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# An Antisense Oligodeoxynucleotide That Depletes $RI_\alpha$ Subunit of Cyclic AMP-dependent Protein Kinase Induces Growth Inhibition in Human Cancer Cells

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## ABSTRACT

**Enhanced expression of the $RI_\alpha$ subunit of cyclic AMP-dependent protein kinase type I has been correlated with cancer cell growth. We provide evidence that $RI_\alpha$ is a growth-inducing protein that may be essential for neoplastic cell growth. Human colon, breast, and gastric carcinoma and neuroblastoma cell lines exposed to a 21-mer human $RI_\alpha$ antisense phosphorothioate oligodeoxynucleotide (S-oligodeoxynucleotide) exhibited growth inhibition with no sign of cytotoxicity. Mismatched sequence (random) S-oligodeoxynucleotides of the same length exhibited no effect. The growth inhibitory effect of $RI_\alpha$ antisense oligomer correlated with a decrease in the $RI_\alpha$ mRNA and protein levels and with an increase in $RII_\beta$ (the regulatory subunit of protein kinase type II) expression. The growth inhibition was abolished, however, when cells were exposed simultaneously to both $RI_\alpha$ and $RII_\beta$ antisense S-oligodeoxynucleotides. The $RII_\beta$ antisense S-oligodeoxynucleotide alone, exhibiting suppression of $RII_\beta$ along with enhancement of $RI_\alpha$ expression, led to slight stimulation of cell growth. These results demonstrate that two isoforms of cyclic AMP receptor proteins, $RI_\alpha$ and $RII_\beta$, are reciprocally related in the growth control of cancer cells and that the $RI_\alpha$ antisense oligodeoxynucleotide, which efficiently depletes the growth stimulatory $RI_\alpha$, is a powerful biological tool toward suppression of malignancy.**

## INTRODUCTION

cAMP[2] functions as a transducer of hormonal signals through binding to its receptor protein, cAMP-dependent protein kinase (1). The tetrameric protein kinase is composed of two C subunits bound to a R subunit dimer. Activation occurs when two cAMP molecules bind to each regulatory subunit of protein kinase, resulting in the release of a C subunit. Thus, all actions of cAMP as second messenger that are implicated in the regulation of various cellular functions, such as metabolism, secretion, cell proliferation, ion channel regulation, and gene induction, have been related to protein phosphorylation through the activation of protein kinase (2). Consequently, the significance of the presence of an R subunit in protein kinase has been confined to its inhibition of the C subunit.

There are, however, two types of cAMP-dependent protein kinases, type I and type II, in mammalian cells. These kinases are distinguished by their different R subunits, RI and RII, that interact with an identical C subunit (3). Through biochemical studies and gene cloning, four isoforms of the R subunits, $RI_\alpha$, $RI_\beta$, $RII_\alpha$, and $RII_\beta$, have been identified (4, 5). These R isoforms differ in tissue distribution (6, 7) and in biochemical properties (3, 8). The two general isoforms of the R subunit also differ in their subcellular localization. RI is found throughout the cytoplasm, whereas RII localizes to nuclei, nucleoli, Golgi, and to the microtubule-organizing center (9–12). Thus, these studies suggest specific roles of the different isoforms of the R subunit, apart from inhibition of the C subunit in the regulatory function of cAMP. An enhanced expression of $RI_\alpha$ subunit has been shown (12, 13) in human cancer cell lines and in primary tumors, as compared with normal counterparts, in cells after transformation with the Ki-*ras* oncogene or transforming growth factor $\alpha$, and upon stimulation of cell growth with GM-CSF or phorbol esters; conversely, a decrease in the expression of $RI_\alpha$ correlates with growth inhibition induced by site-selective cAMP analogues in a broad spectrum of human cancer cell lines (13). Moreover, an inverse relationship between RI (protein kinase type I) and RII (protein kinase type II) has been noted during ontogenic development and cell differentiation (12, 13).

We hypothesize that $RI_\alpha$ is an ontogenic growth-inducing protein, and its constitutive expression disrupts normal ontogenic processes, resulting in a pathogenic outgrowth, such as malignancy. To directly determine whether $RI_\alpha$ has a role in neoplastic cell growth, at least *in vitro*, we used the antisense strategy (14). We exposed human cancer cells to an antisense unmodified oligodeoxynucleotide (15) and to a phosphorothioate analogue complementary to the first 21 bases of the human $RI_\alpha$ mRNA and examined the effects of this treatment on cell growth and morphology. As controls, we used mismatched sequence (random) and $RII_\beta$ antisense (16) oligomers of the same length.

## MATERIALS AND METHODS

The 21-mer oligodeoxynucleotides and their phosphorothioate analogues used in the present study were synthesized (17) using a Milligen Biosearch 8700 DNA synthesizer (Bedford, MA) and purified by reverse-phase high-performance liquid chromatography. The oligomers had the following sequences: human $RI_\alpha$ antisense, 5′-GGC-GGT-ACT-GCC-AGA-CTC-CAT-3′ (15); $RII_\beta$ antisense, 5′-CGC-CGG-GAT-CTC-GAT-GCT-CAT-3′ (16); and the mismatched sequence, random oligomer was a random mixture of all four nucleotides at every position.

8-Cl-cAMP was obtained from the Division of Cancer Treatment, NCI (Bethesda, MD). Pepstatin, antipain, chymostatin, leupeptin, and soybean trypsin inhibitor were obtained from Sigma Chemical Company (St. Louis, MO). 8-$N_3$-[$^{32}$P]cAMP (60.0 Ci/mmol) was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA). Protein A-Sepharose CL-4B was purchased from Pharmacia-LKB (Uppsala, Sweden). Eagle's minimum essential medium, heat-inactivated fetal bovine serum, trypsin-EDTA solution, penicillin-streptomycin solution, L-glutamine, HEPES buffer (1 M stock solution, pH 7.3), and minimum essential medium nonessential amino acids were obtained from GIBCO-BRL (Grand Island, NY).

**Cell Culture.** The LS-174T human colon carcinoma cell line (provided by J. Greiner, National Cancer Institute) and SK-N-SH human neuroblastoma cell line (provided by L. Neckers, National Cancer Institute) were grown in Eagle's minimum essential medium supplemented with 10% heat- inactivated fetal bovine serum, Eagle's minimum essential medium nonessential amino acids, 20 mM HEPES, 2 mM glutamine, and penicillin-streptomycin. MCF-7 human breast cancer cell line was grown in improved minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, 2 mM glutamine, and penicillin-streptomycin. TMK-1 human gastric carcinoma cell line (provided by E. Tahara, Hiroshima University School of Medicine, Hiroshima, Japan) was grown in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 1 mM glutamine, and penicillin-streptomycin.

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[2] The abbreviations used are: cAMP, cyclic AMP; R, regulatory (subunit); C, catalytic (subunit); $RI_\alpha$ and $RII_\beta$, regulatory subunits of cAMP-dependent protein kinase type I and type II, respectively; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; cDNA, complementary DNA; S-oligodeoxynucleotide, phosphorothioate oligodeoxynucleotide.



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For cell growth experiments, 2–3 × $10^5$ cells/60-mm dish were seeded, and oligodeoxynucleotides were added at 4 h postseeding and then every 36 h thereafter without changing medium. At desired times, cell counts in duplicate were performed on a Coulter counter (Coulter Corp., Hialeah, FL) after harvesting cells with gentle trypsinization. Cell viability was assessed by trypan blue dye exclusion.

**Immunoprecipitation of $RI_\alpha$ and $RII_\beta$ cAMP Receptor Proteins after Photoaffinity Labeling with 8-$N_3$-[$^{32}$P]cAMP.** Cell extracts were prepared at 0–4°C. The cell pellets (2 × $10^6$ cells), after 2 washes with phosphate-buffered saline, were suspended in 0.5 ml buffer Ten (16) (20 mM Tris-HCl, pH 7.4; 0.1 M NaCl; 1% NP-40; 0.5% sodium deoxycholate; 5 mM $MgCl_2$; 0.1 mM pepstatin; 0.1 mM antipain; 0.1 mM chymostatin; 0.2 mM leupeptin; 0.4 mg/ml aprotinin; and 0.5 mg/ml soybean trypsin inhibitor, filtered through a 0.45-μm pore size membrane), passed through a 20-gauge needle 5 times using a 1-ml syringe, allowed to sit at 4°C for 15 min, and centrifuged for 5 min in an Eppendorf microfuge at 4°C. The supernatant was used as lysate. Protein concentration (usually between 1 and 5 mg/ml) was determined by the method of Lowry *et al.* (18) using bovine serum albumin as standard. Photoaffinity labeling and immunoprecipitation of cAMP receptor proteins followed the method previously described (16). Anti-RI or anti-RII antiserum (provided by S. O. Doskeland, University of Bergen, Bergen, Norway) and protein A-Sepharose were used for immunoprecipitation, the dissolved immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and the resolved proteins were electrotransferred to nitrocellulose sheets. Radioactivity was detected by autoradiography using Kodak X-Omat film.

**Poly(A)$^+$ RNA Preparation.** Poly(A)$^+$ RNA was extracted from 2 × $10^7$ cells for each treatment by QuickPrep™ mRNA Purification Kit (Pharmacia, Piscataway, NJ), according to the manufacturer's instructions. Briefly, the cells were homogenized with 4 M guanidine thiocyanate and 0.5% Sarkosyl (*N*-lauroyl sarcosine/$Na^+$) and then loaded onto an oligo (dThd) cellulose span column. After washing 3 times with high salt buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5 M NaCl] and twice with low salt buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 M NaCl], poly(A)$^+$ RNA was recovered by washing with elution buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA] at 65°C. The RNA obtained was precipitated in ethanol with glycogen and potassium acetate.

**Northern Blot Analysis.** RNA pellets were suspended in 15 μl denaturing buffer (50% formamide-2.2 M formaldehyde-10 mM sodium phosphate, pH 7.5), incubated at 55°C for 15 min, and quenched on ice. Loading buffer (50% glycerol, 50% formamide, bromophenol blue; 7.5 μl) was added, and 10 μg mRNA were resolved on a 1.5% agarose gel containing 6.7% formaldehyde (v/v) and 20 mM sodium phosphate buffer, pH 6.8. The resolved RNA was blotted onto a Biotrans (ICN) nylon membrane and cross-linked by UV irradiation. After prehybridization in 5 × Denhardt's solution, 5 × SSC, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 250 μg/ml herring sperm DNA, and 50% (v/v) formamide, the membrane was hybridized at 42°C in the same buffer containing nick-translated cDNA probe ($10^7$ cpm probe/ml hybridization solution). The same nitrocellulose filters were sequentially hybridized to the following $^{32}$P-labeled probes: $RI_\alpha$, 1.5 kilobase cDNA clone, containing the entire coding region for the human $RI_\alpha$ (19) (provided by T. Jahnsen, Institute of Pathology, Rikshospitalet, Oslo, Norway); $C_\alpha$, 1.1 kb, containing total open-reading frame of human $C_\alpha$ cDNA (20) (provided by S. K. Hanks, The Salk Institute, San Diego, CA); and human β-actin (Oncor p7000 β-actin). The membrane was washed 4 times in 2 × SSC and 0.1% SDS at room temperature for 5 min followed by 2 washes using 0.1× SSC and 0.1% SDS at 50°C. Autoradiography was performed using XAR2 (Kodak) film.

## RESULTS AND DISCUSSION

Nuclease resistance is one of the major factors to be concerned with in the use of oligodeoxynucleotides for the antisense inhibition of gene expression. The normal unmodified β-oligodeoxynucleotides are far more sensitive to nuclease hydrolysis than are α-oligodeoxynucleotides, methylphosphonate, or phosphorothioate oligodeoxynucleotides (14, 21, 22). In this study, we have chosen S-oligodeoxynucleotide to block the expression of $RI_\alpha$ subunit of protein kinase and examined the effect of $RI_\alpha$ depletion on the cancer cell growth. We also compared the effect of the S-oligodeoxynucleotide antisense on cell growth with that of unmodified oligonucleotide.

LS-174T human colon carcinoma cells were exposed for 6 days to $RI_\alpha$ antisense S-oligodeoxynucleotide at different concentrations, and cell growth was compared with untreated cells. As shown in Fig. 1, the concentration inducing 50% inhibition of cell proliferation of $RI_\alpha$ antisense S-oligodeoxynucleotide was 6.0 μM. Trypan blue dye exclusion test showed that cells exposed to $RI_\alpha$ antisense oligomer up to 10 μM concentration were more than 95% viable, but at 20 μM concentration only 70% of cells were viable, indicating nonspecific cytotoxicity of the oligonucleotide at higher concentrations. $RI_\alpha$ antisense S-oligodeoxynucleotide induced growth inhibition in a time-dependent manner (Fig. 2). While untreated cells demonstrated an exponential rate of growth, cells exposed to 6 μM $RI_\alpha$ antisense oligomer exhibited a reduced growth rate within 4-5 days and eventually stopped proliferating by day 6. Since the half-lives of a S-oligodeoxynucleotide in various biological fluids and sera have been shown to be in the range of 7–19 h (22), and the $RI_\alpha$ antisense oligonucleotide was added to the cell culture every 36 h, it is unlikely that the growth inhibition observed (Fig. 2) was due to a nonspecific cytotoxicity caused by the accumulation of the oligomer. Treatment with mismatched (random) sequence S-oligodeoxynucleotide (6 μM) had no appreciable effect on cell growth (Fig. 2). Interestingly, the growth inhibitory effect of $RI_\alpha$ antisense oligomer was abolished when cells were exposed simultaneously to both $RI_\alpha$ and $RII_\beta$ antisense S-oligodeoxynucleotides; $RII_\beta$ antisense oligomer alone had a slight stimulatory effect on cell growth (Fig. 2).

We next examined the effect of $RI_\alpha$ antisense oligodeoxynucleotide unmodified and S-oligodeoxynucleotide on the growth of other human cancer cell lines, including SK-N-SH neuroblastoma, MCF-7 breast, and TMK-1 gastric carcinoma cells. As shown in Fig. 3, $RI_\alpha$ antisense oligomer induced growth inhibition of these cells as potently as that shown in LS-174T cells. Fig. 3 also shows that the unmodified antisense oligomer at 30 μM produced a growth inhibitory effect similar to that caused by 6 μM S-oligodeoxynucleotide, demonstrating a greater potency of phosphorothioate oligomer as compared with unmodified oligomer. The growth inhibition was not due to nonspecific cytotoxicity, as cells were more than 90% viable after exposure to $RI_\alpha$ antisense oligomer for 7 days. Mismatched (random) sequence oligomer of unmodified or phosphorothioate analogue at 30 and 6 μM

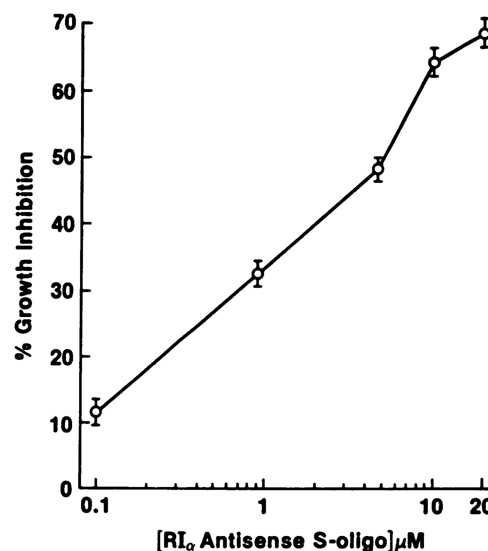


Fig. 1. Concentration dependency of $RI_\alpha$ antisense phosphorothioate oligodeoxynucleotide on the growth inhibition of LS-174T colon carcinoma cells. Cells were exposed for 6 days with $RI_\alpha$ antisense oligomer at various concentrations, and cell counts were performed in duplicate (see "Materials and Methods"). The percentage of growth inhibition was obtained by comparing the growth of cells treated with the antisense oligomer to that of untreated control cells. Data represent the average values ± SD of three separate experiments.



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concentrations, respectively, had no growth inhibitory effect up to 7 days of exposure (data not shown).

The growth inhibition induced by $RI_\alpha$ antisense oligodeoxynucleotide induced a change in cell morphology. SK-N-SH neuroblastoma cells exposed to 6 μM $RI_\alpha$ antisense S-oligodeoxynucleotide for 6 days exhibited a striking change in morphology: cells became flat, exhibiting extremely enlarged cytoplasm (Fig. 4). In the case of HL-60 cells, the $RI_\alpha$ antisense oligodeoxynucleotide (15 μM) treatment for 4–5 days elicited a monocytic morphological change, indicating cell differentiation (15).

To provide evidence that the growth inhibition induced in cells exposed to $RI_\alpha$ antisense oligodeoxynucleotide was directly related to the arrest of $RI_\alpha$ gene expression, we measured $RI_\alpha$ mRNA level. As shown in Fig. 5, the human $RI_\alpha$ cDNA probe detected a major 4.4-kilobase mRNA (23) in growing, untreated LS-174T cells (Fig. 5, $RI_\alpha$, *Lane 4*). Treatment with $RI_\alpha$ antisense S-oligodeoxynucleotide (6 μM) for 6 days resulted in almost complete blockage in the $RI_\alpha$ gene expression (Fig. 5, $RI_\alpha$, *Lane 1*); the steady state level of $RI_\alpha$ mRNA decreased to 10% that of control cells (Fig. 5). Previous studies have shown (24) that LS-174T cells contain mainly type I protein kinase, and thus $RI_\alpha$ is the major, if not only, cAMP receptor in these cells. As cells express R and C in a 1:1 ratio (25), it was expected that $RI_\alpha$ antisense oligomer, which blocks $RI_\alpha$ expression, would also result in suppression of C expression. In fact, $C_\alpha$ mRNA level decreased, in parallel with the decrease in $RI_\alpha$ mRNA in cells exposed to $RI_\alpha$ antisense S-oligodeoxynucleotide (Fig. 5, $C_\alpha$, *Lane 1*). In contrast, exposure of cells to $RII_\beta$ antisense S-oligodeoxynucleotide (6 μM for 6 days), which led to a slight stimulation of cell growth (Fig. 2), brought about an effect on $RI_\alpha$ and $C_\alpha$ mRNA opposite from that induced by $RI_\alpha$ antisense oligomer: cells exhibited an enhanced expression of $RI_\alpha$ and $C_\alpha$ mRNA (Fig. 5, *Lane 2*).

It has been shown (13, 26) that the striking growth-inhibitory effect of site-selective 8-Cl-cAMP demonstrated in cancer cells correlates with its selective modulation of two types of cAMP receptor proteins: a marked down-regulation of $RI_\alpha$ receptor; and an up-regulation of $RII_\beta$ receptor. Present results show that the effect of $RI_\alpha$ antisense oligonucleotide on the reduction of $RI_\alpha$ and $C_\alpha$ mRNA levels was essentially the same as that exerted by 8-Cl-cAMP (Fig. 5, *Lane 3*).

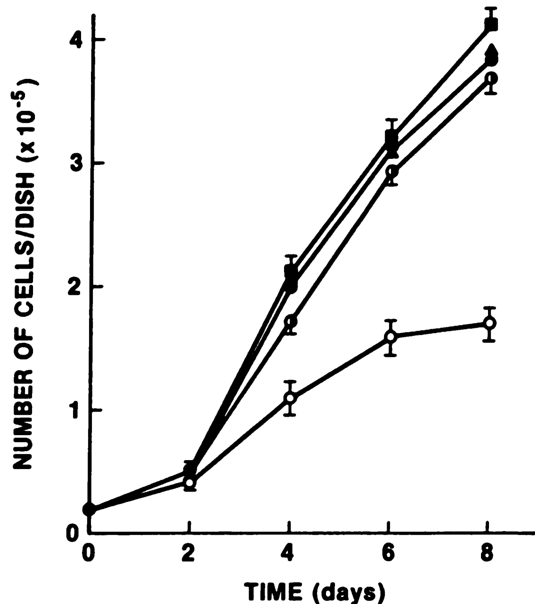


Fig. 2. Time-dependent growth inhibitory effect of $RI_\alpha$ antisense S-oligodeoxynucleotide on LS-174T colon carcinoma cells. Cells were untreated (○) or treated with 6 μM S-oligodeoxynucleotide of $RI_\alpha$ antisense (○), 6 μM $RII_\beta$ antisense (■), 6 μM $RI_\alpha$ antisense + 6 μM $RII_\beta$ antisense (○), or with mismatched (random) sequence (Δ). At indicated times, cell counts in duplicate were performed. Data represent the average values ± SD of three separate experiments.

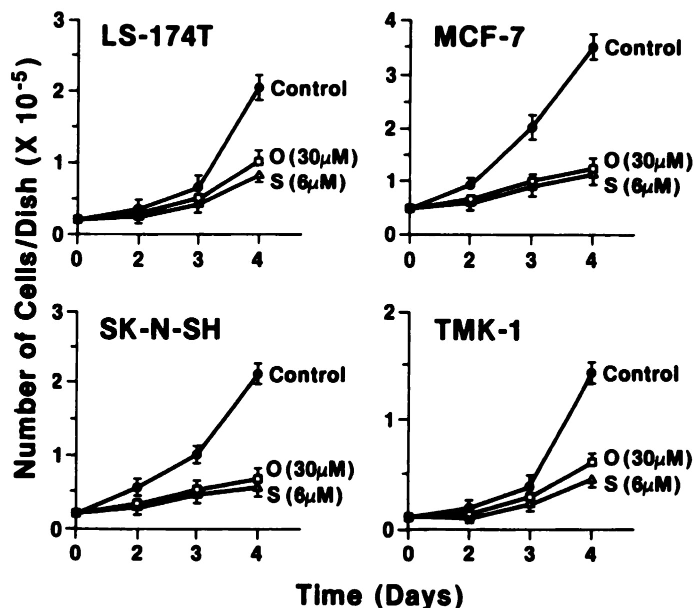


Fig. 3. Effect of $RI_\alpha$ antisense oligodeoxynucleotide on the growth of human cancer cell lines. Cells were grown in the absence (○) and presence of $RI_\alpha$ antisense oligodeoxynucleotide (□) or its phosphorothioate analog (Δ) at indicated doses (see "Materials and Methods"). At indicated times, cell counts in duplicate were performed. Data represent the average values ± SD of three separate experiments.

These changing levels of $RI_\alpha$ and $C_\alpha$ mRNA were the specific effect of the antisense oligonucleotides, as β-actin mRNA level remained fairly constant (Fig. 5).

We next examined the effect of the $RI_\alpha$ antisense oligonucleotide on the levels of $RI_\alpha$ and $RII_\beta$ proteins in these cells using photoaffinity labeling with 8-$N_3$-[$^{32}$P] cAMP followed by immunoprecipitation with anti-RI and -RII antisera and SDS-polyacrylamide gel electrophoresis. In control LS-174T cells, the anti-RI antibody detected the $M_r$ 48,000 protein, which comigrated with rabbit skeletal muscle $RI_\alpha$, that we refer to as $RI_\alpha$ (Fig. 6*A*), while the anti-RII antibody, which cross-reacts with both $RII_\alpha$ and $RII_\beta$ in human cells[3], detected only one species of RII, the $M_r$ 52,000 protein, which did not comigrate with the $M_r$ 54,000 bovine heart $RII_\alpha$, that we refer to as $RII_\beta$ (Fig. 6*B*). That the anti-RII-antibody failed to detect $RII_\alpha$, which exhibits $M_r$ 50,000–51,000 in human cells (9, 10, 27–30) and can be phosphorylated to become $M_r$ 54,000 (30), indicates that these cells contained $RII_\alpha$ at a low undetectable level. Treatment with $RI_\alpha$ antisense S-oligodeoxynucleotide brought about a marked reduction in $RI_\alpha$ level (Fig. 6*A*, *Lane 2*) with an increase in $RII_\beta$ level (Fig. 6*B*, *Lane 2*) as compared with untreated control cells. 8-Cl-cAMP treatment brought about an effect on $RI_\alpha$ and $RII_\beta$ levels similar to that of $RI_\alpha$ antisense oligomer (Fig. 6*A*, *B*, *Lane 4*), whereas $RII_\beta$ antisense S-oligodeoxynucleotide brought about effects opposite those of $RI_\alpha$ antisense oligomer (Fig. 6,*A* and *B*, *Lane 3*) (16). The marked reduction in the band detected with the anti-RII antibody (Fig. 6*B*, *Lane 3*) in cells treated with $RII_\beta$ antisense oligonucleotide further supports that the anti-RII antibody detected $RII_\beta$ but not $RII_\alpha$ in LS-174T cells. These results show that $RI_\alpha$ and $RII_\beta$ antisense oligodeoxynucleotides each exerted a specific effect on their respective target mRNAs and that their targeting of either isoform of R subunit expression resulted in compensatory enhancement of the expression of the other form of R subunit. The arrest of $RI_\alpha$ gene expression was commonly observed in SK-N-SH (Table 1), MCF-7 and TMK-1 (data not shown) cancer cells, those that exhibited growth arrest (Fig. 3) after exposure to $RI_\alpha$ antisense oligonucleotide. Importantly, however, when cells were blocked from the expression of both $RI_\alpha$ and $RII_\beta$ genes by exposure

[3] Cho-Chung *et al.*, unpublished observation.



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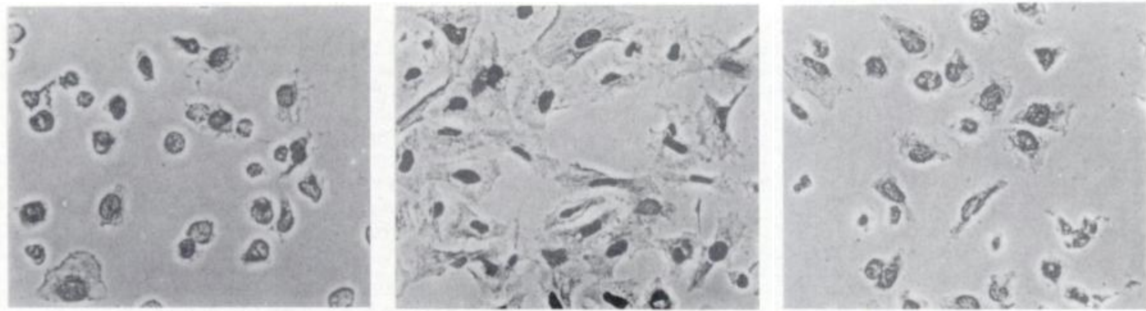
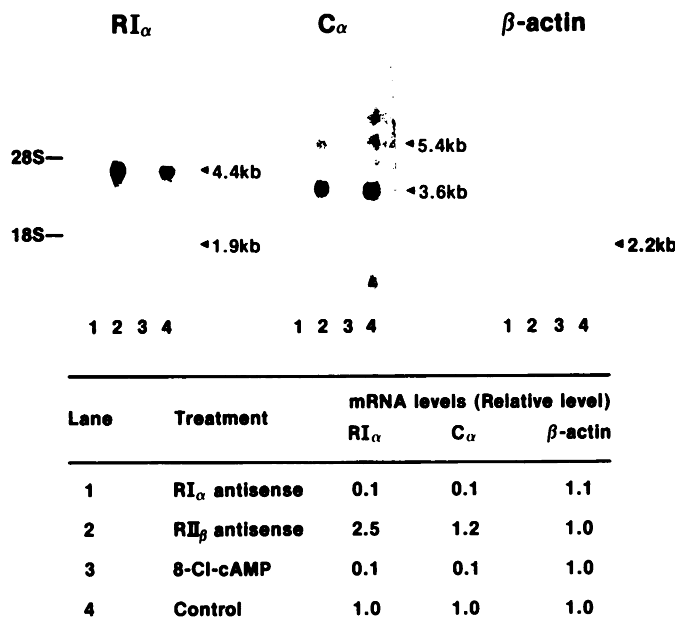



Fig. 4. Change in morphology of SK-N-SH neuroblastoma cells treated with $RI_\alpha$ antisense S-oligodeoxynucleotide. Cells untreated (control) or treated for 6 days with $RI_\alpha$ antisense oligomer, 6 μM, or mismatched sequence oligomer, 6 μM (random), were seeded at $10^4$ cells/ml onto 4-chamber tissue culture slides and grown for 48 h, ± continued treatment with the oligomers. Cells were fixed and stained by Wright's stain × 120.



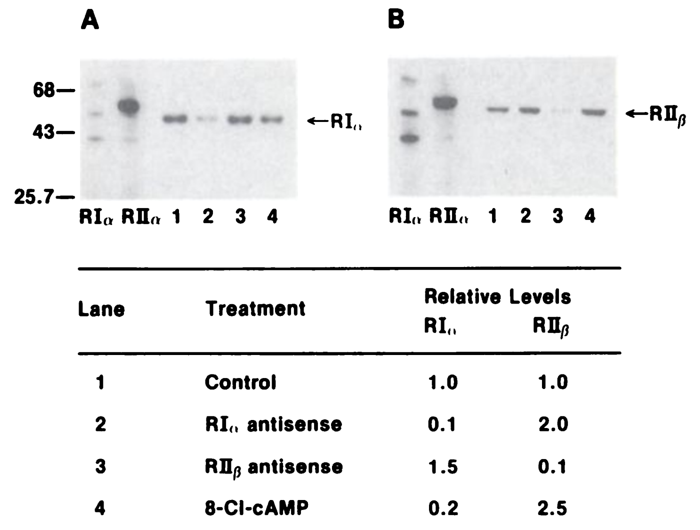

| Lane | Treatment | mRNA levels (Relative level) | | |
|---|---|---|---|---|
| | | $RI_\alpha$ | $C_\alpha$ | β-actin |
| 1 | $RI_\alpha$ antisense | 0.1 | 0.1 | 1.1 |
| 2 | $RII_\beta$ antisense | 2.5 | 1.2 | 1.0 |
| 3 | 8-Cl-cAMP | 0.1 | 0.1 | 1.0 |
| 4 | Control | 1.0 | 1.0 | 1.0 |

Fig. 5. Decreased $RI_\alpha$ and $C_\alpha$ mRNA levels in LS-174T colon carcinoma cells exposed to $RI_\alpha$ antisense oligodeoxynucleotide. Cells were treated for 6 days with $RI_\alpha$ antisense S-oligodeoxynucleotide and $RII_\beta$ antisense S-oligodeoxynucleotide each at 6 μM concentration (see "Materials and Methods") or with 8-Cl-cAMP (10 μM) for 6 days (single treatment at 5 h after seeding). Poly(A)$^+$ RNA was isolated from control (untreated) and treated cells, and Northern blot analysis was performed as described in "Materials and Methods." *Lane 1*, cells exposed to $RI_\alpha$ antisense oligomer; *Lane 2*, cells exposed to $RII_\beta$ antisense oligomer; *Lane 3*, cells treated with 8-Cl-cAMP; *Lane 4*, untreated control cells. The same nitrocellulose filters were sequentially hybridized to the $^{32}$P-labeled nick-translated cDNA probes (see "Materials and Methods"). The data in the table represent quantification by densitometric scanning of the autoradiograms. The data are expressed relative to the levels in control cells, which are set equal to 1 arbitrary unit. The data represent an average value of three separate experiments.

of cells to both $RI_\alpha$ and $RII_\beta$ antisense oligodeoxynucleotides simultaneously, cells were no longer growth inhibited (Table 1), demonstrating a clear reciprocal relationship between $RI_\alpha$ and $RII_\beta$ expression in growth control of these cells.

In LS-174T cells that contained mainly $RI_\alpha$, $RI_\alpha$ antisense oligonucleotide treatment resulted in the suppression of both $RI_\alpha$ and $C_\alpha$ subunits. Thus, growth inhibition induced by $RI_\alpha$ antisense oligomer in LS-174T cells, at least in part, could be due to down-regulation of $C_\alpha$ subunit. However, no direct role of C subunit of protein kinase has been shown (15) in the differentiation induced by $RI_\alpha$ antisense oligonucleotide in HL-60 leukemia cells that contained both $RI_\alpha$ and $RII_\alpha$. Suppression of $RI_\alpha$ by $RI_\alpha$ antisense oligodeoxynucleotide, which brought about a compensatory increase in $RII_\beta$, induced growth inhibition and differentiation in HL-60 cells in which C subunit expression was not down-regulated and remained the same. Differentiation of HL-60 cells was abolished when cells were exposed to both $RI_\alpha$ and $RII_\beta$ antisense oligomers, eliminating both $RI_\alpha$ and $RII_\beta$. However, differentiation was not blocked when $RI_\alpha$ and $RII_\alpha$ were eliminated by $RI_\alpha$ and $RII_\alpha$ antisense oligomers. A situation similar to exposure of cells to both $RI_\alpha$ and $RII_\beta$ antisense oligodeoxynucleotides was demonstrated by overexpression of $RII_\alpha$ in *ras*-transformed NIH3T3 cells in which $RI_\alpha$ and $RII_\beta$ were reduced, but did not produce growth inhibition or reverse transformation (31, 32). Thus, the $RI_\alpha$ antisense oligonucleotide induced growth inhibition with or without $C_\alpha$ suppression. The common effect of $RI_\alpha$ antisense oligonucleotide was, however, down-regulation of $RI_\alpha$ with up-regulation of $RII_\beta$.

As $RII_\alpha$ forms the more favored complex with the C subunit as compared with other R subunits (4, 32), it may serve as a reservoir to




| Lane | Treatment | Relative Levels | |
|---|---|---|---|
| | | $RI_\alpha$ | $RII_\beta$ |
| 1 | Control | 1.0 | 1.0 |
| 2 | $RI_\alpha$ antisense | 0.1 | 2.0 |
| 3 | $RII_\beta$ antisense | 1.5 | 0.1 |
| 4 | 8-Cl-cAMP | 0.2 | 2.5 |

Fig. 6. Effect of $RI_\alpha$ antisense oligodeoxynucleotide on the levels of $RI_\alpha$ and $RII_\beta$ cAMP receptor proteins in LS-174T colon carcinoma cells. Cells were treated with $RI_\alpha$ or $RII_\beta$ antisense S-oligodeoxynucleotide or with 8-Cl-cAMP as described in Fig. 5. Preparation of cell extracts, the photoaffinity labeling with 8-$N_3$-[$^{32}$P]cAMP, immunoprecipitation using the anti-RI or anti-RII antiserum and protein A-Sepharose, and the following SDS-polyacrylamide gel electrophoresis were performed as described in "Materials and Methods." Preimmune serum controls were performed simultaneously, and no immunoprecipitated band was detected. *RIα*, the $M_r$ 48,000 RI (Sigma); *RIIα*, the $M_r$ 56,000 RII (Sigma). *Lanes RIα* and *RIIα* are from photoaffinity labeling only; *A* and *B*, *Lanes 1–4*, photoaffinity labeling followed by immunoprecipitation with anti-RI and anti-RII antisera, respectively. The data in the table represent quantification by densitometric scanning of the autoradiograms. The data are expressed relative to the levels in control (untreated) cells, which are set equal to 1 arbitrary unit. The data represent an average value of three experiments.



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Table 1 *Effect of $RI_\alpha$ and $RII_\beta$ antisense S-oligodeoxynucleotides added singly or in combination on the growth and levels of $RI_\alpha$ and $RII_\beta$ in SK-N-SH neuroblastoma cells*

Cells were treated with $RI_\alpha$ or $RII_\beta$ antisense S-oligodeoxynucleotide, singly or in combination (see "Materials and Methods"). On day 5, preparation of cell extracts, photoaffinity labeling with 8-$N_3$-[$^{32}$P]cAMP, immunoprecipitation using the anti-RI or anti-RII antiserum and protein A-Sepharose, and the following SDS-polyacrylamide gel electrophoresis were performed as described in "Materials and Methods." The data of $RI_\alpha$ and $RII_\beta$ levels represent quantification by densitometric scanning of the autoradiograms of SDS-polyacrylamide gel electrophoresis. The data are expressed relative to the levels in control (untreated) cells, which are set equal to 1 arbitrary unit. The data represent an average value of three separate experiments. On day 5, cell counts in duplicate were performed (see "Materials and Methods"), and the percentage of cell growth was obtained by comparing the growth of cells treated with oligodeoxynucleotide to that of untreated control cells. Data represent the average values of three separate experiments.

| Treatment | Relative levels | | |
|---|---|---|---|
| | $RI_\alpha$ | $RII_\beta$ | % of cell growth |
| Control | 1.0 | 1.0 | 100 |
| $RI_\alpha$AS[a] (5 μM) | 0.2 | 2.0 | 50 |
| $RII_\beta$ AS (5 μM) | 1.3 | 0.2 | 110 |
| $RI_\alpha$ AS (5 μM) + $RII_\beta$ AS (5 μM) | 0.2 | 0.2 | 95 |
| $RI_\alpha$ AS (5 μM) + Random (5 μM) | 0.2 | 1.8 | 45 |
| Random (10 μM) | 1.0 | 1.0 | 92 |

[a] AS, antisense.

sequester the protein kinase in an inactive holoenzyme. Thus, the ratio of type I/type II protein kinase would be regulated mainly by the amounts of $RI_\alpha$ and $RII_\beta$ available, when $RII_\alpha$ remains constant. That the down-regulation of $RI_\alpha$ led to growth inhibition, as shown by $RI_\alpha$ antisense oligodeoxynucleotide or by 8-Cl-cAMP treatment, suggests that $RI_\alpha$ is a positive regulator of cell growth. Down-regulation of both $RI_\alpha$ and $RII_\beta$ or $RII_\beta$ alone leads to continued cell growth, while $RII_\beta$ overexpression results in growth inhibition (33), suggesting that $RII_\beta$ is a negative regulator of cell growth. The $RI_\alpha$ antisense oligodeoxynucleotide, which efficiently down-regulates $RI_\alpha$ and up-regulates $RII_\beta$, in fact induced growth inhibition in a variety of cell types of cancer cells.

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# An Antisense Oligodeoxynucleotide That Depletes $RI_{\alpha}$ Subunit of Cyclic AMP-dependent Protein Kinase Induces Growth Inhibition in Human Cancer Cells

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