Identification of Genes Responsible for Cell Migration by a Library of Randomized Ribozymes

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ABSTRACT

Several genes appear to be associated with metastasis, but the underlying mechanisms of metastasis still remain unclear. In this study, we used a library of randomized ribozymes to identify, by inactivation of transcripts, genes involved in cell migration that is an essential aspect of metastasis. Using a chemotaxis assay, the ribozymes that inhibited cell migration were selected from the library. Among such ribozymes, we found two ribozymes that targeted and cleaved ROCK1 mRNA at independent sites. ROCK1 and ROCK2 are Rho kinases, and it has been demonstrated that they regulate the organization of the actin cytoskeleton and are responsible for cell motility and cytokinesis. The two ribozymes that specifically cleaved ROCK1 mRNA inhibited both the migration and invasion of invasive HT1080 fibrosarcoma, but neither had any effect on cell proliferation. Our analysis indicates that the ribozymes toward ROCK1 can block invasive activity but not the proliferation of HT1080 cells without having any effect on expression of ROCK2. Ribozymes identified in this study, including the ribozymes against ROCK1, might be useful in understanding the mechanisms of cell migration and metastasis.

INTRODUCTION

Metastasis is often one of the most serious problems for cancer therapy, and investigations of the mechanisms of metastasis have involved a variety of approaches (1–4). Comparisons, using DNA microarrays, of highly invasive and weakly invasive cancer cells have suggested that several genes are responsible for metastasis (5–7). There seems to be no doubt that DNA microarray analysis is a powerful tool for the diagnosis and basic research of cancer. However, microarray analysis does not reveal differences among activities of gene products; it only reveals differences in the levels of expression of particular genes (8). Therefore, to clarify the mechanisms of metastasis, a complementary technology is necessary that will allow us to correlate the activity of a gene product with a specific phenotype.

Ribozymes of various types have been used to interfere with the intracellular expression of specific genes via cleavage of the respective mRNAs (9–15). A well-characterized hammerhead ribozyme that consists of recognition arms at its 5′ and 3′ ends and a catalytic core region can bind to and cleave a substrate RNA (16). To create a ribozyme that specifically cleaves the transcript of a particular gene, the nucleotide sequences of the recognition arms are designed to be complementary to the sequence of the mRNA. When we randomize the sequences of the recognition arms, we can prepare a ribozyme library that is targeted to multiple mRNA substrates. Such a library can be used as a “knock down” library to interfere with the activities of numerous gene products (17–20). We can screen ribozymes and identify target genes that appear to be responsible for a particular phenotype by introducing such a ribozyme library into cells. Isolation of cells with the phenotype of interest allows the rescue of the ribozyme(s) of interest. Then, an examination of DNA databases allows rapid identification of the gene(s) responsible for the phenotype of interest using sequences of the rescued ribozyme(s).

Here, we describe the identification of genes responsible for migration and the isolation of ribozymes that would limit the expression of such genes using a library of randomized ribozymes. We focused on the motility of invasive cancer cells because the motility of strongly invasive cancer cells is known to be greater than that of noninvasive or weakly invasive cells, and also, motility is a prerequisite for metastasis (5, 21, 22). Indeed, inhibition of the motility of invasive cancer cells might be expected to prevent metastasis. For the selection of cells with defective migration using ribozymes, we chose a chemotaxis assay in a Boyden chamber (23). Cells that did not migrate toward the chemoattractant as a result of the effect of particular ribozymes were selected, and then ribozymes were isolated from such cells. The sequences of the recognition arms of these ribozymes were used to identify their targets. Among the selected ribozymes, we identified two ribozymes and determined that each cleaved the transcript of ROCK1, one of the genes that regulate the actin cytoskeleton (24, 25). The two ribozymes also inhibited the migration of and invasion by invasive HT1080 fibrosarcoma cells. Two related genes, ROCK1 and ROCK2, are already demonstrated to be responsible for metastasis (26), and we found that ROCK1 was responsible for motility but not for proliferation of HT1080 cells. Moreover, we identified genes other than ROCK1 as candidates for the prevention of cell migration. The selected ribozymes and their alternative inhibitors against the candidate genes might be useful for cancer therapy.

MATERIALS AND METHODS

Construction of a Library of Randomized Ribozymes. Fragments of DNA that encoded randomized hammerhead ribozymes were generated by PCR with, as the template, 5′-TCC CGG GTT CGA AAC CGG GCA GCA CAA CCC ACT TTN NNN NNN CTG ATG AGG CCA AAA GGC CGA AAN NNN NNG GTA CCC CGG ATA TCT TTT TTT TTT TTT-T3′ and primers 5′-TCC CGG GTT CGA AAC CGG GCA GCA-3′ (sense) and 5′-GTC TGC ATG CCT GCA GGT CGA CCC GAT AGA AAA AAA GAT ATC CGG GGT-3′ (antisense). The sequence of the template was based on a tRNAVal-fused hammerhead ribozyme. Construction of the ribozyme expression vector based on a plasmid pPUR (Clontech, Palo Alto, CA) was performed as described previously (27, 28). The plasmid DNA carrying randomized ribozymes was introduced into competent bacteria, *Escherichia coli* DH5α (TOYOBO, Osaka, Japan), and 5 × 10⁶ colonies of bacterial cells were obtained. Then, the plasmid DNA that harbored the ribozymes was purified from the transformants.

Culture and Transfection of Cells. HT1080 human fibrosarcoma cells and B16-BL6 mouse melanoma cells were kindly provided by Dr. Nakajima (Novartis Pharma Research Japan, Tsukuba, Japan). Cells were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO), supplemented with 10% fetal bovine serum and an antibiotics mixture (Life Technologies, Inc., Rockville, MD). Transfection of HT1080 cells was performed using a cationic transfection reagent, Trans-IT LTI (Mirus, Madison, WI), according to the manufacturer’s instructions. For examination of the results of transient transfections, cells were subjected to assays 24 h after transfection. Stable transfectants were selected by culture for 3 weeks in medium that contained puromycin (2.5 μg/ml).
Fig. 1. Chemotaxis assay for gene discovery using randomized hammerhead ribozymes. A, schematic representation of the assay. In B, hammerhead ribozymes are catalytic RNA molecules that bind to target RNA molecules via Watson-Crick base pairing. They cleave their targets enzymatically. The recognition arms of the ribozyme library are randomized. The substrate RNAs should contain NUX triplet (where N and X represent A,U,G,C and A,U,C) for cleavage by hammerhead ribozymes (36, 37). In C, randomized ribozymes were cloned into the plasmid vector pPUR, which carries the promoter of a human gene for tRNA Val . This RNA pol III-dependent expression system is suitable for expression of short RNAs, and ribozymes are expressed as tRNA Val -fused RNAs (28). D, optimization of the chemotaxis assay. The numbers of cells remaining in the top well after a 24-h chemotaxis assay are indicated for each chemoattractant. Human HT1080 cells and mouse B16-BL6 melanoma cells were used as invasive cells. BSA was a negative control for chemoattractant. When we used fibronectin, fresh medium and removal of the migrating cells from the membrane at the midpoint of the assay reduced the number of cells remaining in the top well (**).
Assays of Chemotaxis and Invasion. Cell migration assays were performed using 12-μm-pore Transwell inserts (Costar, Cambridge, MA). By contrast, 8-μm-pore inserts were used for confirmatory assays. Invasion assays were performed with a Cell Invasion Assay Kit (Chemicon, Temecula, CA) as described elsewhere (29). When cells reached 60–70% confluence, they were collected, washed twice with PBS, and suspended in RPMI 1640 plus 0.1% BSA (Sigma) at 2 × 10^5 cells/ml for assays of cell migration. We seeded 1 × 10^5 cells in each transwell insert and performed the invasion assay according to the manufacturer’s instructions.

Plasmid Rescue. The recovery of plasmid DNA from cells that did not migrate to the bottom well was performed by alkaline lysis with SDS (Qiagen GmbH, Hilden, Germany). The resultant plasmid DNAs were introduced into competent E. coli DH5α cells. Colonies appearing on plates were counted and picked up for the determination of sequences of ribozymes.

Immunofluorescence Staining and Microscopy. To visualize the actin cytoskeleton, treated cells were rinsed with PBS and fixed with 3.7% formaldehyde in PBS for 30 min. Cells were rinsed again with PBS and then treated with 100% methanol at −20°C for 30 min. After washing with PBS, cells were labeled with a preparation of FITC-phalloidin (kindly provided by Dr. Nagasaki, Advanced Industrial Science and Technology, Tsukuba, Japan). Phase-contrast and fluorescent images were recorded with an LSM510-V2.01 system (Carl Zeiss, Jena, Germany).

Preparation and Analysis of Cell Extract. Cell lysates were prepared by lysing plated monolayers of cells with lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS], supplemented with a protease inhibitor cocktail (Complete Mini; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. Aliquots of 30 μg of total protein were fractionated on an SDS-polyacrylamide (7.5%) gel, and bands of protein were transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan). Antibodies used were as follows: rabbit polyclonal antibodies against ROCK1, ROCK2, actin (all from Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish peroxidase-linked antibodies against rabbit IgG raised in donkey (Amersham Pharmacia, Piscatway, NJ). Immunoreactive bands were detected with enhanced chemiluminescence plus reagent (Amersham Pharmacia) according to the manufacturer’s instructions.

Assays of Cell Proliferation and Viability. Cells were seeded at 1 × 10^3 cells/well in 96-well optical bottom plates (Nalge Nunc, Rochester, NY). The proliferation and viability of cells were monitored in terms of luminescence with a CellTiter-Glo Assay kit (Promega, Madison, WI) and using a multilabel plate reader (Perkin-Elmer, Turku, Finland).

RESULTS

Identification of Ribozymes that Block Chemotaxis. For the isolation of ribozymes that can inhibit cell migration and identification of the respective target genes, we used the assay system shown in Fig. 1A. First, we treated highly invasive cancer cells with the library of randomized hammerhead ribozymes that had been constructed as shown in Fig. 1, B and C. The treated cells were then subjected to the chemotaxis assay. Most cells migrated toward the chemoattractant, moving from the top well to the bottom well, which contained the chemoattractant. Cells that did not migrate as a result of the inhibitory effects of ribozymes were collected from the top well, and ribozymes were recovered from these cells. The ribozymes that inhibited cell migration were sequenced to allow identification of the genes whose mRNAs had, apparently, bound to the ribozymes via Watson-Crick base pairing and had been cleaved (Fig. 1B) by a DNA sequence database search. A new population of cells was then subjected to the assay after introduction of the newly identified ribozymes to validate their effects.

Optimization of the Assay. In the assay shown in Fig. 1A, cells of interest might be mixed with cells that remain in the top well independently of the effects of ribozymes. We examined how many untreated cells remained in the top well during a 24-h assay, using invasive human fibrosarcoma HT1080 cells and mouse B16-BL6 melanoma cells. We tested fibronectin, fetal bovine serum, and conditioned medium from cultures of NIH3T3 cells as chemoattractants (23). HT1080 cells migrated more efficiently than B16-BL6 toward each chemoattractant (Fig. 1D). Moreover, replacement of the medium in the bottom well and removal of migrated cells from the membrane with a swab at the midpoint of the assay reduced the number of cells that remained in the top well (Fig. 1D, right, column 7). Because cells that had migrated to the bottom well could return to
the top well by random migration known as chemokinesis (30), the removal of migrated cells at the midpoint of the assay might have reduced the number of nonmigrating cells recorded at the end of the assay. This was suitable for our purposes because of the very low background effect attributable to nonmigrating cells. All subsequent assays, therefore, were carried out under these conditions with HT1080 cells and fibronectin.

Recovery of Ribozymes from Nonmigrating Cells. HT1080 cells treated with a plasmid library of randomized ribozymes were subjected to the chemotaxis assay. After a 24-h incubation, we isolated plasmid DNA from nonmigrating cells that remained in the top well. We then introduced the isolated plasmid DNA directly into competent E. coli cells. The colonies that appeared after antibiotic selection were counted (Fig. 2A). Initially, 141 clones were isolated from cells that had been treated with the library of tRNAVal-fused ribozymes [ribozyme (+)]. By contrast, only 20 clones were obtained after cells had been treated with tRNAVal expression plasmids that lacked a ribozyme sequence [ribozyme (−)], which we used as negative controls. The number of ribozyme (+) clones obtained in the assay increased during our confirmatory assays, and we obtained 924 clones in the third round of selection (Fig. 2A). The number of ribozyme (−) clones did not change significantly. Our results indicated that plasmids that encoded the active ribozymes could be selected in a chemotaxis assay. The assay also allowed us to concentrate these plasmid DNAs as the number of rounds of the assay increased.

Morphological Changes of Ribozyme-treated Cells. Cell migration is strictly regulated by reorganization of the actin cytoskeleton and focal adhesion (31, 32). Previous studies of metastasis revealed that strongly and weakly invasive cells differ in terms of morphology (5) and suggested that such differences might be attributable, at least in part, to differences in motile activity, in particular, the reorganization of the actin cytoskeleton, e.g., the dominant-inhibitory Rho (one of the regulators of the actin cytoskeleton) mutant suppressed an elongated morphology of human A375 melanoma cells (5). Because reorganization of the actin cytoskeleton generates intracellular tension, invasive cells might tend to be more elongated than noninvasive cells. Thus, we postulate that such a morphological change can be one of the indicators of the changes in adhesiveness and the actin cytoskeleton. HT1080 cells are usually extended, long, and narrow, as were our wild-type cells and even the cells after transfection with the first ribozyme library (Fig. 2B). Cells that appeared somewhat shrunken were observed among elongated cells after transfection with the ribozymes selected in the first round. The population of shrunken cells was clearly greater after the third round of selection than after the first. These observations suggested ribozymes that inhibited cell migration were selected and concentrated as the number of selections increased.

Isolation of Ribozymes that Cleaved ROCK1 mRNA and Their Effects. We isolated and sequenced the ribozymes obtained during the third round of selection. The sequences of the recognition arms of a ribozyme should be complementary to its specific substrate RNA; in this case, the mRNA that is responsible for cell migration. Among candidate ribozymes selected in our assay, we identified two independent ribozymes that apparently cleaved ROCK1 mRNA at different sites (Fig. 3A and B). ROCK1, as the target gene of two ribozymes, was identified in human DNA databases using the BLAST program with the parameters set automatically to optimize for searching with short sequences (search program for short nearly exact sequences) with the setting of EXPECT threshold = 100 and LIMITATION
Fig. 5. Inhibition of cell migration by the ribozymes toward ROCK1. A, inhibition by ribozymes of migration was correlated with the presence of ribozyme-directed ROCK1 mRNA. HT1080 cells that expressed ROCK1 ribozyme107, ROCK1 ribozyme303, or tRNA<sup>V<sub>I</sub></sup> as a control, were allowed to migrate toward fibronectin for 24 h. In B, migrating cells on the microporous membrane were visualized by Giemsa staining. Scale bar, 200 μm.

Moreover, several redundant pathways or proteins might exist to safeguard the essential processes of cytokinesis and proliferation.

DISCUSSION

To our knowledge, this study provides the first example of use of a chemotaxis assay and a library of randomized ribozymes to identify genes responsible for cell migration. We isolated two independent ribozymes that target ROCK1 mRNA. As noted above, ROCK1 is one of the regulators of the actin cytoskeleton and appears to modulate signals for regulation of the actin cytoskeleton from Rho (a member of the Ras-related family of low molecular weight GTPases) through LIM kinase to cofilin, which can depolymerize actin filaments and reorganize the cytoskeleton (33). Ribozymes against ROCK1 mRNA inhibited cell migration and invasion but did not affect cell proliferation. It remains to be determined whether regulation of the organization of actin by ROCK1 in cytokinesis is distinct from the role of ROCK1 in migration. In our preliminary study, a ribozyme toward LIM kinase inhibited cell migration but not proliferation of invasive HT1080 cells. Therefore, ROCK1 and LIM kinase might play important roles in cell migration but not in cytokinesis. In addition, we can postulate that ROCK1 is more important for cell migration than ROCK2 by the fact that we isolated ribozymes toward ROCK1 by the assay and that ribozymes toward ROCK2 were not identified. We plan to construct expression vectors for ribozymes toward ROCK2 to investigate the effects of the ribozymes on cell migration and invasion. Additional investigations using ribozymes toward ROCK2 will

terms; Homo sapiens and cds (coding DNA sequence). EXPECT threshold is a statistical parameter that regulates the stringency of the database search. As a result of searching with the parameter settings mentioned above, the sequences of the target of the ribozymes were not found in other expressed genes except ROCK1. ROCK is considered to be a regulator of the reorganization of the actin cytoskeleton (25). Therefore, it seems reasonable that our method allowed us to isolate and identify ribozymes that cleaved ROCK1 mRNA.

Next we checked if the ribozymes toward ROCK1 mRNA could indeed cleave ROCK1 mRNA. An examination by RT-PCR<sup>3</sup> and immunoblotting analysis indicated clearly that the two ribozymes specifically cleaved ROCK1 mRNA and reduced the level of expression of ROCK1 itself (Fig. 4A and B). The level of expression of the ROCK2 gene, whose full-length nucleotide sequence is similar to that of ROCK1, did not decrease at all. The ribozymes toward ROCK1 mRNA specifically recognized and cleaved their cognate substrate, ROCK1 mRNA. Furthermore, we observed the effects of transient expression of the ribozymes toward ROCK1 on the morphology of HT1080 cells. As expected, the expression of the ribozymes led to morphological changes in the cells. The ribozymes suppressed the elongated morphology of HT1080 cells (Fig. 4C).

We also examined whether the two ribozymes were actually responsible for the nonmigratory phenotype of invasive HT1080 cells. As shown in Fig. 5A and B, chemotaxis assays confirmed that the ribozymes inhibited the migration of cells. Moreover, inhibition of cell migration by the ribozymes seemed to be correlated with the reduction in the expression of ROCK1 mRNA in cells. This result indicated the importance of ROCK1 in cell motility.

Effects of the Ribozymes on Invasion and Cell Proliferation.

The identified ribozymes toward ROCK1 inhibited cell migration, but it was not clear that they would affect cell invasion. Invasion requires the migration of cells and the dissolution of the extracellular matrix (29). We postulated that the ribozymes that cleaved ROCK1 mRNA should also inhibit cell invasion because they inhibited cell migration. As a result, not only did these ribozymes inhibit cell migration (Fig. 6A), but the inhibition of cell invasion also seemed to be correlated with a reduction in the expression of ROCK1.

ROCK1 contributes to the reorganization of the actin cytoskeleton, and such a reorganization of actin is required both when cells migrate and when they undergo cytokinesis. Therefore, it is possible that ribozymes that cleave ROCK1 mRNA might also inhibit proliferation.

We found, however, no differences in terms of cell proliferation between ribozyme-treated and nontreated HT1080 cells (Fig. 6B). It is possible that migration might be more sensitive than cytokinesis to a reduction in the level of expression of the ROCK1 protein. Indeed, the ribozymes did not totally eliminate expression of ROCK1 (Fig. 4).

<sup>3</sup>The abbreviations used are: RT-PCR, reverse transcription-PCR.

Fig. 6. The ribozymes inhibited invasion without any effect on proliferation. In A, ROCK1 also regulated invasion by HT1080 cells. Cells used in this assay were identical to those used in the chemotaxis assay for which results are shown in Fig. 4A. In B, cell proliferation (cell number) was monitored in terms of luminescence. ROCK1 ribozymes had no effect on cell proliferation and viability. Each column and bar represents the mean ± SD of results of three independent experiments performed in triplicate.
clarify whether ROCK2 contributes to cell migration and whether ROCK1 and ROCK2 play separate and specialized roles or not. The Rho-ROCK system has been implicated in cell mobility, migration, and metastasis in a variety of cell types, e.g., a specific ROCK inhibitor Y-27632 blocked the invasive activity of rat MM1 hepatoma cells (26). Therefore, we expect that the ROCK1 ribozymes may lead to the inhibition of cell migration in several cancer cell lines and that ribozymes and their alternative inhibitors targeted specifically to ROCK1 mRNA might be useful in cancer therapy as low toxicity compounds that inhibit cell motility without affecting cell viability.

As noted above, migration requires regulation of the organization of the actin cytoskeleton. Indeed, using the chemotaxis assay, we isolated ribozymes against genes that are thought to encode proteins involved in the regulation of the organization of actin, such as ROCK1 as discussed above; myosin-IIXb (34), which acts both as a motor protein and as a GTPase-activating protein for the Rho family; and adducin (35), which is a substrate for protein kinase C and a mediator of the reorganization of actin filaments. The genes for these proteins seem to be targets for inhibition of cell migration, which might lead to the inhibition of metastasis. We also isolated ribozymes whose target genes remain to be identified. Additional investigations of these ribozymes and their target genes should help us to understand more clearly the mechanisms of cell migration and metastasis.

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