fig. 3B). In contrast, coexpression of a kinase-dead hLIMK1(D460A)-CFP with LIMK1 siRNA further suppressed the invasive activity of MM1 cells (Fig. 3B). Supplemental Fig. S2 shows the expression and kinase activity of hLIMK1(WT)-CFP and hLIMK1(D460A)-CFP. These results indicate that LIMK1 plays a critical role in the invasive activity of MM1 cells and LIMK1(D460A) functions as a dominant-negative form.

To further examine whether the kinase activity of LIMK1 is required for tumor cell invasion, we analyzed the effect of a cell-permeable peptide inhibitor of LIMK1 (S3} peptide) on the invasive activity of MM1 cells. The S3 peptide is composed of the N-terminal sequence of cofilin, which contains the phosphorylation site (Ser-3), and the cell-permeable
sequence motif of penetratin (12). As a control, the reverse (RV) peptide containing the reverse sequence of cofilin and the penetratin sequence was also tested. Treatment with S3 peptide, but not RV peptide, reduced the P-cofilin/P-ADF levels in MM1 cells (supplemental Fig. S3), indicating that S3 peptide inhibits the kinase activity of LIMK1 in MM1 cells. In the transcellular migration assay, treatment of MM1 cells with S3 peptide, but not RV peptide, significantly decreased the number of the infiltrated cells, as compared with the untreated cells (Fig. 3C). These results further indicate that the kinase activity of LIMK1 is required for the invasive activity of MM1 cells.

**LIMK1 Is Critical for Fibronectin-mediated Cell Adhesion and Focal Adhesion Formation**—A previous study showed that fibronectin-mediated cell adhesion is required for the transcellular migration of MM1 cells through the mesothelial cell layer (34). To elucidate the mechanisms by which cofilin/ADF, SSH1/SSH2, and LIMK1 facilitate the transcellular migration of MM1 cells, we analyzed the effects of knockdowns of these proteins on the fibronectin-mediated cell adhesive activity of MM1 cells. MM1 cells infected with siRNAs targeting cofilin/ADF, SSH1/SSH2, or LIMK1 were seeded on uncoated or fibronectin-coated dishes, and incubated for 30 min. After washing, the number of cells attached to the dishes was estimated by crystal-violet staining. Fibronectin was required for the attachment of MM1 cells to the dishes (Fig. 4A). Whereas knockdowns of cofilin/ADF or SSH1/SSH2 had no apparent effect on the fibronectin-mediated adhesion of MM1 cells, knockdown of LIMK1 significantly decreased the number of cells that adhered to the fibronectin-coated dish (Fig. 4A). These data indicate that LIMK1, but not cofilin/ADF or SSH1/SSH2, is critically involved in fibronectin-mediated cell adhesion.

Stimulation of MM1 cells with LPA induces the formation of focal adhesions (34). To investigate the role of LIMK1 in focal adhesion formation, MM1 cells infected with control or LIMK1 siRNAs were cultured on fibronectin-coated dishes, stimulated with LPA for 1 h, and stained with anti-vinculin antibody. In control siRNA cells, both the number and size of vinculin-positive focal adhesions increased in response to LPA stimulation (Fig. 4B, upper panels). In contrast, in LIMK1 siRNA cells, no increase in vinculin staining was observed after LPA stimulation (Fig. 4B, lower panels). Quantitative analysis of the number of cells possessing vinculin-positive foci revealed that LIMK1 knockdown significantly suppressed LPA-induced focal adhesion formation in MM1 cells (Fig. 4C). These observations suggest that LIMK1 plays a crucial role in LPA-induced focal adhesion formation in MM1 cells.

**Cofilin/ADF, SSH1/SSH2, and LIMK1 Are Critical for Cell Motility and Morphological Change**—We next examined the effects of cofilin/ADF, SSH1/SSH2, and LIMK1 knockdowns on the motility of MM1 cells using Transwell culture chambers. MM1 cells infected with retrovirus coding for each siRNA were seeded on the fibronectin-coated upper chamber and the number of cells that migrated into the lower chamber was counted after a 3-h incubation in the presence or absence of LPA in both chambers. In control siRNA cells, LPA stimulation increased the number of cells migrating into the lower chamber by about
35-fold (Fig. 5A). Knockdowns of SSH1/SSH2, LIMK1, and cofilin/ADF by siRNAs reduced the level of LPA-induced cell migration to 79, 51, and 51%, respectively, of that of the LPA-stimulated control siRNA cells (Fig. 5A), suggesting that SSH1/SSH2, cofilin/ADF, and LIMK1 are all critical for LPA-induced migration of MM1 cells.
To further elucidate the mechanisms by which cofilin/ADF, SSH1/SSH2, and LIMK1 knockdowns impaired the motility of MM1 cells, we traced the morphological changes and migration tracks of MM1 cells in two-dimensional culture in response to LPA stimulation, using time-lapse video microscopy (Fig. 5B and supplemental videos S1–S4). Control siRNA cells plated on the fibronectin-coated dish exhibited a round morphology in the absence of LPA. In response to LPA stimulation, they became elongated with a polarized shape and migrated with the lamellipodial membrane protrusion extended persistently at the leading edge of the cell (Fig. 5B, control siRNA, and supplemental video S1). In contrast, cofilin/ADF siRNA cells displayed multiple membrane blebs around the cell periphery, both before and after LPA stimulation, and barely migrated (Fig. 5B, cofilin/ADF siRNA, and supplemental video S4). SSH1/SSH2 and LIMK1 siRNA cells were round and exhibited no apparent change in overall cell shape prior to LPA stimulation. However, most LIMK1 siRNA cells failed to produce the membrane protrusion even after LPA stimulation (Fig. 5B, LIMK1 siRNA, and supplemental video S3), suggesting that LIMK1 plays a role in the formation of membrane protrusions. SSH1/SSH2 siRNA cells generated the membrane protrusion in response to LPA stimulation, but the protrusions rapidly and repeatedly extended in various directions and retracted and were not retained in one direction (Fig. 5B, SSH1/SSH2 siRNA, and supplemental video S2), indicating that SSH1/SSH2 are involved in maintaining the membrane protrusion persistently at the leading edge of the migrating cell.

We next traced the migration tracks of cofilin/ADF, SSH1/SSH2, and LIMK1 knockdown cells for 3 h after LPA stimulation and measured the total length of the migration path of each cell (Fig. 5C). Cells were categorized into three classes according to their total migration distance (L) and the percentages of cells in each class were calculated (Fig. 5D). Control siRNA cells migrated relatively fast, with 67% of the cells having moved more than 200 μm for 3 h. In contrast, cofilin/ADF and LIMK1 siRNAs markedly suppressed the migration potential, with a total migration distance of less than 200 μm in most of these knockdown cells. SSH1/SSH2 siRNA cells had decreased migration distance but retained much of their migration potential, changing direction more frequently than the control cells. Together, these results suggest that cofilin/ADF and LIMK1 are essential for the migration of MM1 cells andSSH1/SSH2 are required for the effective migration of MM1 cells by maintaining the membrane protrusion to one direction.

**DISCUSSION**

A number of genes that are involved in cell motility and adhesion are deregulated in invasive and metastatic cancer cells, and alterations of the expression patterns of these genes can promote cancer-cell invasiveness and metastasis (35). Expression of cofilin and their regulators, including LIMK and SSH, is upregulated in a variety of invasive cancer cells, compared with less invasive cells (36). In this study, using siRNA knockdowns and an *in vitro* transcellular migration assay, we provide evidence that cofilin/ADF, SSH1/SSH2, and LIMK1 all play critical roles in the transcellular migration of rat ascites hepatoma MM1 cells.

We have shown that cofilin/ADF knockdown significantly suppresses the LPA-induced transcellular migration and two-dimensional culture motility of MM1 hepatoma cells (Figs. 1 and 5). Previous studies have shown that cofilin/ADF siRNAs decreased the motility and chemotaxis of B16F1 melanoma and Jurkat T lymphoma cells, respectively (15, 37). Cofilin/ADF proteins are essential for actin filament dynamics and reorganization (2, 3, 38, 39), and depletion of cofilin/ADF decreases the rate of actin filament turnover and often induces aberrant F-actin accumulation in various types of cells (37, 40, 41). Recent studies have shown that cofilin/ADF contribute to the production of more than half of all actin monomers in the cell (37, 42). These findings suggest that knockdown of cofilin/ADF probably inhibits tumor cell migration by decreasing the actin monomer pool size in the cell and that cofilin/ADF are involved in tumor cell migration by stimulating actin filament turnover in the membrane protrusions via continuously replenishing actin monomers for actin polymerization at the leading edge (39, 42). Our time-lapse analysis shows that cofilin/ADF knockdown changes the cell morphology, causing the formation of multiple membrane blebs around the cell periphery before and after LPA stimulation (Fig. 5B). These membrane blebs are F-actin-rich structures and are also induced by overexpression of active LIMK1 or LIMK2 (43, 44), suggesting that the proper level of cofilin/ADF activities is required for retention of the normal round morphology of MM1 cells. Given the important role of cofilin/ADF in actin cytoskeletal dynamics, the extent of the inhibitory effect of cofilin/ADF knockdown on tumor cell invasion may look too small. This is probably due to the incomplete suppression of cofilin/ADF expression in our experimental conditions. It is likely that the cells, in which cofilin/ADF are severely depleted, are unable to survive during the selection period.

SSH1/SSH2 activate cofilin/ADF by dephosphorylation (6, 7). The roles of SSH1/SSH2 in tumor cell invasion have not been previously investigated. In this study, we have shown that knockdown of SSH1/SSH2 suppresses the LPA-induced transcellular migration and motility of MM1 cells (Figs. 2 and 5), indicating that SSH1/SSH2 are involved in tumor cell migration and invasion. However, the SSH1/SSH2 knockdown had less of an inhibitory effect on the motility of MM1 cells in the Transwell assay and time-lapse analysis than did the cofilin/ADF and LIMK1 knockdowns. Time-lapse analysis shows that SSH1/SSH2 siRNA cells generate unstable membrane protrusions in various directions in response to LPA stimulation (Fig. 5B), suggesting that SSHs are required for maintaining membrane protrusion in one direction during cell migration. We have previously shown that SSH1 knockdown in Jurkat cells causes the formation of multiple protrusions around the cell and suppresses chemokine-induced directional cell migration (15). SSH1 is localized in the lamellipodium after chemokine stimulation and is activated by association with F-actin (11). These results suggest that SSH1/SSH2 are involved in tumor cell migration and invasion by stabilizing the lamellipodium membrane protrusion at the leading edge of the migrating cell via local activation of cofilin/ADF and stimulation of actin filament turnover in the front of the cell. A recent study suggests that cofilin accumulates in the invadopodium and plays a crit-
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tical role in the stabilization and maturation of the invadopodia of mammary tumor cells (45), further supporting the hypothesis that SSH1-mediated local activation of cofilin/ADF is important for the maintenance of membrane protrusions.

Knockdown of LIMK1 expression or treatment with a LIMK1 inhibitor (S3 peptide) suppresses the LPA-induced transcellular migration of MM1 cells (Fig. 3), indicating that the kinase activity of LIMK1 is required for LPA-induced transmigration. Because Rho and ROCK, known regulators of LIMK1, are also required for the LPA-induced transcellular migration of MM1 cells (26–28), LIMK1 probably acts downstream of a Rho–ROCK signaling pathway (9, 10). It is likely that the Rho–ROCK pathway is involved in LPA-induced transcellular migration by cooperatively increasing both LIMK1-mediated actin filament stabilization and actomyosin-based contractility via myosin light chain phosphorylation (46). LIMK1 knockdown suppresses LPA-induced cell attachment to fibronectin-coated dishes and cell motility in two-dimensional culture (Figs. 4 and 5), indicating that LIMK1 is required for both cell adhesion and motility processes in MM1 cells. It has previously been shown that overexpression of LIMK1 or LIMK2 induces the formation of stress fibers and focal adhesions (9, 31, 47). Together, these results suggest that LIMK1 is involved in LPA-induced cell adhesion and focal adhesion formation by stabilizing actin filaments at the attachment sites via inhibition of actin-disassembling activity of cofilin/ADF. Time-lapse microscopic analysis shows that LIMK1 knockdown results in a marked decrease in LPA-induced cell motility of MM1 cells and suppression of membrane protrusion formation (Fig. 5B). Likewise, chemokine-induced cell migration and membrane protrusion formation are impaired by LIMK1 siRNA in Jurkat T cells (15). Thus, LIMK1 seems to play a crucial role in the formation of the membrane protrusion that is an essential step for cell migration in most cells.

Previous studies have shown that ectopic expression of LIMK1 increases migration and invasiveness of breast and prostate cancer cells and that suppression of LIMK1 expression or activity by antisense LIMK1 or dominant-negative LIMK1 decreases migration and invasiveness (18, 19). Together with our observations, these results support the view that LIMK1 is required for the migration and invasion of various types of cancer cells. In contrast, other groups have reported that overexpression of LIMK1 decreases the motility and invasion of Rastransformed fibroblasts and mammary tumor cells (21, 22). These seemingly contradictory results may be explained by differences in expression levels of ectopic LIMK1 or the different cell types used in these studies, in which the expression levels of endogenous LIMK1 or related genes, such as cofilin/ADF, LIMK2, TESKs (48), SSHs, Rho GTPases, or other actin regulators, may differ. It is expected that the proper level of LIMK1 activity is required for cell migration: either excessive increases or decreases in LIMK1 expression may prevent tumor cell migration and invasion. If LIMK1 expression is too high, cofilin/ADF activity is depleted and the cells fail to reorganize their actin cytoskeletons, resulting in cell immobilization. If LIMK expression is too low, cells are unable to generate the lamellipodial membrane protrusions and focal adhesions, both of which are required for cell migration. Thus, the balanced phos-

phoregulation of cofilin/ADF activity is important for tumor cell migration and invasion. In addition, a recent report found that overexpression of LIMK1 increases tumor metastasis of breast cancer cells by up-regulating the expression of urokinase-type plasminogen activator and its receptor (49). The protease cascade initiated by urokinase-type plasminogen activator appears to promote the degradation of the intercellular connections of the cell monolayer during the transcellular migration of tumor cells. Furthermore, because LIMK1 is critical for VEGF-induced migration and tubule formation of vascular endothelial cells and promotes angiogenesis in vitro and in vivo (16, 49), LIMK1 may play a role in metastasis via tumor angiogenesis.

In summary, our data indicate that both SSH1/SSH2 and LIMK1 are required for tumor cell migration and invasion. It is likely that the spatial and temporal regulation of cofilin/ADF activities by the balanced control of LIMK-catalyzed phosphorylation and SSH-catalyzed dephosphorylation is critical for tumor cell migration and invasion. Further studies on the mechanism by which cofilin/ADF activities are regulated in tumor cell invasion will contribute to a better understanding of the mechanism of tumor invasion and the development of novel therapeutic strategies for preventing tumor metastasis.

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