The Neurofibromatosis Type 1 Gene Product Neurofibromin Enhances Cell Motility by Regulating Actin Filament Dynamics via the Rho-ROCK-LIMK2-Cofilin Pathway*§

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Neurofibromin is a neurofibromatosis type 1 (NF1) tumor suppressor gene product with a domain that acts as a GTPase-activating protein and functions, in part, as a negative regulator of Ras. Loss of neurofibromin expression in NF1 patients is associated with elevated Ras activity and increased cell proliferation, predisposing to a variety of tumors of the peripheral and central nervous systems. We show here, using the small interfering RNA (siRNA) technique, that neurofibromin dynamically regulates actin cytoskeletal reorganization, followed by enhanced cell motility and gross cell aggregation in Matrigel matrix. NF1 siRNA induces characteristic morphological changes, such as excessive actin stress fiber formation, with elevated negative phosphorylation levels of cofilin, which regulates actin cytoskeletal reorganization by depolymerizing and severing actin filaments. We found that the elevated phosphorylation of cofilin in neurofibromin-depleted cells is promoted by activation of a Rho-ROCK-LIMK2 pathway, which requires Ras activation but is not transduced through three major Ras-mediated downstream pathways via Raf, phosphatidylinositol 3-kinase, and Raf-GEF. In addition, the exogenous expression of the NF1-GTPase-activating protein–related domain suppressed the NF1 siRNA-induced phenotypes. Neurofibromin was demonstrated to play a significant role in the machinery regulating cell proliferation and in actin cytoskeletal reorganization, which affects cell motility and adhesion. These findings may explain, in part, the mechanisms of multiple neurofibromatosis formation in NF1 patients.

Neurofibromatosis type 1 (NF1), 2 also called von Recklinghausen disease, is autosomal dominant and one of the most common inherited disorders, affecting 1 in 3500 individuals (1). The phenotype of NF1 is highly variable, with several organ systems being affected, including bones, skin, irises, and central and peripheral nervous systems. The disease commonly manifests with “cafe au lait” macules in the skin, iris Lisch nodules, and learning disability (2, 3). The hallmark of NF1 is benign tumors that develop in the peripheral nervous system accompanied by an increased risk of malignancies.

The NF1 gene lies on chromosome 17q11.2 and encodes a large 2818-aminoacid protein, termed neurofibromin. Sequence analysis of neurofibromin revealed that a region centered around the 360 amino acids encoded by the NF1 gene shows significant homology to the known catalytic domains of mammalian Ras GTPase-activating protein (p120GAP), which interacts with Ras and promotes hydrolysis of Ras-bound GTP (active form) to GDP (inactive form), resulting in inactivation of the Ras protein. Accordingly, loss and/or mutations of neurofibromin elevate Ras activity and are followed by activation of various Ras effectors. Ras activation has been considered to be the causative event for tumor formation and other clinical manifestations in NF1 patients (2, 3).

Recent studies have suggested that neurofibromin has additional functions besides regulating cell proliferation via Ras pathways. For instance, mast cells in NF1/+ mice exhibited increased motility through hyperactivation of the Ras-P13K-Rac2 pathway in response to KitL, which is secreted by homozygous NF1 mutant (NF1/−/) Schwann cells, leading to formation of cell aggregations, which become neurofibromas (4, 5). In addition, as we have shown previously, neurofibromin regulates neuronal differentiation via its GAP function. In PC12 cells, time-dependent increases in the GAP activity of cellular neurofibromin (NF1-GAP) were detected after nerve growth factor stimulation and were correlated with the down-regulation of Ras activity during neurite extension (6). Although cell migration and neurite extension are known to be regulated by cytoskeletal reorganization in cells, the molecular mechanisms by which neurofibromin is involved in these cytoskeletal dynamics are understood poorly.

Coordinated regulation of actin cytoskeleton dynamics is required for several fundamental cellular events such as cell movement, cell adhesion, and cytokinesis. Actin reorganization is often initiated by extracellular stimuli and is regulated by diverse actin-binding proteins. Among these, the actin-depolymerizing factor (ADF)/cofilin family proteins play an essential role in promoting actin depolymerization at pointed ends and severing long actin filaments, which leads to rapid turnover of actin filaments (7). In mammals, the activity of ADF/cofilin is repressed by phosphorylation at Ser3, leading to actin cytoskeletal reorganization. Four kinases responsible for this phosphorylation have been identified: LIM-kinase 1 and 2 (LIMK1/2) and TES-kinase 1 and 2. LIMK1/2 are serine/threonine/tyrosine kinases characterized structurally by two NH2-terminal LIM domains and a PDZ domain. Recent studies have revealed that activities of LIMK are regulated by the downstream effec-

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‡ The abbreviations used are: NF1, neurofibromatosis type 1; DN, dominant negative; GAP, GTPase-activating protein; GRD, GAP-related domain; LIMK, LIM-kinase; MAPK, mitogen-activated protein kinase; P13K, phosphatidylinositol 3-kinase; RT, reverse transcriptase; ADF, actin-depolymerizing factor; ROCK, Rho-associated, coiled-coil forming protein kinase; siRNA, small interfering RNA; LPA, lysophosphatidic acid.

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**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—HeLa and HT1080 cells were cultured under 5% CO₂ at 37 °C in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 Ham (Sigma) with 10% fetal bovine serum. For transient transfection of plasmids, cells in 6-well plates were transfected using a FuGene6 transfection reagent following the manufacturer’s instructions (Roche Applied Science).

**Plasmids and Constructs**—Mammalian expression plasmids for NF1-GT-Pase-activating protein-related domain (GRD) types 1 and 2 (pcDNA3-FLAG-GRD1 and pcDNA3-FLAG-GRD2) were prepared as described previously (6). An expression plasmid for a nonphosphorylatable form of cofilin (pcDNA3-FLAG-CofilinS3A) was previously constructed (8). The pE-BOS-hemagglutinin RhoN19 plasmid and the pBj-1/DN-Ras(S17N) plasmid were kindly provided by Dr. K. Kaibuchi (Nagoya University, Japan) and Dr. A. Kikuchi (Hiroshima University, Japan), respectively. Antibodies and Chemicals—Polyclonal antibodies against the C-terminal (D) portion of neurofibromin, LIM kinase 2 (LIMK2), RhoA, phospho-cofilin (Ser³), and a monoclonal antibody against H-Ras were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An anti-hemagglutinin monoclonal antibody was obtained from Roche Applied Science. Polyclonal antibodies against Akt, phospho-Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2, LIM kinase 1 (LIMK1), cofilin, and phospho-cofilin (Ser³) were purchased from Cell Signaling Technology. Monoclonal antibody against paxillin was purchased from Transduction Laboratories. Monoclonal antibody against Ras and RalA were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antibodies against the β-actin and polyclonal antibodies against LIMK1, cofilin, and FLAG came from Sigma. Secondary antibodies linked to horseradish peroxidase and Cy5 were purchased from Amersham Biosciences. Texas Red, rhodamine phalloidin, and Alexa Fluor 488 phalloidin were from Molecular Probes, Inc. (Eugene, OR). Mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitors U0126 and PD98059 were purchased from Promega and Cell Signaling Technology, respectively. The PI3K inhibitor LY294002 and wortmannin were from Sigma. ROCK inhibitors Y27632 and HA1077 were supplied by Calbiochem and Upstate Biotechnology, respectively. Dispase was purchased from BD Biosciences.

**siRNA**—We designed the following three target sequences for human NF1 siRNA: 5'-AAC TTC GCA ATT CTG CCT GCT-3' and 5'-AAG GTT GCG CAG TTA GCA GAT GGT-3' and 5'-AAC TAG CTC GAG TCG TGG TTA-3'. Annealing of the component strands of each siRNA and transfection were performed as described (16, 17). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The above three siRNA oligonucleotides gave similar results. In addition, we designed target sequences for LIMK1 siRNA (1178AAG AGC ATG GAC AGC CAG TAC-1298) for LIMK2 siRNA (460AAC TAC GCC ACC ACT GTG CAA-480) and for RalA siRNA (157AAG AAG GTA GTG CTA CTA GAG-177). Double-stranded RNA targeting the luciferase gene (GL-2) (17) and the rodent NF1 gene (rNF1 siRNA; 249 AAC AAG GAG TGT CAC CTG AAC-269) were used as controls.

**RT-PCR Analysis of siRNA**—To evaluate NF1 mRNA expression after RNA interference, we performed RT-PCR using a set of primers: 5'-CGTGAAGGAAAACCAGCTGACGCT-3' (NFS1-S) and 5'-TGTCAAGCTGCCTACTTCCCTCATG-3' (NFS1-D). β-ACTIN primers, 5'-TCCCTGGGAAGAAAGCTCAGGC-3' (ACT-S) and 5'-GAGTTTCTCTGGATGCCCACAGG-3' (ACT-AS), were used as internal controls. First strand cDNA, which served as the PCR template, was synthesized from 2 μg of total RNA purified using an RNeasy mini kit (Qiagen). The reverse transcription (RT) reaction was performed using an oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen). PCR was performed with 1 μl of the RT reaction, 1.25 units of Taq DNA polymerase (Takara), 2 mM MgCl₂, and 0.8 mM dNTP mixture in a final volume of 50 μl. PCR conditions were 94 °C, , following a 2 min initial denaturation step followed by 25 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. PCR products were resolved by electrophoresis in a 1.5% agarose gel with ethidium bromide.

**Small G-protein Pull-down Assays**—Ras activity was measured by detecting Ras protein bound to the Ras-binding domain of Raf-1 immobilized to agarose with a Ras activation assay kit (Upstate Biotechnology) used according to the manufacturer’s instructions and as described previously (18). The activity of Raf was also measured by a method similar to the Ras activity assay. In place of the Raf-1 Ras-binding domain, the Raf binding domain of Raf-1BP1 (Ral-BP1-Ral-binding domain) was used to measure Raf activity (Upstate Biotechnology).

**Immunofluorescence**—HeLa or HT1080 cells grown on a 35-mm culture dish were fixed with 4% paraformaldehyde/phosphate-buffered saline for 15 min at room temperature and then permeabilized with 0.2% Triton X-100/phosphate-buffered saline for 5 min. After being washed with phosphate-buffered saline, the cells were incubated with primary antibodies diluted in phosphate-buffered saline containing 1% bovine serum albumin, followed by a secondary antibody conjugated with a fluorescent dye for 60 min at room temperature, respectively. Analysis was performed by confocal microscopy (Fluoview, FV300; Olympus).

**Matrigel Assays**—HeLa or HT1080 cells were transfected with siRNA and/or plasmids. At 24 h after transfection, ~5000 cells were placed on a layer of Matrigel (3.5 mg/ml in culture medium; BD Biosciences) in a 35-mm culture dish. The thickness of the Matrigel layer was usually ~1000 μm. Cells on the Matrigel were observed by phase-contrast microscopy.

For time lapse video analysis, cells were placed on Matrigel in ΔΔT 0.15-mm dishes (Biopheces). Before observation, the culture medium was replaced with dye-free L-15 medium, pH 7.2 (Sigma), supplemented with 10% fetal calf serum and overlaid with mineral oil. Dishes were maintained at 37 °C using the ΔΔT Culture Dish System (Biopheces) and imaged on an Olympus IX 70 microscope equipped with a TCS SP2 confocal microscope.
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FIGURE 1. Reduction of neurofibromin expression by siRNA. A, Immunoblot analysis of whole cell lysates extracted from untreated HeLa cells (lane 1), control siRNA-transfected cells (lane 2), and cells transfected with NF1 (609) (lane 3), NF1 (827) (lane 4), or NF1 (3924) (lane 5) siRNA. Cells were harvested 72 h after transfection and were detected with an anti-NF1 C-terminal antibody. β-Actin was used as a loading control. B, RT-PCR analysis of NF1 mRNA expression in untreated HeLa cells (lane 1), control siRNA-transfected cells (lanes 2 and 5) and cells transfected with NF1 (609) (lane 3), NF1 (827) (lane 4), or NF1 (3924) (lane 6) siRNA. RT-PCR of β-actin transcript was used as an internal standard. C, Immunocytochemical analysis of neurofibromin expression. HeLa cells transfected with control siRNA or NF1 (827) siRNA were immunostained with an anti-NF1 C-terminal antibody followed by a fluorescein isothiocyanate-conjugate secondary antibody. The stained cells were observed by confocal microscopy.

RESULTS

Suppression of NF1 Expression Leads to Changes in Cell Shape and Cytoskeletal Organization—To examine the roles of neurofibromin in cell growth and morphology, we attempted to reduce the expression of neurofibromin in HeLa cells by an RNA interference strategy. The levels of both NF1 mRNA and protein were suppressed beginning at 9 h after siRNA transfection and were reduced up to 95% at 72 h. Three siRNA oligonucleotides against NF1 showed similar effects in reducing expression of neurofibromin (Fig. 1, A and B). In addition, immunocytochemical analysis also confirmed the reduction of neurofibromin expression in cytoplasm by NF1 siRNA transfection, whereas siRNA controls showed no effects (Fig. 1C).

Immunocytochemical analysis showed that NF1 siRNA induced dynamic morphological changes according to the level of neurofibromin suppression with time. Neurofibromin-depleted HeLa cells tended to display the flattened shapes having excessive actin stress fiber formation from 12 h after transfection, and these changes were enhanced from 24 to 48 h (Fig. 2, J–O). Control siRNA induced no discernible alterations in cell morphology and cytoskeletal structure during the observation period (Fig. 2, D–I). Similar morphological changes and actin stress fiber formation were observed when other siRNA oligonucleotides against NF1 were used (supplemental Fig. S1).

The formation of actin stress fibers is usually accompanied by assembly of focal adhesions at cell margins. Immunostaining of paxillin, a major component of focal adhesions, revealed that NF1 siRNA promotes formation of focal adhesions at 24 and 48 h after transfection, when excess actin stress fibers were formed (Fig. 2, K and N). In contrast, control siRNA had no effect on formation of stress fibers and focal adhesions (Fig. 2, E and H).

We examined the effects of neurofibromin depletion on another NF1-relevant tumor cell, HT1080 human fibrosarcoma. HT1080 fibrosarcoma cells exhibit similar characteristics to malignant peripheral nerve sheath tumors (MPNSTs) frequently found in NF1 patients. As observed in HeLa cells, siRNA treatment effectively reduced the expression of neurofibromin (supplemental Fig. S2) and induced dynamic morphological changes with the robust stress fiber formation and focal adhesion assembly in HT1080 cells (Fig. 2, j–o). These observations suggest that neurofibromin plays a role in regulation of cytoskeletal organization.

Effect of NF1 siRNA on Cell Migration in Extracellular Matrix—Migration of cells in the extracellular matrix requires morphological changes with dynamic remodeling of the actin cytoskeleton. Therefore, we performed a Matrigel assay to assess the growth and motility of neurofibromin-depleted cells in the extracellular matrix. The growth of neurofibromin-depleted HeLa cells was about 1.5–2.0 times faster than that of control cells after contact with Matrigel for 3 days (data not shown). However, the number of colonies formed in the Matrigel was obviously fewer in neurofibromin-depleted cells compared with control cells (Fig. 3A), because neurofibromin-depleted cells tended to generate cell aggregation. After culturing for 24 h, the control cells became rounded shape and migrated into the Matrigel (Fig. 3B, a and c) and then formed a small spherical mass in Matrigel at 96 h in culture (Fig. 3B, i and m). In contrast, ~30% of the neurofibromin-depleted cells showed an elongated morphology (Fig. 3B, b–d and f–h). The neurofibromin-depleted cells migrated into the Matrigel and formed gross irregular and insular spherical masses around 96 h in culture (Fig. 3B, j–i and n–p). Time lapse, real time recording analysis revealed that neurofibromin-depleted cells actively move and rapidly invade in the Matrigel and aggregate to form gross spherical masses (Fig. 3C, lower panels, and supplemental Movie 2), whereas control cells move slowly and experience less aggregation (Fig. 3C, upper panels, and supplemental Movie 1). HT1080 cells treated with NF1 siRNA also tended to form cell aggregation in the Matrigel matrix (Supplemental Fig. S3). These results indicate that the reduction of neurofibromin by siRNA promotes peculiar cell migration and cell-cell adhesion, forming cell aggregates in the Matrigel matrix.

Morphological Changes Induced by Reduction of NF1 Expression Are Due to Cofilin Phosphorylation—The morphological changes and active motility of neurofibromin-depleted cells suggested that neurofibromin is involved in regulation of actin cytoskeletal reorganization. Thus, we

with a sensitive SenSys-1401E CCD camera (Roper Scientific). Images were obtained using a ×20 UPlan Apo objective (Olympus). The camera, shutters, and filter wheel were controlled by MetaMorph imaging software (Universal Imaging), and the images were collected every 5 min with exposure times of 100 ms.
investigated various signal molecules that regulate actin cytoskeletal reorganization in the neurofibromin-depleted cells and found that phosphorylation of ADF/cofilin is significantly elevated with time in the cells, whereas their levels in control siRNA transfectants had no changes (Fig. 4A and supplemental Fig. S4). The activity of ADF/cofilin is negatively regulated by phosphorylation at Ser3 through a RhoGTPase-ROCK-LIMK pathway that is activated in response to extracellular stimuli, such as lysophosphatidic acid (LPA), and followed by stabiliza-
tion and accumulation of actin filaments (11). Therefore, we hypothesized that the excessive actin stress fiber formation in neurofibromin-depleted cells was due to the enhancement of ADF/cofilin phosphorylation through Rho activation. It was shown that components of serum, such as LPA, activate Rho-GTPase, leading to the formation of actin stress fibers and focal adhesion, and subsequently Rho become inactivated in adherent cells (19, 20). Therefore, we investigated whether LPA and serum stimulation affect the level of phosphocofilin (Fig. 4B) and the formation of actin stress fibers in neurofibromin-depleted cells (Fig. 4C). Control cells after serum stimulation exhibited prompt cofilin dephosphorylation after transient phosphorylation with the enhancement of cortical actin (Fig. 4, B (upper panels) and C (b and c)), followed by attenuation to be consistent with the dephosphorylation of cofilin. In contrast, after serum stimulation, neurofibromin-depleted cells exhibited sustained levels of phosphorylated cofilin with the enhancement of actin stress fibers in the cell body (Fig. 4, B (upper panels) and C (g and h)). The induced actin stress fibers persisted, consistent with the sustained phosphorylation of cofilin. In addition, when cells were stimulated by LPA, control cells exhibited biphasic cofilin phosphorylation (dephosphorylation at 10 min and rephosphorylation at 60 min after the LPA treatment) with enhancement of cortical actin and fine stress fibers formed in the cell body (Fig. 4, B (lower panels) and C (d and e)), followed by the attenuation consistent with the dephosphorylation of cofilin. In contrast, neurofibromin-depleted cells exhibited a high level of phosphorylated cofilin with enhancement of actin stress fiber polymerization in the center of the cell body (Fig. 4, B (lower panels) and C (i and j)). These results supported our hypothesis that the sustained cofilin phosphorylation by serum and LPA stimulation induces the formation of stable actin stress fibers in cells where neurofibromin was down-regulated.

Nonphosphorylatable Cofilin-S3A Mutant Rescues the Phenotypes of Neurofibromin-depleted Cells—To confirm whether NF1 siRNA-induced morphological effects are attributable to inactivation of cofilin, we co-transfected NF1 siRNA with a cofilin-S3A mutant that functions as a constitutively active form of cofilin. Immunocytochemical analysis after 24 h of co-transfection revealed that the cofilin-S3A mutant suppresses excessive stress fiber formation in neurofibromin-depleted cells. HeLa cells co-transfected with FLAG-cofilin S3A and either control or NF1 siRNA were stained with Alexa 488 phalloidin and anti-FLAG antibody at 24 h after transfection.

**FIGURE 4. Promotion of cofilin phosphorylation in neurofibromin-depleted cells.** A, effect of neurofibromin depletion on the levels of phosphocofilin. Either control or NF1 siRNA were transfected into HeLa cells. After 24 and 48 h, cells were harvested and examined for the levels of Ser(P)3-cofilin by immunoblotting analysis. B, neurofibromin-depleted cells exhibited a high level of phosphorylated cofilin. After 24 h of siRNA transfection in a serum-free condition, HeLa cells were stimulated with 10% serum or 10 μM LPA for the indicated periods and then examined for levels of Ser(P)3-cofilin by immunoblot analysis. C, enhancement of actin stress fiber formation in neurofibromin-depleted cells. After 24 h of siRNA transfection under a serum-free condition, HeLa cells were stimulated with serum or LPA at the indicated times and then stained with rhodamin phalloidin to visualize the actin cytoskeleton. D, the nonphosphorylatable cofilin S3A mutant suppresses excessive stress fiber formation in neurofibromin-depleted cells. HeLa cells co-transfected with FLAG-cofilin S3A and either control or NF1 siRNA were stained with Alexa 488 phalloidin and anti-FLAG antibody at 24 h after transfection.
Pathways but Is Not Transduced through Three Major Ras-mediated Downstream ROCK-LIMK2 pathway.

rofibromin regulates cofilin-induced actin reorganization via the Rho-robimin-depleted cells. The changes observed in neurofibromin-depleted cells by the transfection of a dominant negative mutant of RhoA, N19RhoA, with supplemental Fig. S5A). These results suggest that neurofibromin regulates actin dynamics via a LIMK2-cofilin pathway.

Neurofibromin Regulates Cofilin-induced Actin Reorganization via the Rho-ROCK-LIMK2 Pathway—It is known that LIMK2 is directly activated by ROCK, which is a downstream effector of small GTPase Rho (8, 11, 21, 22). Therefore, to examine whether the elevated phosphorylation of cofilin is regulated through a ROCK-LIMK2 pathway in neurofibromin-depleted cells, we analyzed the levels of phosphocofilin after treating the cells with the ROCK inhibitor, Y27632. Y27632 effectively suppressed the levels of phosphocofilin (Fig. 6A) and the formation of excessive actin stress fibers (Fig. 6B) in neurofibromin-depleted cells. Another ROCK inhibitor, HA1077, showed similar effects (data not shown). We also found that treatment with Y27632 significantly reduced the formation of spherical masses of neurofibromin-depleted cells in the Matrigel matrix (Fig. 6C and supplemental Fig. S5B). In addition, we examined the involvement of RhoA in the phenotypic changes observed in neurofibromin-depleted cells by the transfection of a dominant negative mutant of RhoA, N19RhoA, with NF1 siRNA. N19RhoA suppressed not only the elevated phosphorylation of cofilin but also excess actin stress fiber formation in neurofibromin-depleted cells (Fig. 6, D and E). Taken together, these results suggested that neurofibromin regulates cofilin-induced actin reorganization via the Rho-ROCK-LIMK2 pathway.

NF1-mediated Regulation of Rho Signaling Requires Ras Activation but Is Not Transduced through Three Major Ras-mediated Downstream Pathways—To investigate whether the NF1 siRNA-induced effects were dependent on the Ras activity, we transfected a plasmid for expression of a dominant negative form of Ras, DN-Ras(S17N), into neurofibromin-depleted cells. The NF1 siRNA-induced cofilin phosphorylation and stress fiber formation were partially suppressed by expression of the DN-Ras mutant (Fig. 7, A and B), suggesting that Ras activation is involved in the phenotypic changes observed in NF1-depleted cells.

Ras activates various downstream signaling molecules, such as Raf, PI3K, and RaGEF, and is known to be a potent inducer of cell motility through the regulation of focal adhesion and stress fiber turnover (23). The ability of activated Ras to stimulate PI3K in addition to Raf is particularly essential in Ras-induced cytoskeletal organization (24). Therefore, we analyzed the activation of MAPK and Akt in neurofibromin-depleted cells. Neurofibromin-depleted cells exhibited more constitutive activation of the MAPK and PI3K pathways than control cells (supplemental Fig. S4). In addition, to examine correlations with the status of cofilin phosphorylation after serum and LPA stimulation, we also analyzed their time-dependent alteration of Ras activities and MAPK and Akt phosphorylation levels after stimulation with serum and LPA. In both control and neurofibromin-depleted cells, the active form of Ras was apparently increased at an early phase of serum and LPA stimulation. However, the levels of active Ras were significantly higher in neurofibromin-depleted cells than in control cells (Fig. 7C). The phospho-Akt levels increased only in neurofibromin-depleted cells, and their levels were higher and remained elevated longer than those in the control cells. Interestingly, although the phospho-ERK1/2 levels also increased concomitantly with Ras activation after serum stimulation, levels were no different between control and neurofibromin-depleted cells. On the other hand, LPA stimulation induced a slight activation of ERK1/2 in neurofibromin-depleted cells compared with control cells (Fig. 7C).

We presented evidence that NF1 siRNA induced cofilin inactivation through the RhoA-ROCK-LIMK2-cofilin pathway, in addition to activating Ras and its downstream pathway by serum and LPA stimulation. Therefore, to examine whether Ras-MAPK or Ras-PI3K activity is involved with NF1 siRNA-induced cofilin phosphorylation, we treated the neurofibromin-depleted cells with the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitors U0126 and PD98095 and the PI3K inhibitors LY294002 and wortmannin. However, any inhibitor of the Ras-related signaling pathway did not inhibit NF1 siRNA-induced cofilin phosphorylation (Fig. 7D) and excessive actin stress fiber formation (data not shown). Moreover,
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FIGURE 6. Neurofibromin regulates cofilin-induced actin reorganization via Rho-ROCK pathway. A, ROCK inhibitor Y27632 suppresses NF1 siRNA-induced cofilin phosphorylation. After 24 h of siRNA transfection, HeLa cells were treated with (lanes 3 and 4) or without 10 μM Y27632 (lanes 1 and 2) for 1 h and then examined for levels of Ser(P)3-cofilin by immunoblot analysis. B, Y27632 suppresses NF1 siRNA-induced stress fiber formation. After 24 h of siRNA transfection, cells were treated with or without 10 μM Y27632 and stained with Alexa 488 phalloidin. C, number of colonies that formed on Matrigel matrix. After transfecting HeLa cells with the indicated siRNA in the presence or absence of Y27632, the number of colonies formed on Matrigel at 96 h was counted under a phase-contrast microscope. Each datum represents the average of three independent experiments. Error bars represent S.D. Statistical differences were determined with a t test; *, p < 0.001. D, N19RhoA suppresses NF1 siRNA-induced cofilin phosphorylation. NF1 or control siRNA was co-transfected with mock (lanes 1 and 2) or N19RhoA (a dominant negative form of RhoA) plasmid (lanes 3 and 4). The levels of Ser(P)3-cofilin were analyzed by immunoblot after 24 h. E, N19RhoA suppresses the NF1 siRNA-induced stress fiber formation. Cells treated as described in D were stained with Alexa 488 phalloidin (actin; green) and anti-hemagglutinin antibody (N19RhoA; red) after 24 h of siRNA transfection.

Although it has been shown previously that both PI3K and MEK inhibitors are combined to inhibit Ras signaling to cofilin (25), the combined inhibition of both PI3K and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase pathways also showed no effect on the cofilin phosphorylation induced by NF1 depletion (supplemental Fig. S6).

Next, to investigate whether decreased neurofibromin expression by the NF1 siRNA affects the Ras-Ral pathway, we analyzed Ral activity in neurofibromin-depleted cells. Ral activity in neurofibromin-depleted cells was not significantly different than in control cells (supplemental Fig. S7A). Furthermore, co-transfection with RaA siRNA also had no effect on cofilin phosphorylation induced by the depletion of neurofibromin (supplemental Fig. S7B).

Taken together, these results raised the possibility that Ras activity is partially required for NF1 siRNA-induced effects, although three major Ras-mediated pathways via Raf, PI3K, and RafGEF are not involved in the effects.

The NF1-GRD Region Is Required for NF1-mediated Regulation of Rho-ROCK-LIMK2-Cofilin Pathways—To confirm whether the loss of NF1-RasGAP function is involved in cofilin phosphorylation and cytoskeletal reorganization observed in neurofibromin-depleted cells, we transfected NF1-GRD into neurofibromin-depleted cells. The NF1-GRD was identified as having two different alternative splicing isoforms, NF1-GRD type 1 and type 2 (26). Type 2 contains an additional 63-bp insertion (exon 23a) that encodes 21 amino acids in the center of NF1-GRD type 2. The specific expression patterns of these isoforms in several organs and cells provide a basis for implicating differential expression of NF1-GRD type 1 and type 2 transcripts in the regulation of neuronal differentiation and development (6). The expression of these GRD variants was not affected by the NF1 siRNA (609 and 827), because the target sequences of those NF1 siRNAs are not included in the variants. The expression of NF1-GRD type 2 significantly suppressed not only cofilin phosphorylation but also morphological changes induced by NF1 siRNA, whereas NF1-GRD type 1 expression showed fewer effects (Fig. 8, A and B). These findings suggest that not only RasGAP function but also other unknown mechanisms of NF1-GRD type 2 may be necessary for the NF1-mediated regulation of Ras signaling.

Finally, we examined the effect of NF1-GRD on the invasive activity of neurofibromin-depleted cells. Co-transfection with NF1-GRD type 2 significantly suppressed the formation of spherical cell aggregates in Matrigel found among neurofibromin-depleted cells, leading to an increased number of colonies (Fig. 8, C and D). This finding suggested that the peculiar migration activity of neurofibromin-depleted cells is inhibited by the expression of NF1-GRD2. NF1-GRD type 1 expression also partially suppressed the formation of cell aggregates but did not inhibit the elongated morphology. These results further support our notion that NF1-GRD, especially GRD type 2, is required for regulating cell invasion.

DISCUSSION

In this study, we demonstrated a novel function of neurofibromin on actin cytoskeletal reorganization using siRNA. Depletion of neurofibromin activates the Rho-ROCK-LIMK2 pathway, leading to inactivation of cofilin by phosphorylation, which alters actin dynamics and, consequently, induces a highly invasive phenotype in HeLa and HT1080 cells. These results suggest that neurofibromin is a key regulator for not only Ras-mediated cell proliferation but also cell migration via actin cytoskeletal reorganization in human cells.

Several previous studies have suggested that neurofibromin is involved in cytoskeletal reorganization (27–29). However, the related biological significance and the signaling pathways are poorly understood. Our present data show that depletion of neurofibromin activates a Rho-ROCK-LIMK2-cofilin pathway to alter the actin cytoskeleton reorganization and promote cell motility, invasiveness, and cell-cell

Although it has been shown previously that both PI3K and MEK inhibitors are combined to inhibit Ras signaling to cofilin (25), the combined inhibition of both PI3K and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase pathways also showed no effect on the cofilin phosphorylation induced by NF1 depletion (supplemental Fig. S6).

Next, to investigate whether decreased neurofibromin expression by the NF1 siRNA affects the Ras-Ral pathway, we analyzed Ral activity in neurofibromin-depleted cells. Ral activity in neurofibromin-depleted cells was not significantly different than in control cells (supplemental Fig. S7A). Furthermore, co-transfection with RalA siRNA also had no effect on cofilin phosphorylation induced by the depletion of neurofibromin (supplemental Fig. S7B).

Taken together, these results raised the possibility that Ras activity is partially required for NF1 siRNA-induced effects, although three major Ras-mediated pathways via Raf, PI3K, and RafGEF are not involved in the effects.

The NF1-GRD Region Is Required for NF1-mediated Regulation of Rho-ROCK-LIMK2-Cofilin Pathways—To confirm whether the loss of NF1-RasGAP function is involved in cofilin phosphorylation and cytoskeletal reorganization observed in neurofibromin-depleted cells, we transfected NF1-GRD into neurofibromin-depleted cells. The NF1-GRD was identified as having two different alternative splicing isoforms, NF1-GRD type 1 and type 2 (26). Type 2 contains an additional 63-bp insertion (exon 23a) that encodes 21 amino acids in the center of NF1-GRD type 2. The specific expression patterns of these isoforms in several organs and cells provide a basis for implicating differential expression of NF1-GRD type 1 and type 2 transcripts in the regulation of neuronal differentiation and development (6). The expression of these GRD variants was not affected by the NF1 siRNA (609 and 827), because the target sequences of those NF1 siRNAs are not included in the variants. The expression of NF1-GRD type 2 significantly suppressed not only cofilin phosphorylation but also morphological changes induced by NF1 siRNA, whereas NF1-GRD type 1 expression showed fewer effects (Fig. 8, A and B). These findings suggest that not only RasGAP function but also other unknown mechanisms of NF1-GRD type 2 may be necessary for the NF1-mediated regulation of Ras signaling.

Finally, we examined the effect of NF1-GRD on the invasive activity of neurofibromin-depleted cells. Co-transfection with NF1-GRD type 2 significantly suppressed the formation of spherical cell aggregates in Matrigel found among neurofibromin-depleted cells, leading to an increased number of colonies (Fig. 8, C and D). This finding suggested that the peculiar migration activity of neurofibromin-depleted cells is inhibited by the expression of NF1-GRD2. NF1-GRD type 1 expression also partially suppressed the formation of cell aggregates but did not inhibit the elongated morphology. These results further support our notion that NF1-GRD, especially GRD type 2, is required for regulating cell invasion.

DISCUSSION

In this study, we demonstrated a novel function of neurofibromin on actin cytoskeletal reorganization using siRNA. Depletion of neurofibromin activates the Rho-ROCK-LIMK2 pathway, leading to inactivation of cofilin by phosphorylation, which alters actin dynamics and, consequently, induces a highly invasive phenotype in HeLa and HT1080 cells. These results suggest that neurofibromin is a key regulator for not only Ras-mediated cell proliferation but also cell migration via actin cytoskeletal reorganization in human cells.

Several previous studies have suggested that neurofibromin is involved in cytoskeletal reorganization (27–29). However, the related biological significance and the signaling pathways are poorly understood. Our present data show that depletion of neurofibromin activates a Rho-ROCK-LIMK2-cofilin pathway to alter the actin cytoskeleton reorganization and promote cell motility, invasiveness, and cell-cell
adhesion in Matrigel matrix, leading to consequent formation of large cell aggregates. This phenotype is reminiscent of multiple neurofibroma formation in NF1 patients. Neurofibromas are characterized by excessive extracellular matrix deposition and aggregation of the multiple cell types within the tumors, including Schwann cells, fibroblasts, endothelial cells, and mast cells (2, 30–33). A recent study using a mouse model revealed that NFI1/−/− Schwann cells secrete chemotactic factors, such as KitL, and promote recruitment of inflammatory cells to the tumor microenvironment, generating neurofibromas (4). Our findings suggest that not only the expression of chemotactic factors but also a high motility phenotype of neurofibromin-depleted cells contributes to forming cell aggregates.

Cell migration requires coordination of several processes, such as membrane protrusion of the leading edge, adhesion to the substratum, retraction of the trailing edge, and detachment (34). Cofilin has been shown to be involved in regulating membrane protrusions in THP-1 monocytes and tail retraction in primary monocytes via RhoA-ROCK signaling (35, 36). Therefore, actin stress fibers formed by cofilin phosphorylation through the activation of Rho/ROCK/LIMK2 signaling in neurofibromin-depleted cells might induce successful tail retraction, leading to the increased cell motility. It should be noted that phosphorylation of cofilin is induced not only by activation of LIMK but also by misregulation of cofilin phosphatases, such as slingshot. Therefore, the role of neurofibromin in regulation of cofilin phosphatases is under investigation.

Rho GTPases play a critical role in the cell shape changes and adhesion dynamics that drive migration. It is known that the activities of LIMK1 and LIMK2 are regulated by the distinct Rho family GTPases, and the signal transduction pathways, such as Cdc42/Rac-PAK1-LIMK1, Rho-ROCK-LIMK2, and Cdc42-myotonic dystrophy kinase-related Cdc42-binding kinase α-LIMK, play distinct roles in the regulation of cofilin-mediated actin dynamics. Our findings show that LIMK2, but not LIMK1, is activated by depletion of neurofibromin, resulting in suppression of cofilin, so enhancing stress fiber formation and cell motility. Some recent studies have suggested that LIMK2 regulates cell motility and migration activity. Transforming growth factor-β induces actin cytoskeletal reorganization via the transforming growth factor-β type 1 receptor-Rho-ROCK-LIMK2-cofilin pathway (37). Suppression of LIMK-2 expression by hammerhead ribozymes in metastatic human fibrosarcoma (HT1080) cells limits their migration and dense colony-forming efficiency without affecting cell proliferation rate or viability (38). LIMK might play a significant role in actin dynamics via cofilin inactivation with the spatial and temporal regulation by distinct Rho family GT Pases in migrating cells. Cells might acquire an excessive invasive property in cases where the leading and rear edges of the cell are unbalanced. The imbalance of cofilin activity by LIMK might cause
incidental cell invasiveness or akinesis. Although there is, as yet, no direct evidence for a role of deregulated ADF/cofilin activity in the etiology of human cancers, altered cofilin production levels have been demonstrated in some cancer tissues (39). Our findings thus support the hypothesis that deregulation of cofilin is involved in tumor cell motility and invasiveness.

NF1-GRD is known to act as a negative regulator of Ras (2). Our data showed that the expression of dominant negative form of Ras partially suppressed the cofilin phosphorylation and stress fiber formation induced in neurofibromin-depleted cells, suggesting that Ras activation is somehow involved in the NF1-mediated Rho signaling. We thus examined whether Ras downstream pathways (Ras-MAPK, Ras-PI3K, and Ras-Ral pathways) participate in phenotypes found in neurofibromin-depleted cells. However, MAPK and PI3K inhibitors did not restore those phenotypes, although the MAPK and PI3K pathways were constitutively activated in neurofibromin-depleted cells. The co-transfection with RalA siRNA also had no effect on the phenotypes. These results indicate that Ras activation is involved in the NF1 siRNA-induced effects, but three major Ras-mediated pathways do not play a role in those effects.

Neurofibromin-depleted cells acquired high motility in Matrigel matrix and increase the likelihood of encountering neighbor cells, followed by gross insular mass formation with the assistance of haptotaxis (supplemental movie 2). This characteristic phenotype in the neurofibromin-depleted cells was suppressed by overexpression of NF1-GRD. This finding suggests that NF1-GRD plays a significant role in the suppression of the invasive characteristics of cells. NF1-GRD type 2 expression could restore the gross insular cell mass formation induced by depletion of neurofibromin. On the other hand, NF1-GRD type 1 expression also could be restored partially, although an irregular tumor mass remained. Given that NF1-GRD type 1 has a higher Ras-GAP catalytic activity than NF1-GRD type 2 (6), the NF1-GRD type 2 mutant may convey not only RasGAP function but also other unknown function, leading to the NF1-mediated regulation of Rho signaling. In a recently published study, it was reported that neurofibromin can interact with syndecan via their GRD and C-terminal lesions, respectively, and colocalizes in the axon and synapses of neurons (40). The syndecans, an ancient family of transmembrane proteoglycans, bind to extracellular matrix proteins, such as laminin and fibronectin, and are implicated in cell matrix adhesion, cell motility, focal adhesion assembly, and morphogenesis in several cell types (41). Although we do not yet understand the biological significance of this interaction, it has been suggested that the NF1-GAP domain has a novel function as a binding motif besides the catalytic activity. The cytoskeletal reorganization induced by
depletion of neurofibromin might be caused by not only Ras-related signaling but also another form of direct signaling via a membrane receptor.

Recently, several lines of evidence implicate the ROCK-LIMK-cofilin pathway in the regulation of axon growth and guidance in neuronal cells (42). ROCK mediates the effects of Rho on neurite retraction in neuroblastoma N1E-115 cells, on preventing the initiation of axon outgrowth in cultured cerebellar granule neurons, and on dendrite retraction in rat hippocampal pyramidal neurons in cultured brain slices, respectively (43–45). Increased cofilin activity by overexpression of Xenopus ADF/cofilin promotes process extension and neurite outgrowth in primary neurons (46). This evidence indicates that cofilin activity is indispensable for actin filament dynamics and reorganization in not only neuronal but also neuronal cells. The potential importance of cofilin in neuronal development is also underscored by findings that the impairment of visuospatial constructive cognition found in Williams syndrome may be attributable to hemizygosity of the LIM kinase 1 gene (47). NF1 patients, as well as NF1+/− mice and Drosophila NF1 mutants, frequently exhibit a variety of cognitive deficits as learning disabilities beyond the tumorigenic symptoms (48–50). Therefore, we speculate that NF1 might regulate neural morphogenesis via the NF1-GRD/Rho/ROCK/LIMK/cofilin signaling pathway, as shown in our present findings, and the abnormal regulation in this pathway might contribute to the initiation of a cognitive deficit.

In summary, we have demonstrated that neurofibromin is involved in not only proliferation but also cytoskeletal organization and cell motility through regulation of the Rho–ROCK-LIMK2-cofilin pathway. Various clinical manifestations of NF1, such as multiple neurofibroma formation and learning disabilities, may be partly explained by abnormal regulation of this novel pathway. Therefore, inhibition of Rho-ROCK-LIMK2-cofilin signals may provide a new therapeutic approach for this disease.

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REFERENCES

1. Stephens, K., Riccardi, V. M., Rising, M., Ng, S., Green, P., Collins, F. S., Rediker, K. S., Wang, M. J., Diaz, B., Travers, J. B., Hood, A., Marshall, M., Williams, D. A., and Clapp, D. W. (2003) J. Clin. Invest. 112, 1519–1532.

2. Nobes, C. D., and Hall, A. (1999) J. Cell Biol. 144, 1235–1244.

3. Li, C., Cheng, Y., Gutmann, D. A., and Mangoura, D. (2001) Brain Res. Dev. Brain Res. 130, 231–248.

4. Sumi, T., Matsumoto, K., Nakamura, T. (2003) J. Biol. Chem. 278, 670–676.

5. Meberg, P. J., and Bamburg, J. R. (2000) J. Biol. Chem. 275, 708–718.

6. Yunoue, S., Tokuo, H., Fukunaga, K., Feng, L., Ozawa, T., Nishi, T., Kikuchi, A., Noble, M., Atkinson, D. L., Odelberg, S. J., and Keating, M. T. (1996) EMBO J. 15, 585–595.

7. Pollard, T. D., and Borisy, G. G. (2003) Nat. Rev. Mol. Cell. Biol. 4, 926–937.

8. Lu, L. (2000) Nat. Rev. Neurosci. 1, 173–180.

9. Sumi, T., Matsumoto, K., Shibuya, A., and Nakamura, T. (1999) J. Biol. Chem. 147, 1519–1532.

10. Edwards, D. C., Sanders, L. C., Bokoch, G. M., and Gill, G. N. (1999) Nat. Cell Biol. 1, 253–259.

11. Bito, H., Furuyashiki, T., Ishihara, H., Shibasaki, Y., Ohashi, K., Levin, V. A., Tanase, S., Morino, Y., and Haya, S. (1991) Oncogene 6, 1555–1559.

12. Xu, H., and Gutmann, D. H. (1997) Brain Res. 759, 149–152.

13. Arber, S., Barbayannis, F. A., Hansen, H., Schneider, C., Stanyon, C. A., Bernard, O., and Caron, P. (1998) Nature 393, 805–809.

14. Toshima, J., Toshima, J. Y., Takeuchi, K., Mori, R., and Mizuno, K. (2001) J. Biol. Chem. 276, 31449–31458.

15. Toshima, J., Toshima, J. Y., Amano, T., Yang, N., Narumiya, S., and Mizuno, K. (2001) Mol. Biol. Cell 12, 1131–1145.

16. Sumi, T., Matsumoto, K., and Nakamura, T. (2003) J. Biol. Chem. 278, 1519–1532.