Biochemistry

 Volume 30, Number 34 August 27, 1991

Accelerated Publications

Antisense Inhibition of *ras* p21 Expression That Is Sensitive to a Point Mutation†

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Received May 24, 1991; Revised Manuscript Received July 8, 1991

ABSTRACT: Many genetic disorders result from a single point mutation, and many tumor oncogenes have been found to be altered by a point mutation. The ability to inhibit selectively the expression of the mutated form of a protein without affecting its normal counterpart is central to many therapeutic strategies, since the normal protein may serve indispensable functions. Antisense oligonucleoside methylphosphonates and their psoralen derivatives directed at either normal human Ha-*ras* p21 or *ras* p21 that is mutated at a single base in codon 61 have been examined for their efficacy and specificity as inhibitors of p21 expression. Mixed cultures of cells expressing both forms of p21 were treated with the antisense oligomer complementary to the normal p21 or with the antisense oligomer complementary to the point-mutated p21. Each of the antisense oligomers specifically inhibited expression of only the form of *ras* p21 to which it was completely complementary and left the other form of p21 virtually unaffected.

ras p21 proteins resemble G proteins in sequence homology and function and have been considered as potential intermediaries in the signal transduction pathway. Point mutations appear to be mainly responsible for the association of *ras* with the initiation and development of many human neoplasias. A point mutation in the 12th or 61st amino acid codon of c-Ha-*ras* has been found in numerous chemical- and radiation-induced tumors as well as in naturally occurring malignant mammalian tumors. These mutations are found to occur in regions of the protein that are GTP/GDP binding domains (Bishop, 1987; Weinberg, 1988; Barbacid, 1987, 1990). Amplification of the *ras* genes and overexpression of the *ras* gene product, p21, have been detected in many human tumors derived from different tissue types (Heighway & Hasleton, 1986).

It has been demonstrated in several animal tumor models that Ha-*ras* genes are the direct targets of chemical carcinogens, such as dimethylbenz[*a*]anthracene (DMBA), *N*-nitroso-*N*-methylurea (NMU), or dibenzacridine (DBACR). A transition of A to T in the second base of codon 61 of Ha-*ras* has been repeatedly observed (Barbacid, 1987, 1990). This same mutation has also been reported in a human lung carcinoma (Yuasa et al., 1983), in a melanoma (Sekiya et al., 1984), and in 30% of human keratoacanthomas (Leon et al., 1988). The A to T nucleotide transition leads to an amino acid change of glutamine to leucine in the protein. This single amino acid change results in a slightly increased gel electrophoretic mobility of *ras* p21 relative to that of its normal counterpart.

Methods to control selectively the expression of the normal or mutated form of the Ha-*ras* oncogene could be of great value in studying the function of p21 in normal and malignant cells and would possibly lead to new therapeutic approaches for the treatment of malignancies involving this gene. In theory the synthesis of normal and mutated p21 could be controlled by antisense oligonucleoside methylphosphonates (ONMPs), which have been shown to be sequence-specific inhibitors of gene expression at the mRNA level in cells in culture (Miller & Ts'o, 1988; Miller, 1989, 1990; Uhlmann & Peyman, 1990).

† This work was supported in part by NIH Grant CA42672, a program project grant to E.H.C., P.S.M., and P.O.P.T., Grant GM74CA from the Strategic Defense Initiative Organization Medical Free Election Laser Program to E.H.C., and Grant HU0001 from the National Foundation for Cancer Research to E.H.C.

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0006-2960/91/0430-8283$02.50/0

These oligonucleotide analogues contain nuclease-resistant methylphosphonate internucleotide linkages and are taken up intact by mammalian cells, possibly by a passive diffusion mechanism (Miller et al., 1979). ONMPs form stable hydrogen-bonded duplexes with complementary nucleic acid sequences with a high degree of base-pairing specificity.

In previous studies we have shown that *ras*-specific ONMPs arrest the synthesis of the encoded p21 (Brown et al., 1989; Yu et al., 1989). For example, an anti-*ras* ONMP complementary to the first 11 nucleotides of the initiation codon region of the Balb-*ras* has been shown to almost completely inhibit p21 translation in a cell-free system at an ONMP concentration of 100 μM (Brown et al., 1989). The specificity of inhibition was evaluated by using two additional ONMPs, whose sequences were identical with that of the first ONMP with the exception of a single or double base mismatch. The inhibitory efficacies of these ONMPs were reduced in proportion to the number of base mismatches.

The ONMP targeted against the first 11 nucleotides of the initiation codon region of Ha-*ras* was also tested in RS485 cells (Brown et al., 1989), a transformed NIH 3T3 cell line that overexpresses the normal human Ha-*ras* p21 (Chang et al., 1982). After treatment with 50 μM ONMP for 60 h, *ras* p21 was reduced more than 90% relative to an untreated control. We have also demonstrated the inhibitory effectiveness of an anti-*ras* ONMP complementary to the splice junction of the first intron 1/exon 2 splice site of Ha-*ras* precursor mRNA. This oligomer inhibits the expression of *ras* p21 in RS485 cells, a cell line that has been transformed with genomic c-Ha-*ras* (Yu et al., 1991).

The inhibitory effects of a series of 8-mer ONMPs whose sequences are complementary to a region including the 12th amino acid codon of normal human c-Ha-*ras* or activated c-Ha-*ras*-EJ have been studied in a cell-free translation system (Yu et al., 1989). Even though normal and mutated c-Ha-*ras* differ by only a single nucleotide, the complementary antisense ONMPs display specific inhibitory activities. Thus the ONMP targeted against the normal c-Ha-*ras* inhibits synthesis of the normal *ras* gene product more efficiently than does the ONMP complementary to the mutated Ha-*ras*. Conversely, the ONMP targeted against the mutated Ha-*ras* mRNA shows greater inhibition of synthesis of the mutated *ras* gene product than of the normal p21. These results demonstrate that the specifics of anti-*ras* ONMPs are sufficiently stringent that the oligomer can discern a single nucleotide change in its nucleic acid target sequence.

Materials and Methods

Synthesis of Underderivatized and Psoralen-Derivatized Anti-ras ONMPs. ONMPs were synthesized on controlled pore glass supports by using suitably protected 5′-(dimethoxytrityl)nucleoside 3′-[*N*,*N*-diisopropylamino)methyl]-phosphonamidite synthons (Agarwal & Goodchild, 1987). The oligomers were deprotected, purified, and phosphorylated as previously described (Miller et al., 1986; Murakami et al., 1985). The phosphorylated oligomers were derivatized with 4′[[*N*-(aminoethyl)amino]methyl]-4,5′,8-trimethylpsoralen as previously described, and the derivatized oligomers were purified by reversed-phase high-performance liquid chromatography (Bhan & Miller, 1990).

Cell Lines. RS 504 cells, a transformed NIH 3T3 line, contain multiple copies of a cloned human c-Ha-*ras* gene that contains a mutation in the 12th amino acid codon. The 61st amino acid codon is normal in this cloned *ras* gene (Tabin et al., 1982). The 453 cell line contains multiple copies of a human c-Ha-*ras* gene that contains a point mutation in the 61st amino acid codon (Yuasa et al., 1982). Both cell lines express elevated levels of p21. The cell lines were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% heat-inactivated calf serum and 50 μg/mL each penicillin, streptomycin, and neomycin.

Biosynthetic Labeling, Immunoprecipitation, and Gel Analysis of ras p21. Cell lysis, immunoprecipitation of 2×10^7 cpm of TCA-precipitable protein, SDS–PAGE, and autoradiography were as described previously [Harford (1984) and see above]. Briefly, 453 and RS504 cells were labeled with 150 μCi/mL [^{35}S]methionine in DMEM, containing 2% dialyzed fetal bovine serum and 10% of the normal amount of methionine, for 6 h. The labeled cells were lysed with ice-cold lysis buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, and 0.1% bovine serum albumin and were made 0.1 mM in phenylmethanesulfonyl fluoride (PMSF), 1% in aprotinin, and 10 μg/mL in leupeptin fresh each time. The lysed cells were centrifuged at 13000*g* for 20 min. The supernatant fluid was removed and stored at −70 °C.

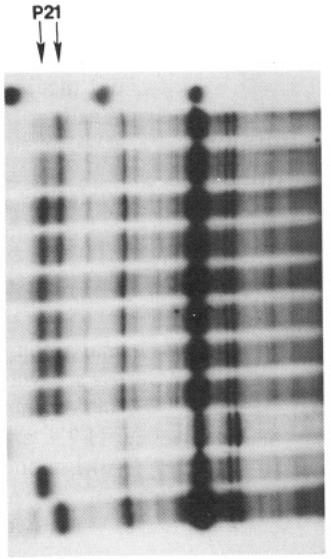
Monoclonal antibody directed to *ras* p21, Y13–259 (Furth et al., 1982) and then protein A-Sepharose beads coated with goat anti-rat IgG (Pharmacia) were sequentially added to the 2×10^7 cpm of TCA-precipitable protein. Immune complexes were washed, eluted, and applied to a 13% acrylamide gel cross-linked with acrylaide (FMC Bioproducts). Electrophoresis was carried out in the buffer system described by Laemmli (1970). After being fixed in methanol, the gels were soaked in Enlightning for 20 min (New England Nuclear), dried, and fluorographed at −70 °C. The extent of inhibition was measured by densitometric scanning of the autoradiogram.

Treatment of RS504 and 453 Cells with Anti-ras ONMPS. A mixture of 453 and RS504 cells in the ratio of 3:1 were seeded in 6-well plates at a total cell density of 2×10^5 cells/well, 48 h prior to treatment with the oligonucleotide. The anti-*ras* ONMP was then added at various concentrations to duplicate wells. The cells were incubated with the oligomer for 48 h to allow for sufficient uptake of the compound. Cells treated with ONMP were labeled with [^{35}S]methionine and the levels of p21 were analyzed as described above. The labeling medium was not supplemented with fresh oligomer. One member of each pair of duplicate wells treated with psoralen-derivatized ONMP was irradiated at 365 nm. Irradiation was carried out by using a laser (Questek 2000 excimer laser) at a frequency of 40 Hz with a power setting of 23 mJ/pulse for 100 s. Following irradiation, the cells were labeled with [^{35}S]methionine and the levels of p21 synthesis were determined.

Results

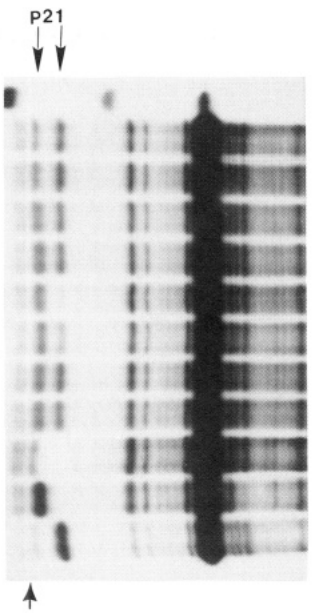
In the present study, we extend our previous observations to cells in culture. Two anti-*ras* 11-mers, 5′ Tp<u>CCTCCTGGCC</u> 3′ (*ras*-T) and 5′ Tp<u>CCTCCAGGCC</u> 3′ (*ras*-A), were synthesized. The underlined bases indicate the position of the methylphosphonate linkages. *ras*-T is completely complementary to a region including the 61st amino acid codon of normal human c-Ha-*ras* (Capon et al., 1983), and *ras*-A is complementary to the same region of a human lung carcinoma c-Ha-*ras*, Hs242 (Yuasa et al., 1983). The predicted secondary structures of these two mRNAs suggest that binding sites for the oligomers are partially single-stranded.

A pair of NIH 3T3 *ras*-transformed cell lines, RS504 and 453, were employed to evaluate the effects of *ras*-T and *ras*-A on p21 synthesis. The 453 line contains multiple copies of the mutated human Ha-*ras* gene, which was isolated from DNA



Cells	Oligo	Conc. (μM)	Ab.
Mix	RAS-A	150	+
Mix	RAS-A	75	+
Mix	RAS-A	37.5	+
Mix	RAS-A	0	+
Mix	RAS-T	150	+
Mix	RAS-T	75	+
Mix	RAS-T	37.5	+
Mix	RAS-T	0	+
Mix	———	0	−
453	———	0	+
504	———	0	+

FIGURE 1: Inhibition of *ras* p21 expression in 453 and RS504 cells by sequence-specific anti-*ras* ONMPs. Twenty-four hours prior to treatment, 2×10^5 cells (453:RS504 = 3:1) were seeded in each well of a 6-well tissue culture dish. Cells were treated with the indicated concentrations for *ras*-A or *ras*-T for 48 h and then incubated in the presence of 150 μCi/mL [^{35}S]methionine in low methionine medium. The cell lysate was preincubated with protein A–Sepharose beads coated with goat anti-rat IgG. After removal of the beads, 2×10^7 cpm of TCA-precipitable counts was immunoprecipitated with a monoclonal antibody (Ab.) Y13-259 directed against p21 (Furth et al., 1982) and analyzed by SDS–13% polyacrylamide gel electrophoresis followed by autoradiography as described (Harford, 1984).



Cells	Oligo	Conc. (μM)	Ab.
Mix	psRAS-A	15	+
Mix	psRAS-A	7.5	+
Mix	psRAS-A	3.75	+
Mix	psRAS-A	0	+
Mix	psRAS-T	15	+
Mix	psRAS-T	7.5	+
Mix	psRAS-T	3.75	+
Mix	psRAS-T	0	+
Mix	———	0	−
453	———	0	+
504	———	0	+

FIGURE 2: Inhibition of *ras*-p21 expression in 453 and RS504 cells by psoralen-derivatized anti-ras ONMPs. Twenty-four hours prior to treatment, 2×10^5 cells (453:RS504 = 3:1) were seeded in each well of a 6-well tissue culture dish. The psoralen-modified anti-*ras* ONMP ps*ras*-A or ps*ras*-T was then added at the indicated concentrations to duplicate wells. After incubation for 48 h, one member of each pair of oligomer-treated cells was irradiated with a XeF laser (Questek 2000 excimer laser) at 40 Hz and 23 mJ/pulse for 110 s. This irradiation step was followed by metabolic labeling with 150 μCi of [^{35}S]methionine/well in 0.8 ml of low-methionine medium/well for 6 h. The labeling medium was not supplemented with fresh oligomer. Cells were lysed and the lysates were immunoprecipitated with a monoclonal antibody Y13-259 (Furth et al., 1982) directed against p21 and then with protein A-Sepharose beads coated with goat anti-rat IgG. The immunoprecipitates were analyzed by SDS–13% polyacrylamide gel electrophoresis followed by autoradiography as described (Harford, 1984).

from a human lung carcinoma (Yuasa et al., 1983). The point mutation is located in the second base of the 61st codon in Ha-*ras*. The RS504 cells contain a point mutation in the 12th codon (Capon et al., 1983; Tabin et al., 1982). However, the sequence in the 61st codon region is identical with that of normal human Ha-*ras*. The RS504 cell line was chosen to serve as the control because, as shown in Figure 1, *ras* p21 from RS504 cells has a lower gel electrophoretic mobility than that of *ras* p21 from 453 cells.

When mixed cultures of 453 and RS504 cells were treated with 37.5, 75, or 150 μM *ras*-T, a dose-dependent decrease in the intensity of the band corresponding to p21 from RS504 cells was observed, whereas no significant decrease of the p21 from 453 cells was observed (see Figure 1). Conversely, treatment with 37.5, 75, or 150 μM *ras*-A resulted in a dose-dependent decrease of p21 from 453 cells and no decrease in p21 from RS504 cells. The only difference between *ras*-T and *ras*-A is a single nucleotide (indicated by boldface type in the above sequences) in the middle of the oligomer sequence. The autoradiogram of the gel was scanned with a densitometer (LKB; Ultroscan XL enhanced laser densitometer). At a concentration of 150 μM, the *ras*-A oligomer inhibited 453 p21 synthesis 97% and RS504 p21 synthesis 26%, whereas the *ras*-T oligomer inhibited RS504 p21 synthesis 89% and stimulated 453 p21 synthesis 3%.

The inhibitory activity of antisense oligonucleoside methylphosphonates can be significantly increased by derivatization with the photoreactive cross-linking group 4′-[[*N*-(aminoethyl)amino]methyl-4,5′,8-trimethylpsoralen (Lee et al., 1988a,b; Kean et al., 1988; Bhan et al., 1990). The psoralen group is attached to the 5′ end of the methylphosphonate oligomer via a nuclease-resistant phosphoramidate linkage. This modification enables the oligomer to form a covalent bond with a pyrimidine residue of the targeted mRNA when irradiated with long-wavelength ultraviolet light.

ras-A and *ras*-T ONMPs were each derivatized with 4′-[[*N*-(aminoethyl)amino]methyl]-4,5′,8-trimethylpsoralen. Mixed cultures of 453 and RS504 cells were treated with 3.75, 7.5, or 15 μM anti-ras oligomer for 48 h and the cultures were then irradiated with a XeF laser (Questek 2000 excimer laster) at 351 nm. As shown in Figure 2, each oligomer specifically inhibited p21 synthesis in a manner identical with that of the underivatized oligomers but at 10-fold lower oligomer concentrations. Thus, at 15 μM, psoralen-derivatized *ras*-A inhibited 453 p21 synthesis by 100% and slightly stimulated synthesis RS504 p21 synthesis by 8%, whereas psoralen-derivatized *ras*-T inhibited RS504 p21 synthesis 96% and 453 p21 synthesis 15%.

A number of nonspecific protein bands as well as the specific *ras* p21 band are immunoprecipitated by the anti-*ras* antibody Y13-259, as indicated by the arrow on the left side of Figure 2. The lane not treated with antibody showed a nonspecific 20-kDa band that migrates close to the 453 p21 band. This band can be removed by incubating the cell lysate with protein A–Sepharose beads prior to the addition of Y13-259 anti-*ras* as shown in Figure 1 for the lane not treated with antibody. The intensity of this nonspecific band did not change as a result of treatment with ONMP *ras*-A or *ras*-T; however, the *ras*-specific p21 band did decrease in a dose-dependent manner. Separate control experiments showed that laser irradiation had no effect on synthesis of either of the *ras* p21 proteins. The remarkable specificity of these psoralen-derivatized oligomers suggests that their interaction with *ras* mRNA is primarily dependent upon the hydrogen-bonding interactions between the oligomer and the target mRNA and that these specific

interactions are not perturbed by the addition of the psoralen ring.

Discussion

There are a number of potential target sites for antisense oligonucleotides in the *ras* mRNA. Daaka and Wickstrom (1990) showed that normal antisense pentadecadeoxynucleotides complementary to the 5′ cap region, the initiation codon region, and the untranslated region of human c-Ha-*ras* effectively inhibit colony formation by transformed cells in semisolid agar (Daaka & Wickstrom, 1990). An oligomer complementary to the 5′ cap region was found to be the most effective among the three.

ONMPs complementary to the initiation codon region or splice junction of Ha-*ras* mRNA were both found to be effective as inhibitors of *ras* p21 expression in c-Ha-*ras*-transformed mouse NIH 3T3 cells, line RS485 (Brown et al., 1989). These oligomers do not appear to have inhibitory effects on the synthesis of other cellular proteins. In addition, cells in culture have been treated with relatively high concentrations, 150–200 μM, of anti-*ras* ONMP for up to 14 days with no apparent cytotoxic effects.

Because the sequences of the 5′ cap region, the initiation codon region, and the untranslated region of normal and mutated *ras* p21 mRNAs are identical, antisense oligonucleotides directed at these regions would not be expected to be specific for either form of *ras* p21. However, as demonstrated in this report, not only are ONMPs directed to the coding region of *ras* mRNA effective inhibitors of p21 synthesis but both psoralen-derivatized and nonderivatized oligomers show remarkable specificity in their ability to selectively inhibit synthesis of proteins containing a single point mutation. A similar specificity has been recently reported for acridine- or dodecanol-derivatized oligodeoxyribonucleotides directed against the G to T point mutation in the 12th codon of Ha-*ras* mRNA (Saison-Behmoaras et al., 1991).

The observed sequence specificity of inhibition by the ONMP methylphosphonates suggests that these oligomers, which differ by only a single base, interact effectively only with the *ras* mRNA targets for which they are completely complementary. The ability to selectively inhibit expression of a mutated form of p21 without affecting its normal counterpart provides a powerful method for studying the role of this protein in normal and transformed cells and also suggests a potentially useful strategy for treating diseases whose etiology involves a point mutation.

Acknowledgments

We thank Mr. M. Smith of the Laser Biophysics Center, USUHS, for his excellent technical support and Mrs. S. Taylor and Miss D. Friedman for manuscript prepration. We also thank Dr. J. B. Harford for his helpful discussions. The opinions or assertions contained herein are the private views of the authors and should not be construed as official or necessarily reflecting the views of the Uniformed Services University of the Health Sciences or the Department of Defense.

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