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Enzymatic and antisense effects of a specific anti-Ki-*ras* ribozyme *in vitro* and in cell culture

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ABSTRACT

Due to their mode of action, ribozymes show antisense effects in addition to their specific cleavage activity. In the present study we investigated whether a hammerhead ribozyme is capable of cleaving mutated Ki-*ras* mRNA in a pancreatic carcinoma cell line and whether antisense effects contribute to the activity of the ribozyme. A 2'-*O*-allyl modified hammerhead ribozyme was designed to cleave specifically the mutated form of the Ki-*ras* mRNA (GUU motif in codon 12). The activity was monitored by RT–PCR on Ki-*ras* RNA expression by determination of the relative amount of wild type to mutant Ki-*ras* mRNA, by 5-bromo-2'-deoxy-uridine incorporation on cell proliferation and by colony formation in soft agar on malignancy in the human pancreatic adenocarcinoma cell line CFPAC-1, which is heterozygous for the Ki-*ras* mutation. A catalytically inactive ribozyme was used as control to differentiate between antisense and cleavage activity and a ribozyme with random guide sequences as negative control. The catalytically active anti-Ki-*ras* ribozyme was at least 2-fold more potent in decreasing cellular Ki-*ras* mRNA levels, inhibiting cell proliferation and colony formation in soft agar than the catalytically inactive ribozyme. The catalytically active anti-Ki-*ras* ribozyme, but not the catalytically inactive or random ribozyme, increased the ratio of wild type to mutated Ki-*ras* mRNA in CFPAC-1 cells. In conclusion, both cleavage activity and antisense effects contribute to the activity of the catalytically active anti-Ki-*ras* hammerhead ribozyme. Specific ribozymes might be useful in the treatment of pancreatic carcinomas containing an oncogenic GTT mutation in codon 12 of the Ki-*ras* gene.

INTRODUCTION

Antisense oligonucleotides and ribozymes are powerful tools for the specific suppression of gene expression. In contrast to antisense oligonucleotides, ribozymes possess an additional catalytic activity to cleave RNA without the help of an enzyme (1). In most applications the hammerhead-type ribozyme is used. It consists of a catalytic loop and three stems, two of which contain the sequence complementary to the target sequence (2). Hammerhead ribozymes were first recognized as a sequence motif responsible for self-cleavage of satellite RNAs of certain viruses (3). They can cleave complementary substrate RNAs in *cis* and *trans* (4). Cleavage occurs through transesterification which produces RNA with terminal 5'-hydroxyl and 2',3'-cyclic phosphate groups. An important requirement for hammerhead ribozyme-mediated target cleavage is the presence of an NUX-base triplet in the target RNA sequence (N corresponds to any ribonucleotide and X corresponds to A, C and U) adjacent to the 3' cleavage site (5).

The three members of the *ras* gene family (Ha-*ras*, Ki-*ras* and N-*ras*) have been identified as active oncogenes in many different human tumors (6,7). About 90% of human pancreatic carcinomas possess a point mutation in codon 12 of the Ki-*ras* gene, leading to continuous proliferation and transformation by the expression of p21 Ras oncoprotein. Approximately 30% of these Ki-*ras* mutations are G to T transversions in the second position of codon 12. Inhibition of mutated *ras* expression by ribozymes has been shown to suppress the malignant phenotype of Ha-*ras* transformed NIH 3T3 cells (8). To investigate if an anti-Ki-*ras* ribozyme could be used in the treatment of pancreatic cancer, we studied the effects of a 2'-*O*-allyl modified hammerhead ribozyme on Ki-*ras* mRNA expression and cell proliferation. The ribozyme was designed to cleave specifically the mutated form of the Ki-*ras* mRNA with the GUU motif at codon 12. The human pancreatic adenocarcinoma cell line CFPAC-1 is heterozygous for Ki-*ras* and expresses both the wild type (GGU) and the mutated mRNA with the GUU motif at codon 12. This cell line is therefore an excellent model to differentiate between the antisense and cleavage activity of the anti-Ki-*ras* ribozyme.

MATERIALS AND METHODS

Materials

FCS, Iscove's modified Dulbecco's medium, Lipofectin, penicillin, streptomycin, fungizone and reverse transcriptase (Superscript) were obtained from Life Technologies, Inc. (Eggenstein, Germany). The 5-bromo-2'-deoxy-uridine (BrdU) Labeling and Detection Kit III, DOTAP, *Taq* DNA polymerase and RNase inhibitor were from Boehringer Mannheim (Mannheim,

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Germany). The pGEM-T vector PCR cloning system was from Promega (Madison, WI). The Dye Deoxy Terminator Kit was from Applied Biosystems (Weiterstadt, Germany).

Ribozyme synthesis and sequence characteristics

The hammerhead ribozyme, the catalytically inactive ribozyme and a random ribozyme were chemically synthesized (Tib Molbiol, Berlin, Germany). The hammerhead ribozyme and the catalytically inactive ribozyme contain binding arms (8 and 9 nt) to anneal to the Ki-*ras* mRNA and an invariant core sequence (22 nt) required for catalytic activity. The anti-Ki-*ras* hammerhead ribozyme (5'-ccuacgcccu**G**AuGAggccgugag-gccGAAAcagcucca-3') was designed to cleave specifically the mutated form of the Ki-*ras* mRNA with the GUU motif at codon 12. Lowercase letters indicate 2'-*O*-allyl modifications and uppercase characters indicate unmodified ribonucleotides. The catalytically inactive ribozyme (5'-ccuacgcccu**g**AuGAg-gccgugaggccGAAAcagcucca-3') had the same sequence and modifications as the catalytically active ribozyme except for a 2'-*O*-allyl modification in position 5 (bold letter), according to the numbering system for hammerhead ribozymes (9). The random ribozyme (5'-accccuuacu**G**AuGAggccgugaggccGAAG-uccgaccc-3') contained the core sequence of the hammerhead ribozyme, but the binding arms were randomly chosen sequences. All backbone linkages are normal phosphodiesters.

Cell culture

The human pancreatic carcinoma cell line CFPAC-1 (European Collection of Animal Cell Cultures, Salisbury, UK) was used (10). Cells express the wild type and the mutated form of the Ki-*ras* mRNA with a GGU and GUU motif at codon 12, respectively. The cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% FCS and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone). All incubations were carried out at 37°C, 5% CO_2, in humidified atmosphere.

In vitro cleavage assay

Two target RNAs of 281 nt in length were synthesized by T7 polymerase transcription from Ki-*ras* cDNA templates. The RNAs represent the coding region (nucleotide position 1–257) of the Ki-*ras* mRNA with the GGU and GUU motif at codon 12, respectively. Fifty picomoles of the hammerhead ribozyme, catalytically inactive ribozyme or random ribozyme were mixed with 10 pmol of the respective target RNA in a total volume of 10 µl containing 100 mM Tris–HCl, pH 7.5, and 2 mM EDTA. The mixture was heated to 95°C for 10 min and subsequently chilled on ice. The cleavage was started by adding $MgCl_2$ to a final concentration of 10 mM in a reaction volume of 20 µl followed by an incubation at 37°C. Aliquots at time points 0, 2, 4, 16 and 24 h were mixed with formamide, heat denatured at 95°C for 10 min and visualized by denaturing 6% polyacrylamide–urea gel electrophoresis and silver staining. The intensity of the bands was determined densitometrically.

Stability of the ribozymes in cell culture

Hammerhead ribozyme, catalytically inactive ribozyme or random ribozyme (2 µM each) were incubated in a 6-well plate with and without 10^5 CFPAC-1 cells for 0, 1, 2, 3, 4, 5, 6 and 7 days. Aliquots of the supernatant collected at each time point were mixed with formamide, heat denatured at 95°C for 10 min, analyzed by denaturing 12% polyacrylamide–urea gel electrophoresis and visualized by silver staining.

RT–PCR and amplicon analysis

Total RNA was isolated by the acid guanidinium isothiocyanate–phenol–chloroform method from 10^5 CFPAC-1 cells which had been incubated for 48 h with 0.05, 0.5, 1, 2 and 5 µM hammerhead ribozyme, catalytically inactive ribozyme, random ribozyme, or without oligoribonucleotides. Total RNA (0.5 µg) was denatured in the presence of 50 pmol oligo-d$(T)_{16}$ primer at 70°C for 10 min and then chilled on ice. First strand buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.4 mM deoxynucleotide mix, 10 mM DTT, 20 U RNase inhibitor and 200 U Superscript were added to a final volume of 23 µl. The mixture was incubated at 37°C for 45 min. The first strand cDNA was divided into two aliquots and used for two separate PCRs. One PCR was performed with the sense primer (5'-ATGACTGAATATAAACTTGTGGTA-3'), that anneals to position 1–24 of the Ki-*ras* cDNA coding region and the antisense primer (5'-TGTCTTGTCTTTGCTGAT-GTTTCA-3'), that anneals to position 426–449 of the Ki-*ras* cDNA coding region. This primer set generated a 449 bp RT–PCR product which was designed to detect Ki-*ras* mRNA including the ribozyme binding region. This amplicon is called the 'long' amplicon. The other RT–PCR generated a product of 338 bp and was designed to detect Ki-*ras* mRNA excluding the ribozyme binding site. The primer set consisted of the sense-112 (5'-GATTCCTACAGGAAGCAAGTAGTA-3') annealing to position 112–135 of the Ki-*ras* cDNA coding region) and the antisense primer (5'-TGTCTTGTCTTTGCTGATGTTTCA-3'). This amplicon is called the 'short' amplicon. Specific cleavage of the mutated Ki-*ras* mRNA by the catalytically active ribozyme should preclude RT–PCR amplification from position 1 to 449 ('long' amplicon), but not from position 112 to 449 ('short' amplicon). Due to the loss of the cap-structure, however, the cleaved Ki-*ras* mRNA will rapidly be degraded. Thus, ribozyme-mediated cleavage of the Ki-*ras* mRNA will specifically and primarily affect the amplification of the 'long' Ki-*ras* RT–PCR product, but indirectly and to a lesser extent also the amplification of the 'short' Ki-*ras* RT–PCR product.

The PCR mix contained 11 µl of the RT reaction mixture, 21 µl water, 5 µl 10× PCR-buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl), 1.6 mM $MgCl_2$, 5 pmol sense or sense-112 primer, 5 pmol antisense primer and 1 U *Taq* DNA polymerase in a final volume of 50 µl. PCR was run for 40 cycles (30 s at 95°C, 30 s at 55°C and 30 s at 72°C). The RT–PCR products were visualized on a 3% agarose gel stained by ethidium bromide. The intensity of the bands was determined densitometrically. The Ki-*ras* RT–PCR achieved a sensitivity of 100 RNA molecules.

To disclose unspecific effects of the ribozymes, an actin-specific RT–PCR was performed. The sequences of the actin primers were: actin-sense (5'-GCCGCGCTCGTCGTCGACA-ACGGC-3') and actin-antisense (5'-TTCTCCATGTCGTCC-CAGTTGGTG-3'). All other experimental conditions were identical to those for amplification of Ki-*ras*. The RT–PCR amplification of the 'short' and 'long' amplicon of Ki-*ras* mRNA was investigated in the absence and presence of increasing amounts of catalytically active, catalytically inactive or random ribozyme. Neither ribozyme inhibited the RT–PCR at concentrations applied in the cell culture assays, excluding unspecific inhibition of the RT–PCR.

To determine the relative amount of wild type to mutant RNA, the 449-bp amplicons were cloned and sequenced before and after treatment of CFPAC-1 cells with the ribozymes (pGEM-T Kit, according to the manufacturer's instructions). Sequencing was performed by an automat (Applied Biosystems 373 A DNA Sequencer, Weiterstadt, Germany) using the PCR-primers for the 'long' amplicon according to the instructions of the Dye Deoxy Terminator protocol.

Cell proliferation assay

Cells were seeded in 96-well plates (1000 cells per well) in 100 µl complete cell culture medium. After preincubation for 3 days, the medium was replaced and the cells were incubated for 24 and 48 h with hammerhead ribozyme, catalytically inactive ribozyme, random ribozyme, or without oligoribonucleotides in quadruplicate, respectively. The proliferation assay was performed with the BrdU Labeling and Detection Kit III according to the manufacturer's manual. 5-bromo-2'-deoxyuridine was added and after 3 h the optical density of the incorporated dye was measured at 405 nm.

Cationic lipid-mediated uptake of the ribozymes

To investigate the benefit of cationic lipid mediated uptake of the ribozymes, CFPAC-1 cells were incubated with 0.88 µg DOTAP and 0.88 µg (corresponding to 0.5 µM) of the ribozymes or genomic, ultrasonic fragmentated DNA as a control for 24 or 48 h, respectively. According to the instructions of the manufacturer, we have incubated the cells with DOTAP for 3 h without FCS and added serum only for the remaining incubation period. The DOTAP concentration was chosen according to previous studies (11). In addition, we complexed ribozymes with DOTAP in ratios of 4:1 to 1:4. We found that a ratio above 1:1 was toxic to the cells, while the effects of the anti-Ki-*ras* ribozymes on cell proliferation were not influenced by the addition of DOTAP in ratios from 4:1 to 1:1.

Colony formation in soft agar

Untreated cells, cells treated with 2 µM catalytically active, catalytically inactive or random ribozyme were seeded in duplicate in 24-well plates (1000 cells per well) in 0.33% soft agar and layered on top of a 0.5% soft agar base layer with complete cell culture medium. After the first and second week, 200 µl of complete medium with or without 2 µM catalytically active, catalytically inactive or random ribozyme, respectively, was added. After 3 weeks, single cells and colonies were counted and the ratios between single cells and colonies determined. Data are expressed as mean ±SEM and compared by Fisher's exact test.

RESULTS

The 2'-*O*-allyl modified anti-Ki-*ras* hammerhead ribozyme was designed to cleave the mutant Ki-*ras* mRNA with a GUU motif at codon 12, but not wild type Ki-*ras* mRNA which has a GGU motif at this position. The catalytically inactive ribozyme has the same sequence and modifications as the catalytically active ribozyme except for a 2'-*O*-allyl modification of the ribonucleotide in position 5. Through this modification the catalytically inactive ribozyme retains antisense properties. The random ribozyme as a negative control has the core sequence of the hammerhead ribozyme, but random sequences as binding arms.

In vitro cleavage assay

An *in vitro* cleavage assay was performed to confirm the catalytic activity of the hammerhead ribozyme and to exclude cleavage activity of the catalytically inactive and the random ribozyme. Catalytically active, catalytically inactive and random ribozymes were incubated with the *in vitro* transcribed wild type and mutated target Ki-*ras* RNA which represented the coding region from position 1 to 257 of the Ki-*ras* cDNA. The hammerhead ribozyme cleaved the mutated, but not wild type Ki-*ras* target RNA (Fig. 1). A large proportion of mutated target RNA (~50%) was still intact after an incubation for 24 h, indicating a relatively low *in vitro* cleavage activity of the hammerhead ribozyme (12). The catalytically inactive ribozyme and the random ribozyme showed no detectable cleavage activity (Fig. 1).

Stability of the ribozymes in cell culture

It has been shown that 2'-*O*-allyl modified hammerhead ribozymes are resistant to nuclease degradation in cell culture systems (13). To investigate the stability of our oligoribonucleotides, the catalytically active anti-Ki-*ras* ribozyme, the catalytically inactive ribozyme and the random ribozyme were incubated with or without CFPAC-1 cells, respectively, in complete cell culture medium (including 10% non-heat inactivated FCS) for up to 7 days. In each case no degradation of the ribozymes was observed after incubation for up to 3 days (data not shown). At least 60% of the ribozymes were still intact after an incubation for 7 days. In contrast, unmodified ribozymes have a half-life of only ~6 s (14).

Ki-*ras* mRNA analysis

RT–PCR analysis of Ki-*ras* mRNA was performed to investigate the effects of the ribozymes on the Ki-*ras* mRNA level in CFPAC-1 cells. Two different RT–PCRs were performed, which generated either a 'long' amplicon including the ribozyme hybridization and cleavage site, or a 'short' amplicon of the 3'-end of the 'long' amplicon which lacks the hybridization site (Fig. 2A). This RT–PCR design serves as a model to show specific activity of the ribozymes (Materials and Methods).

As illustrated in Figure 2B and C, the levels of the Ki-*ras* mRNA of both the 'long' and the 'short' amplicon decreased in cells treated with catalytically active or catalytically inactive ribozyme in a concentration-dependent manner over a range of 0.05–5 µM. The generation of the 'long' amplicon was more sensitive to inhibition by ribozyme treatment than the 'short' amplicon. Complete inhibition of the long Ki-*ras* mRNA amplification was observed at 2 µM (catalytically active ribozyme) and 5 µM (catalytically inactive ribozyme), indicating suppression of both mutant and wild type Ki-*ras* mRNA expression. The random ribozyme revealed no inhibition of Ki-*ras* mRNA amplification. As further control, actin RT–PCR was performed and showed equal amounts of amplicons in all reactions, excluding unspecific effects of the ribozymes.

The ratio of wild type and mutated form of the Ki-*ras* mRNA in CFPAC-1 cells was determined by cloning of the Ki-*ras* cDNA products generated from RNA of untreated cells and cells treated with 1 µM catalytically active, catalytically inactive

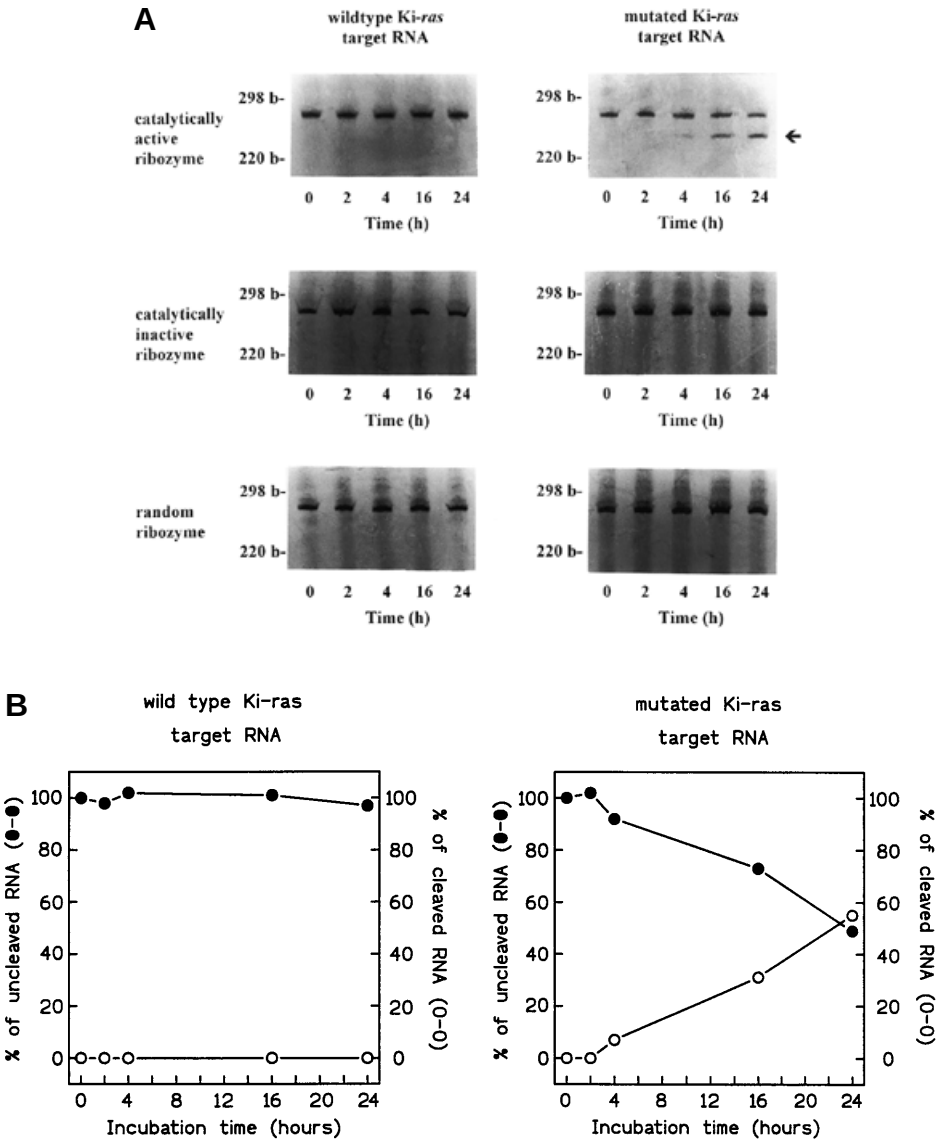

Figure 1. *In vitro* cleavage activity of the catalytically active, catalytically inactive anti-Ki-*ras* ribozyme and a random ribozyme. Fifty picomoles catalytically active, catalytically inactive or random ribozyme were mixed with 10 pmol of wild type or mutant target RNA (position 1–257 of Ki-*ras* mRNA plus 24 nt poly-T tail). The cleavage was started by adding $MgCl_2$ (10 mM) as described in Materials and Methods. Aliquots at time points 0, 2, 4, 16 and 24 h were mixed with formamide, heat denatured at 95°C for 10 min and visualized by denaturing 6% polyacrylamide–urea gel electrophoresis and silver staining (**A**). Cleavage at position 35 results in a band of 246 (arrow) and 35 ribonucleotides (not visualized on the gel). Bands referring to the cleavage activity of the catalytically active ribozyme were quantified by laser densitometry (**B**).

or random ribozyme, respectively. At this concentration the 'long' Ki-*ras* RT–PCR amplicon can still be detected in the presence of all ribozymes (Fig. 2B). From each group, 50 clones were sequenced. Sequence analysis of the Ki-*ras* clones from untreated CFPAC-1 cells showed that 38% of the clones represented wild type Ki-*ras* mRNA, probably due to hyperdiploidy of the cells (65–75 chromosomes) (10). Moreover, cells with multiple mutated Ki-*ras* alleles may have a growth advantage as compared with cells possessing only one mutated Ki-*ras* allele, implying a mutational pressure to gain multiple copies of the mutated allele. As shown in Table 1, wild type sequence of the Ki-*ras* mRNA was obtained in 52, 36 and 36% of cDNA clones derived from cells treated with catalytically active ribozyme, inactive ribozyme or random ribozyme, respectively. Thus, the catalytically active ribozyme, but not the catalytically inactive or random ribozyme, shifted the relation of wild type and mutant Ki-*ras* mRNA towards the wild type mRNA.

Effect of the ribozymes on cell proliferation

When CFPAC-1 cells were incubated with catalytically active or catalytically inactive ribozyme, cell proliferation was inhibited in a dose-dependent manner (Fig. 3). At a concentration of 2 μM the catalytically active ribozyme was ~2-fold more

Table 1. Wild type and mutant Ki-ras mRNAs amplified from untreated cells or cells treated with catalytically active, catalytically inactive or random ribozyme

	Wild type clones (GGU)	Mutant type clones (GUU)	Wild type/ total clones (%)
Untreated control	19	31	38
Catalytically active ribozyme	26	24	52
Catalytically inactive ribozyme	18	32	36
Random ribozyme	18	32	36

CFPAC-1 cells were incubated with catalytically active ribozyme (1 μM), catalytically inactive ribozyme (1 μM), random ribozyme (1 μM) or without oligoribonucleotides for 48 h. Total RNA was extracted from the cells and reverse transcribed into cDNA. The 'long' amplicon of Ki-*ras* mRNA was PCR amplified and the products were cloned. For each condition, 50 clones were sequenced.

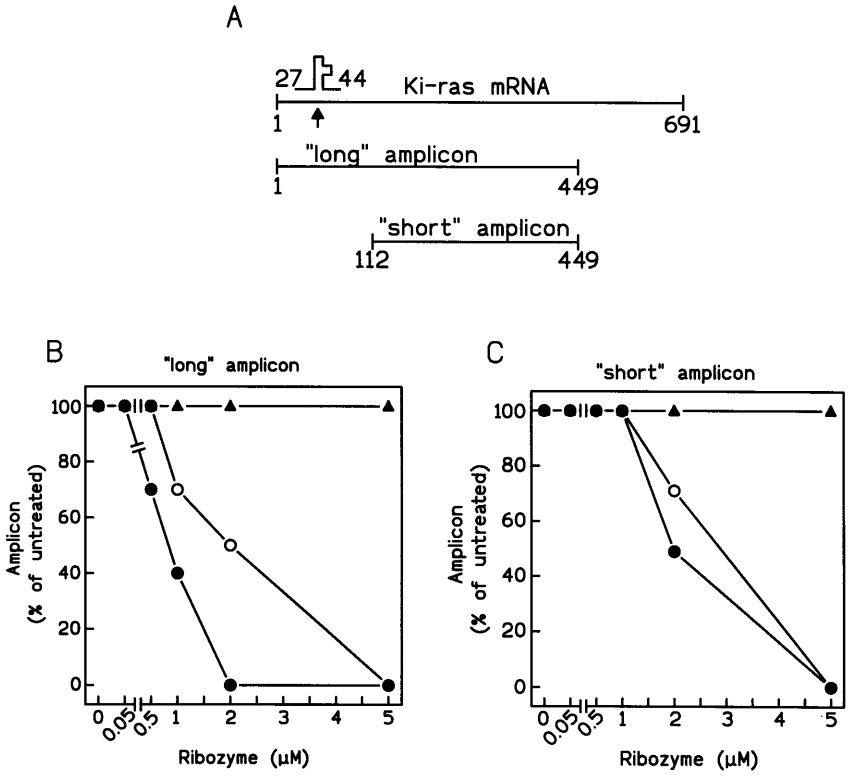

Figure 2. Effect of the catalytically active, catalytically inactive ribozyme and random ribozyme on Ki-*ras* mRNA. CFPAC-1 cells were incubated for 48 h without and with catalytically active ribozyme (closed circles), catalytically inactive ribozyme (open circles) or random ribozymes (triangles), respectively. Total RNA was extracted from the cells and reverse transcribed into cDNA as described in Materials and Methods. As illustrated in (**A**) two different PCRs were performed generating either a 'long' amplicon including the ribozyme hybridization and cleavage site (arrow tip) or a 'short' amplicon excluding the ribozyme hybridization site. 'Long' and 'short' amplicons were separated on agarose gels and bands subsequently analyzed by laser densitometry (**B** and **C**). The data shown are representative for two independent experiments.

potent than the catalytically inactive ribozyme. The dose–response curves of the catalytically active and catalytically inactive ribozyme on cell proliferation were shifted to higher concentrations after incubation for 48 h compared with the 24 h assay (Fig. 3A and B). The random ribozyme in concentrations up to 5 μM showed no effects on cell proliferation.

Cationic lipids have been described to facilitate the uptake of oligonucleotides into cells (15). To investigate if DOTAP increase the potency of the catalytically active or catalytically inactive ribozymes, we examined the effect of this cationic lipid on cell proliferation at the threshold concentration of the oligoribonucleotides (0.5 μM). At a ratio of 1:1 DOTAP itself

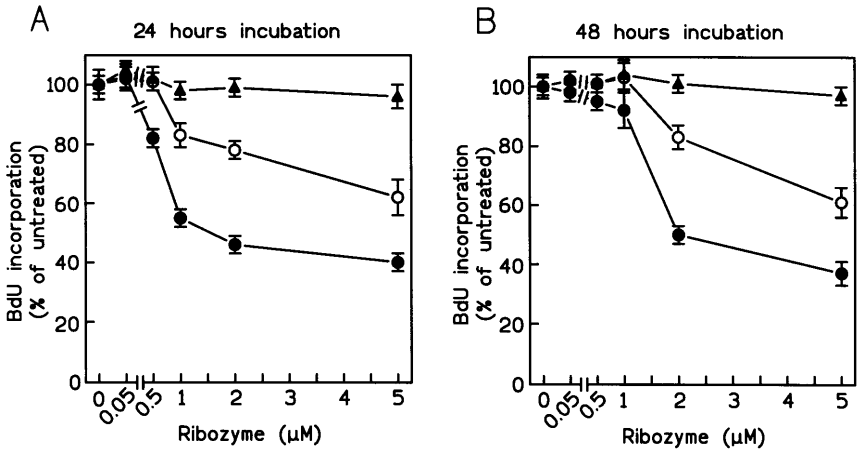

Figure 3. Dose–response curve for the effect of the ribozymes on cell proliferation. After preincubation for 3 days, CFPAC-1 cells were incubated without and with increasing concentrations of catalytically active (closed circles), catalytically inactive (open circles) and random ribozyme (triangles) for 24 and 48 h, respectively. Then, proliferation was measured by BrdU incorporation into synthesized DNA as described in Materials and Methods. The data shown are mean ±SEM of four to six independent experiments.

Table 2. Effect of catalytically active, catalytically inactive or random ribozyme on colony formation in soft agar

	Single cells	Colonies	Colonies/ (single cells + colonies) (%)
Untreated control	89 ± 5	411 ± 5	82
Catalytically active ribozyme	269 ± 7	231 ± 7	46
Catalytically inactive ribozyme	147 ± 12	353 ± 12	71
Random ribozyme	97 ± 8	403 ± 8	81

Untreated cells and cells treated with 2 μM catalytically active, catalytically inactive or random ribozyme were seeded in duplicate in 24-well plates (1000 cells per well) in 0.33% soft agar, layered on top of a 0.5% soft agar base layer with complete cell culture medium. After the first and the second week, 200 μl of complete medium with or without 2 μM catalytically active, catalytically inactive or random ribozyme, respectively, were added. After 3 weeks the ratio between single cells and colonies was determined. Results are means ±SEM of three independent experiments.

inhibited cell proliferation by ~20%. Higher DOTAP concentrations were toxic to the cells, while the effects of the anti-Ki-*ras* ribozymes on cell proliferation were not influenced by the addition of DOTAP in ratios from 4:1 to 1:1 (data not shown). Another cationic lipid, Lipofectin, inhibited cell proliferation completely after 48 h (data not shown). Thus, the use of DOTAP and Lipofectin did not improve the effectiveness of the ribozymes in CFPAC-1 cells under the applied experimental conditions.

Colony formation in soft agar

We next investigated whether inhibition of expression of Ki-*ras* oncogene results in a less malignant phenotype of the CFPAC-1 cells. Therefore, we studied the effect of the ribozymes on colony formation of CFPAC-1 cells in soft agar. A ribozyme concentration of 2 μM was chosen because at this concentration the 'long' amplicon of Ki-*ras* mRNA could not be amplified from cells treated with the catalytically active ribozyme (Fig. 2B). From 1000 cells that had been initially seeded, a total of 500 single cells/colonies were counted after an incubation for 3 weeks. Colony formation was found in 82% of untreated cells, in 81% of cells treated with the random ribozyme, and in 71 and 46% of cells treated with the catalytically inactive or catalytically active ribozyme, respectively ($P < 0.0001$ for untreated cells versus cells treated with catalytically inactive and catalytically active ribozyme, respectively; Table 2). Thus, similar to the effect on Ki-*ras* expression and cell proliferation, the catalytically active ribozyme was more potent than the catalytically inactive ribozyme.

DISCUSSION

Hammerhead ribozymes possess catalytic activity in addition to antisense effects, the latter being defined as hybridization of the oligoribonucleotide with the target RNA leading to translation arrest or degradation of the target mRNA through dsRNases (16). Earlier studies investigating the properties of ribozymes did not carefully differentiate and quantitate the antisense and

catalytic effects (17,18). Therefore, the aims of the present study were (i) to demonstrate that the anti-Ki-*ras* hammerhead ribozyme cleaves the target mRNA in cultured pancreatic carcinoma cells and (ii) to quantitate the ratio of antisense and cleavage activity. The relationship between antisense and cleavage activity is important with respect to a complete inhibition of oncogene expression without influencing the expression of the wild type Ki-*ras* mRNA. CFPAC-1 cells express both wild type and mutated Ki-*ras* mRNA with a GUU mutation in codon 12 and are therefore well suited to differentiate between cleavage and antisense activity of a ribozyme that specifically cleaves mutant Ki-*ras* mRNA.

Ki-*ras* mutations in codon 12 are observed in ~90% of human pancreatic carcinomas. The frequency of yet published mutations are GAU (47%), GUU (27%), CGU (15%), UGU (9%), AGU (1%) and GCU (1%) (19). Hammerhead ribozymes require an NUX motif (N corresponds to any ribonucleotide and X to A, C or U) in the target RNA for catalytic cleavage activity. Since the first position of codon 13 in Ki-*ras* gene contains a guanine, only the GUU mutation in codon 12 can be approached by a hammerhead ribozyme specifically designed to cleave oncogenic Ki-*ras* mRNA. Among the different sequences fulfilling the required NUX cleavage motif for hammerhead ribozymes GUC appears to be the optimal triplet (20). *In vitro* the efficacy of cleavage of the GUC motif is generally higher compared with the cleavage efficiency of the GUU motif (5,12,21). Differences in cleavage activity of ribozymes can also be related to different flanking sequences and/or different secondary structures of the target RNAs (17,22). The *in vitro* catalytic activity of ribozymes, however, might be different from *in vivo* experiments in cultured cells (23). For instance, it has been reported that a ribozyme that showed a low catalytic activity *in vitro* effectively degraded its target RNA in a cell culture system (24). Most, if not all, RNAs normally exist *in vivo* as ribonucleoprotein (RNP) complexes and not as free molecules. Such RNP complexes may inhibit ribozyme activity by steric hindrance or, alternatively, enhance activity via annealing and strand-exchange activities. Furthermore, RNA hybridization may require 'chaperones' in the sense of hybrid promoting proteins or RNA helicases, which can dissolve intramolecular structures in favor of intermolecular structures (25).

This study provides several lines of evidence that the catalytically active ribozyme cleaves the target RNA in a cell culture system. (i) The catalytically active ribozyme, but not the catalytically inactive or the random ribozyme shifted the relation between wild type and mutated Ki-*ras* mRNA towards wild type mRNA. (ii) The catalytically active ribozyme was ~2-fold more potent than the catalytically inactive ribozyme to decrease expression of Ki-*ras* mRNA and to inhibit cell proliferation and colony formation in soft agar.

The dose–response curves for the effects of the catalytically active and catalytically inactive anti-Ki-*ras* ribozymes on cell proliferation were shifted to higher concentrations after incubation for 48 h compared with 24 h incubation. It remains speculative whether this observation is due to an adaptation of the cells in the presence of the ribozymes (e.g. activation of alternative signal transduction pathways) or due to intracellular degradation of the ribozymes. The latter explanation, however, appears less likely because (i) the stability of the ribozymes in cell culture medium with non-heat-inactivated FCS was high with a half-life of ~7 days and (ii) Ki-*ras* mRNA was undetectable after a 48 h incubation with a catalytically active ribozyme at a concentration of ≥2 μM.

In a previous study, a 2'-*O*-allyl modified ribozyme and a 2'-*O*-allyl modified antisense oligoribonucleotide designed to degrade amelogenin mRNA were injected into the proximity of the developing mandibular molar teeth in newborn mice and resulted in a prolonged and specific arrest of amelogenin synthesis (26). In that study the antisense oligoribonucleotide was 80% as effective as the hammerhead ribozyme, but its effect was of shorter duration. A hammerhead ribozyme designed to degrade mutant Ha-*ras* mRNA has been shown to suppress the transformed phenotype of NIH 3T3 cells (27). In another study, the effects of phosphorothioate-modified antisense oligonucleotides directed against Ki-*ras* mRNA were investigated in the human urinary bladder carcinoma cell line T24 and were found to reduce the level of Ki-*ras* mRNA as well as cell proliferation (28). However, phosphorothioate-modified oligonucleotides are especially prone to cause unspecific effects by binding to various proteins (29–31). Although some of these side effects will almost certainly have clinical value, they make it hard to produce drugs that act primarily through true antisense mechanisms and complicate the use of phosphorothioate-modified antisense oligonucleotides as research reagents (23). We have also studied phosphorothioate-modified antisense oligonucleotides in CFPAC-1 cells, and could not observe specific effects on cell proliferation (unpublished data).

It is generally anticipated that chemically synthesized antisense oligoribonucleotides act through translation arrest (32). The data of the present study show that the catalytically active ribozyme is more effective than the catalytically inactive ribozyme. However, both anti-Ki-*ras* ribozymes but not the random control ribozyme lead to degradation of wild type and mutated Ki-*ras* mRNA indicating an indirect RNA degradation process in addition to translation arrest. As shown by the specifically designed RT–PCR assay, both the catalytically active and catalytically inactive ribozyme are more effective in decreasing the levels of the 'long' Ki-*ras* mRNA compared with the 'short' Ki-*ras* mRNA. This indicates that the catalytically active and catalytically inactive ribozyme specifically hybridize at the predicted site, excluding unspecific effects.

Hybridization of the anti-Ki-*ras* ribozymes at the predicted site could lead to degradation of both wild type and mutated Ki-*ras* mRNA by dsRNases. Recently, Wu *et al.* investigated antisense oligoribonucleotides with 2'-*O*-methyl modifications targeted to point mutated Ha-*ras* mRNA and found a dose-dependent reduction of the Ha-*ras* mRNA (16). Neither single strand nucleases nor mammalian or *Escherichia coli* RNase HI cleaved the RNA duplex. The dsRNase activity resulted in cleavage products with 5'-phosphate and 3'-hydroxyl termini. Wu *et al*. partially purified the dsRNase activity and found an enzyme with a molecular weight of 50 000–65 000 Da. Other enzymes with dsRNase activity have been described and partially purified (33,34).

The anti-Ki-*ras* ribozyme used in the present study was three times more effective in reducing colony formation in soft agar than the catalytically inactive ribozyme. This could be interpreted to mean that the catalytically active ribozyme affects malignancy of CFPAC-1 cells. However, it is also possible that this reflects an increased growth inhibitory effect of the

catalytically active ribozyme compared with the catalytically inactive ribozyme rather than a loss of malignancy. Colony formation was not completely blocked, although the 'long' amplicon of Ki-*ras* mRNA was undetectable in the cells treated with catalytically active ribozyme. Most likely, the activating Ki-*ras* mutation is not the only oncogene causing proliferation and malignant phenotype in CFPAC-1 cells. As has been shown for other tumor cell lines, additional oncogenes and mutated tumor suppressor genes contribute to the malignant phenotype of these cells (35).

Cationic lipids are widely used to facilitate uptake of oligonucleotides into cells. In some (36–38), but not all, systems (39–41) cationic lipids increase the potency of oligonucleotides. In addition, hammerhead ribozymes may enter cells by endocytic mechanisms similar to that reported for a variety of antisense oligonucleotides (42). In the present study, the specific effects of the ribozymes in the absence of cationic lipids suggest efficient transport of the oligoribonucleotides into CFPAC-1 cells. This might be facilitated by the increased hydrophobicity of 2'-*O*-allyl modified ribozymes compared with other modifications.

From the results obtained *in vitro* and in cell culture, we cannot predict the biological consequences when wild type Ki-*ras* mRNA would be affected in humans by a mutation-specific therapeutic anti-Ki-*ras* ribozyme. However, in preclinical trials, farnesyltransferase inhibitors, which are designed to inhibit membrane insertion of wild type and mutated Ras proteins, were well tolerated (43).

In conclusion, the results of the present study show that the cleavage activity of the Ki-*ras* directed 2'-*O*-allyl modified catalytically active ribozyme strongly increases the ability to degrade the target RNA as compared with a catalytically inactive ribozyme possessing only antisense effects. Anti-Ki-*ras* hammerhead ribozymes may be a promising tool for the treatment of pancreatic carcinomas with an oncogenic GTT mutation in codon 12 of the Ki-*ras* gene.

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