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SUPPRESSION OF H-*ras*-MEDIATED TRANSFORMATION IN NIH3T3 CELLS BY A *ras* RIBOZYME

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Abstract—Murine NIH3T3 cells were used to study the effect of ribozymes on H-*ras*-mediated transformation. Parental 3T3 cells were transfected with the activated H-*ras* gene. H-*ras*-transformed cells had altered morphology and increased colony formation in soft agar in contrast to untransfected 3T3 cells. A hammerhead ribozyme (site-specific ribonuclease) designed to cleave codon 12 (GUC) of the activated H-*ras* RNA was expressed in transformed cells. 3T3 clones expressing the *ras* ribozyme displayed decreased expression of activated H-*ras* RNA. The *ras* ribozyme reversed the transformed phenotype to resemble that of untransfected 3T3 cells. Furthermore, 3T3 cells containing the *ras* ribozyme were shown to suppress transformation when they were subsequently transfected with activated H-*ras*. Insertion of a mutant ribozyme largely devoid of cleaving capacity into H-*ras*-transformed cells resulted in smaller reductions in H-*ras* gene expression and colony formation in soft agar when compared with the *ras* ribozyme. Finally, the *ras* ribozyme alone did not perturb normal 3T3 cell growth. This study suggests the possible utility of anti-oncogene ribozymes as suppressors of tumor cell growth as well as inhibitors of cellular transformation.

Key words: oncogenes; activated H-*ras*; mutated ribozymes; cellular transformation

Expression of activated *ras* oncogenes has been shown previously to stimulate proliferation and induce transformation in a variety of cell types [1, 2]. A single point mutation can be responsible for this transforming potential [3]. The *ras* gene product, p21, participates as a molecular switch in the early steps of the signal transduction pathway, leading ultimately to cell growth and differentiation [4, 5]. Activated *ras* oncogenes have also been implicated in the malignant phenotype and are thought to play an important role in tumorigenesis, invasion and metastasis [6–8]. Transfection studies have shown that 3T3 cells can acquire both a tumorigenic phenotype and metastatic ability in nude mice following transfection with activated H-*ras* [9, 10]. The 3T3 cell line has been used to study the reversion of the transformed phenotypes induced by activated H-*ras* genes and to study the transforming process of the recipient cell. One approach has been to micro-inject antibodies for p21 that transiently reverse the neoplastic phenotype in morphologically transformed 3T3 cells [11]. Another approach has been the utilization of antisense *fos* RNA to effect partial reversion of the transformed phenotype of *ras*-transformed 3T3 cells [12]. However, only a partial or transient reversal of tumorigenicity was achieved with these strategies.

Ribozymes are catalytic RNAs that represent another option for the manipulation of gene expression [13]. The class of hammerhead ribozymes, cleaving immediately 3′ to any XUX^{-G} sequence (where X: G, C, A, or U), consists of a conserved catalytic core flanked by sequences complementary to the desired target RNA sequence to confer specificity [14]. Hammerhead ribozymes in mammalian cells have thus far been shown to cleave HIV-1 [15], c-*fos* [16] and H-*ras* [17–20] RNAs by transfection of the plasmids containing the respective DNA sequences encoding the ribozyme. In this study, codon 12 of H-*ras* was the ribozyme target site, and we demonstrate that transfection of 3T3 cells with the ribozyme inhibits subsequent transformation by activated H-*ras*.

MATERIALS AND METHODS

Genes. cDNAs were obtained as follows: pEJ6.6 (H-*ras*), the plasmid containing the human c-H-*ras* activated codon 12, from Dr. C. Shih (Massachusetts Institute of Technology); and human c-H-*ras1* (pT24-C3, No. 41000) and the human c-K-*ras* (No. 41026) plasmids from the American Type Culture Collection (Rockville, MD). cDNAs were isolated as previously described [16, 17, 20]. Synthetic nucleotides and their sequences for the primers used in this study were described previously [17].

RNA sequences. The RNA sequence of the activated H-*ras* gene targeted by the ribozymes spans codons 1696–1714 and is encoded by the following

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‡ Abbreviations: pHβ, plasmid pHβAPr-1 neo; G418, geneticin; Rz, H-*ras* ribozyme; and mRz, mutant ribozyme.

Table 1. Growth characteristics of 3T3 cells

3T3 Cell lines	Ribozyme expression	Generation time*(hr)	[^{3}H]dThd incorporation†	Colonies‡ 10^4 Cells 1%/20%	Colonies‡ 10^5 Cells 1%/20%
1. Vector only	−	34.8	100.0	0/2	0/10
2. *ras*	−	21.6	477.0	16/109	97/211
3. *ras*/Rz	+	33.6	105.0	0/31	9/43
4. *ras*/mRz	+	26.0	244.0	8/102	57/181
5. Rz	+	36.4	100.4	0/6	0/13
6. Rz/*ras*	+	34.4	106.4	1/16	4/47

Subconfluently growing 3T3 cells were obtained from the American Type Culture Collection and transfected by electroporation with either vector only (pHβApr-1) (No. 1), or vector containing H-*ras* (No. 2, 3, 4 and 6), vector containing *ras* ribozyme (Rz) (No. 3, 5 and 6) or a vector containing mutant *ras* ribozyme (mRz) (No. 4). Cells were selected in growth medium containing 500 μg/mL G418 for 4 weeks [16]. Individual G418-resistant colonies were picked, grown and screened for expression of the *ras* ribozyme by the PCR assay [17].

* Proliferation of cells in Dulbecco's modified essential medium (Gibco) supplemented with 10% fetal bovine serum (Sigma) were followed every other day for 7 days by counting the number of cells using a hemocytometer.

† To determine the rate of [^{3}H]dThd incorporation in acid-soluble material, cells (2.5×10^3 cells/35 mm^2 dish) were grown for 48 hr, then incubated for 2 hours with [^{3}H]dThd (10^9 dpm/dish), washed, acid precipitated and counted. The 3T3 cell control (100%) represented 1.5 ($\pm$0.25) fmol/mg DNA/hr.

‡ 3T3 cells were plated at 10^4 and 10^5 cells/35 mm^2 dish in 0.3% agar and 1% to 20% fetal bovine serum. Colonies were counted 9 days later with Giemsa stain as previously described [20, 26]. This data represent the average of three separate experiments.

nucleotides: 5′-GGG CGCC *GUC** GGU GUG GUG GGC-3′ (the * denotes the cleavage site). The hammerhead ribozyme is encoded by: 5′-GCC CAC ACC CUG AUG AGU CCG UGA GGA CGA AAC GGC GCC C-3′. The mutated ribozyme is encoded by: 5′-GCC CAC ACC CUG AUG AGU CCG UGA GGA CGA ACC GGC GCCC-3′. The underlined base represents the A to C mutation yielding the mutant ribozyme.

3T3 Cells. The 3T3 cells were obtained from the American Type Culture Collection. They were grown in Dulbecco's modified essential medium (Gibco, Grand Island, NY) and supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and penicillin-streptomycin (Gibco). 3T3 Cells were plated at 10^4 and 10^5 cells/35 mm^2 dish in 0.3% agar and 1% to 20% fetal bovine serum. Colonies were counted 9 days later with Giemsa stain as previously described [17]. Thymidine uptake studies were used to determine the rate of [^{3}H]dThd incorporation in acid-soluble material. 3T3 Cells (2.5×10^3 cells/35 mm^2 dish) were grown for 48 hr, then incubated for 2 hr with [^{3}H]dThd (10^6 dpm/dish; New England Nuclear, Boston, MA), washed, acid precipitated and counted as previously described [17].

Plasmid construction. pHβ was obtained from Dr. L. Kedes (USC, Los Angeles, CA) [21]. SV40 splicing and polyadenylation sequences are downstream from the multiple cloning site on this plasmid. The H-*ras* ribozyme [17] and the mutant ribozyme [19], prepared from two synthetic single-stranded oligodeoxyribonucleotides, contained a 77-base pair sequence with flanking *Sal* I (BRL) and *Hind* III (BRL) restriction sites in the multiple cloning site.

Transfection studies. Subconfluently growing 3T3 cells were transfected by electroporation according to a protocol provided by IBI (New Haven, CT). Cells were selected in growth medium containing 500 μg/mL geneticin (G418-sulfate, Gibco), and clones were picked up after 4 weeks. Individual G418-resistant colonies were picked up, grown and screened for expression of the H-*ras* ribozyme by the polymerase chain reaction assay as previously described [17]. These clones expressed the *ras* ribozyme for over 12 months.

RNA expression. RNA isolation using the guanidium isothiocyanate method, electrophoresis on horizontal agarose gels, hybridization and densitometric analysis (Ambis, San Diego, CA) were performed as previously described [16]. The probes (25 ng) were labeled with 10^9 dpm/μg DNA (New England Nuclear) by using a random primers DNA labeling system (BRL) [22]. PCR analysis of ribozyme expression followed that of a commercially available protocol (Perkin–Elmer–Cetus, Norwalk, CT) and was performed as previously described [17].

Western blot analysis. For immunoblotting, the BioRad Mini-Protean II gel electrophoresis apparatus and the BioRad ImmunoBlot Assay kit were used according to the specifications of the manufacturer. The H-*ras*, K-*ras* and N-*ras* specific monoclonal antibodies (Ab-1) were obtained from Oncogene Science, Inc. (Manhasset, NY). Purification of the proteins was performed as previously described [23–25]. α-Tubulin antibody (Oncogene Science, Inc.) was used as an intrinsic control.

RESULTS

Previous studies have characterized the optimal

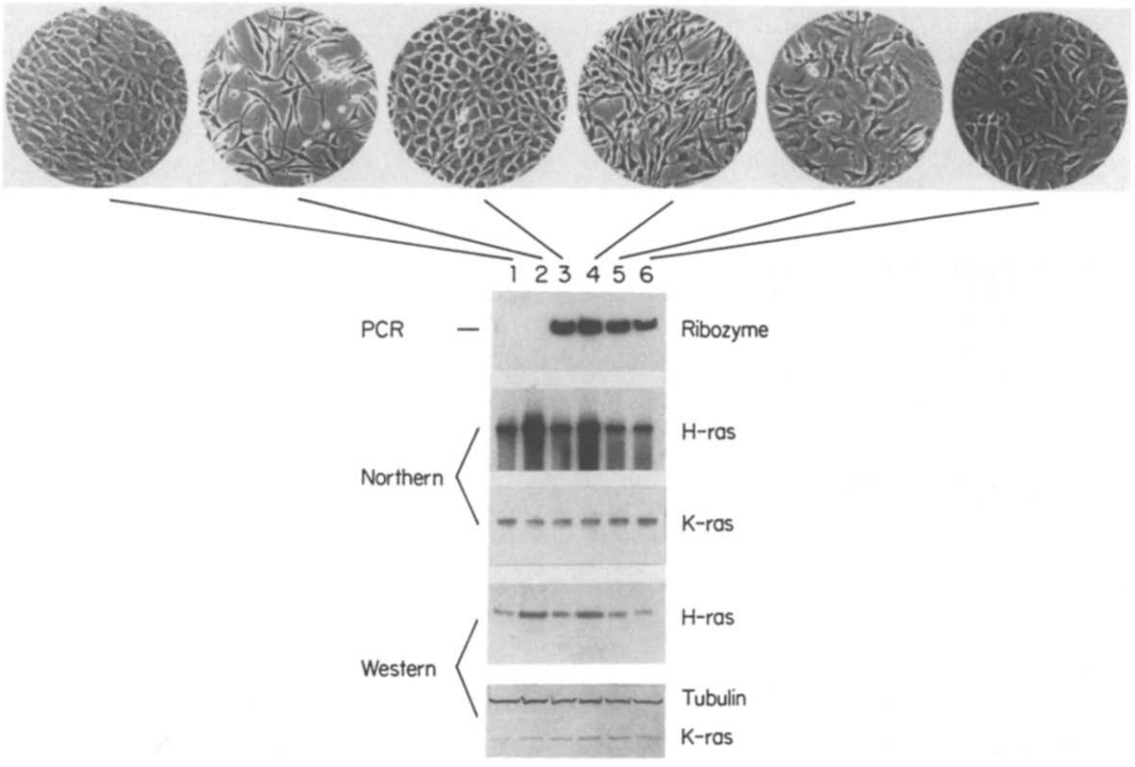

Fig. 1. Morphology of (1) 3T3 vector only cells, (2) 3T3 cells transfected with *ras*, (3) 3T3*ras* cells with Rz, (4) 3T3*ras* cells with mRz, (5) 3T3Rz only cells, and (6) 3T3 Rz cells transfected with *ras* (top panel). Gene and ribozyme expressions were analyzed in the various clones (mRNA 2 μg/lane). The PCR assay yielded a signal at 118 bases. Quantitation of the northern blots was performed by Radioanalytic Imaging Systems (Ambis). The 3T3 vector only cells (lane 1) were used to normalize the data. The values for gene expression are for the following lanes (1–6, respectively): ribozyme RNA (X, X, 2.0, 2.5, 1.8 and 1.0); H-*ras*RNA (1.0, 3.6, 1.1, 2.1, 0.9 and 0.8) and K-*ras* RNA (1.0, 0.8, 0.9, 0.9, 1.0 and 1.1).

conditions for *in vitro* cleavage of H-*ras* RNA by the ribozyme [17, 19]. The pEJ6.6 plasmid containing activated H-*ras* was transfected into 3T3 cells by electroporation yielding 3T3*ras* cells. The nucleotide sequence of codon 12 in the H-*ras* gene was confirmed in the 3T3*ras* cells by dideoxynucleotide sequencing (data not shown). 3T3*ras* Cells had significant changes in morphology, growth characteristics and H-*ras* expression when compared with control clones transfected with the pHβ vector but lacking the ribozyme (3T3 vector only cells) (Table 1, Fig. 1). 3T3*ras* Cells displayed altered morphology (elongated and spindly) (Fig. 1) and showed increased soft agar colony formation with 20% serum in contrast to untransformed 3T3 cells (Table 1). Northern and western blot analyses both showed that the level of H-*ras* expression was increased in transformed 3T3*ras* cells when compared with 3T3 vector only cells (Fig. 1, lanes 1 and 2).

The *ras* ribozyme, cloned into the pHβ vector, was transfected into the *ras*-transformed 3T3 cells (3T3*ras*/Rz). Expression of the ribozyme was demonstrated in isolated G418-resistant clones (3T3*ras*/Rz) by the RNA PCR assay (Fig. 1). The clones with ribozyme vectors were rechallenged with G418 every 3 months to ensure antibiotic resistance, and after 12 months the clones were still stably expressing the transfected gene. In Fig. 1, the clones containing the ribozyme (3T3*ras*/Rz) expressed H-*ras* (lane 3) at levels similar to those of 3T3 cells (lane 1). As a control, c-K-*ras* expression was not altered significantly in all six clones (Fig. 1). All clones expressing the *ras* ribozyme (Fig. 1, lanes 3, 5 and 6; Table 1, No. 3, 5 and 6) displayed growth characteristics and morphology similar to the parent 3T3 cells (Fig. 1, lane 1, and Table 1, No. 1). The generation time of 3T3*ras*/Rz cells (Table 1, No. 3) was substantially longer (1.56 times), ranging from 33.6 hr to 21.6 hr in 3T3*ras* cells (Table 1, No. 2); the plating efficiency was lower in 3T3*ras*/Rz cells at 1% or 20% serum; and the rate of DNA synthesis was also reduced significantly in comparison to 3T3*ras* cells (Table 1). Thus, 3T3*ras*/Rz cells exhibited a longer doubling time *in vitro*, a slower rate of DNA synthesis, and a significant decrease in colony formation in soft agar than that of 3T3*ras* cells.

The mutant ribozyme-containing clone expressed similar levels of ribozyme RNA as the ribozyme-containing clone (Fig. 1, lanes 3 and 4), but there was only a slight decrease in H-*ras* mRNA and p21 in the 3T3*ras*/mRz cells that corresponded to

only modest changes in morphology and growth characteristics. Therefore, 3T3*ras*/mRz cells exhibited morphology, generation time, plating efficiency in soft agar, and [^{3}H]dThd uptake into DNA similar to that of 3T3*ras* cells. The mutant ribozyme did partially reduce H-*ras* mRNA levels by northern analysis (Fig. 1, lane 4). However, it was not as effective as the *ras* ribozyme.

Furthermore, to study the effect of inhibiting the transforming activity of activated H-*ras*, 3T3 cells were transfected with the *ras* ribozyme, and these transformants (3T3Rz) were then transfected with the pEJ6.6 plasmid (3T3Rz/*ras*). With the exception of a longer generation time, 3T3 Rz/*ras* cells (Table 1, No. 6) exhibited growth properties similar to those of the 3T3 cells and 3T3 Rz cells (Table 1, No. 1 and 5). The 3T3 Rz/*ras* clone continued to express the *ras* ribozyme (Fig. 1, lane 6). Activated H-*ras* DNA was present in 3T3 Rz/*ras* clones as determined by Southern analysis (data not shown). The morphology, growth characteristics, and northern and western blotting of 3T3 Rz and 3T3 Rz/*ras* clones were similar to those of 3T3 cells and 3T3*ras*/Rz cells (Table 1, Fig. 1).

DISCUSSION

A ribozyme was directed against activated H-*ras* mRNA to reverse the *ras*-transformed phenotype in 3T3 cells. The levels of H-*ras* mRNA and p21 were predictably decreased in ribozyme-containing transformants as compared with those of transformed 3T3 cells. These decreases were associated with reduced growth parameters of the transformed cells. These results suggest that the *ras* ribozyme acts as a suppressor of *ras*-mediated cell growth *in vitro*. Moreover, the *ras* ribozyme also reversed the morphology of the transformed cells to resemble that of parent 3T3 cells. Thus, the suppression of H-*ras* mRNA and p21 by the ribozyme resulted in the reversal of the transformed phenotype conferred by activated H-*ras*.

Using the same criteria for morphology and growth characteristics, cells containing the *ras* ribozyme also showed suppression of transformation when subsequently transfected with activated *ras*. The H-*ras* ribozyme was shown to cleave activated H-*ras* RNA, with no deleterious effects on normal growth of 3T3 cells. This may have important clinical implications as conventional chemotherapeutic agents are unable to discriminate between normal and neoplastic growth. Although there are dramatic morphological changes with a ribozyme in 3T3*ras*Rz cells, the effects of ribozyme action were limited by the presence of both normal and activated H-*ras* RNA. Thus, the ribozyme could maximally reduce total H-*ras* gene expression by 70%. The 3T3 system is unlike EJ human bladder carcinoma cells, which express only activated H-*ras* RNA [17] and in which more dramatic changes were observed at the molecular and phenotypic level upon ribozyme activation.

This study suggests the possibility of anti-oncogenic ribozymes as tumor suppressors, as well as inhibitors of transformation. This adds ribozymes to the growing list of agents that inhibit transformation and reverse the transformed phenotype, such as antibodies [27], antisense RNA [12, 28, 29], and tumor suppressor genes [30]. In recent reports, antisense oligonucleotides were used against H-*ras* point mutations [31–35]. These studies have demonstrated that antisense molecules targeted against the H-*ras* activated codon 12 are more effective than at other regions of the H-*ras* RNA molecule. Our results showed that the ribozyme had greater efficacy in reducing H-*ras* gene expression and in inhibiting transformed 3T3 cell growth than a mutant ribozyme with little cleavage capacity. Ribozymes may have an advantage over antisense with the potential to operate both as antisense molecules and as catalytic RNAs. Moreover, for antisense molecules to achieve optimal efficacy a molar excess of antisense over target RNA is required. The ribozyme in this study caused greater reductions in H-*ras* RNA than its mutant counterpart despite the presence of more mutant ribozyme RNA in 3T3*ras*/mRz cells. One advantage of using the present system is that the ribozyme acts specifically to cleave mutated codon 12 (GUC) but not normal H-*ras* RNA, leaving normal cell growth unperturbed [17, 36]. These results also parallel studies performed with tumor suppressor genes demonstrating suppression of oncogene-mediated transformation of 3T3 cells [26]. However, they also indicate that the presence of the ribozyme can inhibit subsequent activated H-*ras*-mediated transformation. These findings suggest possible clinical implications for utilizing ribozymes as a distinct class of anticancer agents.

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