Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2011–2015, March 1991
Medical Sciences

Differentiation of HL-60 leukemia by type I regulatory subunit antisense oligodeoxynucleotide of cAMP-dependent protein kinase

(cAMP kinase)

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Communicated by Van Rensselaer Potter, November 19, 1990 (received for review October 5, 1989)

ABSTRACT **A marked decrease in the type I cAMP-dependent protein kinase regulatory subunit (RI_α) and an increase in the type II protein kinase regulatory subunit (RII_β) correlate with growth inhibition and differentiation induced in a variety of types of human cancer cells, *in vitro* and *in vivo*, by site-selective cAMP analogs. To directly determine whether RI_α is a growth-inducing protein essential for neoplastic cell growth, human HL-60 promyelocytic leukemia cells were exposed to 21-mer RI_α antisense oligodeoxynucleotide, and the effects on cell replication and differentiation were examined. The RI_α antisense oligomer brought about growth inhibition and monocytic differentiation, bypassing the effects of an exogenous cAMP analog. These effects of RI_α antisense oligodeoxynucleotide correlated with a decrease in RI_α receptor and an increase in RII_β receptor level. The growth inhibition and differentiation were abolished, however, when these cells were exposed simultaneously to both RI_α and RII_β antisense oligodeoxynucleotides. The RII_β antisense oligodeoxynucleotide alone has been previously shown to specifically block the differentiation inducible by cAMP analogs. These results provide direct evidence that RI_α cAMP receptor plays a critical role in neoplastic cell growth and that cAMP receptor isoforms display specific roles in cAMP regulation of cell growth and differentiation.**

cAMP-dependent protein kinase is composed of two genetically distinct subunits, catalytic (C) and regulatory (R). The activating ligand cAMP, which binds to the R, induces conformational changes and dissociates holoenzyme R_2C_2 into an $R_2(cAMP)_4$ dimer and two free C that are catalytically active (1, 2). There are two different classes of cAMP-dependent protein kinase designated as type I and type II, which contain distinct R, RI and RII, respectively, but share a common C (3, 4). Four different regulatory subunits [RI_α (RI) (5), RI_β (6), RII_α (RII_{54}) (7), and RII_β (RII_{51}) (8)] have been identified at the gene/mRNA level. Two distinct C [C_α (9) and C_β (10, 11)] have also been identified; however, preferential coexpression of either one of these C with either the type I or type II protein kinase R has not been found (11).

It has been shown that the ratio of cAMP-dependent protein kinases type I and type II varies among tissues and that an increased expression of protein kinase type I or RI correlates with active cell growth, cell transformation, or early stages of differentiation (12).

We hypothesized that RI 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 has a role in neoplastic cell growth, at least *in vitro*, we used the antisense strategy (13). We used 21-mer synthetic oligodeoxynucleotides complementary to either the sense or antisense strands of the human RI_α (14) mRNA transcript starting from the first codon (15–17). We then exposed human HL-60 leukemia cells to these oligodeoxynucleotides and examined the effects of this treatment on cell proliferation and differentiation. As additional controls, we also synthesized RII_α (18) and RII_β (19) antisense and a random-sequence oligodeoxynucleotide of identical size.

MATERIALS AND METHODS

Oligodeoxynucleotides. The 21-mer oligodeoxynucleotides used in the present studies were synthesized at Midland Certified Reagent (Midland, TX) and had the following sequences: human RI_α (14) antisense, 5′-GGC-GGT-ACT-GCC-AGA-CTC-CAT-3′; human RII_β (19) antisense, 5′-CGC-CGG-GAT-CTC-GAT-GCT-CAT-3′; human RII_α (18) antisense, 5′-CGG-GAT-CTG-GAT-GTG-GCT-CAT-3′; and the random-sequence oligodeoxynucleotide was made of a mixture of all four nucleotides at every position.

Cell Growth Experiment. Cells grown in suspension culture in RPMI 1640 medium/10% fetal bovine serum/penicillin at 50 units/ml/streptomycin at 500 μg/ml/1 mM glutamine (GIBCO) were seeded at 5×10^5 cells per dish. Oligodeoxynucleotides were added after seeding and every 48 hr thereafter. Cell counts were performed on a Coulter Counter. Cells unexposed or exposed to oligodeoxynucleotides for 4 days were reseeded (day 0) at 5×10^5 cells per dish, and cells preexposed to the oligodeoxynucleotide were further treated with the oligomer at day 0 and day 2. cAMP analogs (provided by R. K. Robins, Nucleic Acid Research Institute, Costa Mesa, CA) or phorbol 12-myristate 13-acetate (PMA) were added one time at day 0. Cell counts were performed on day 4.

Immunoprecipitation of RI_α and RII_β cAMP Receptor Proteins After Photoaffinity Labeling with 8-Azidoadenosine 3′,5′-Cyclic [^{32}P]Monophosphate ([^{32}P]8-N_3-cAMP). Cell extracts were prepared at 0–4°C. The cell pellets (2×10^6 cells), after two washes with phosphate-buffered saline, were suspended in 0.5 ml of buffer Ten (0.1 M NaCl/5 mM $MgCl_2$/1% Nonidet P-40/0.5% sodium deoxycholate/bovine aprotinin at 2 kallikrein inhibitor units per ml/20 mM Tris·HCl, pH 7.4) containing proteolysis inhibitors (17), Vortex-mixed, passed through a 22-gauge needle 10 times, allowed to stand for 30 min at 4°C, and centrifuged at $750 \times g$ for 20 min; the resulting supernatants were used as cell lysates. The photoactivated incorporation of [^{32}P]8-N_3-cAMP (60.0 Ci/mmol; 1 Ci = 37 GBq) and the immunoprecipitation using the anti-RI_α or anti-RII_β antiserum (provided by S. O. Døskeland, University of

Abbreviations: C, catalytic subunit(s); R, regulatory subunit(s); PMA, phorbol 12-myristate 13-acetate; RI and RII, type I and type II R, respectively; [^{32}P]8-N_3-cAMP, 8-azidoadenosine 3′,5′-cyclic [^{32}P]monophosphate; 8-Cl-cAMP, 8-chloroadenosine 3′,5′-cyclic monophosphate.

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Bergen, Bergen, Norway) and protein A–Sepharose and SDS/PAGE of solubilized antigen–antibody complex followed the method described (17, 20).

cAMP-Dependent Protein Kinase Assays. After two washes with Dulbecco's phosphate-buffered saline, cell pellets (2×10^6 cells) were lysed in 0.5 ml of 20 mM Tris, pH 7.5/0.1 mM sodium EDTA/1 mM dithiothreitol/0.1 mM pepstatin/0.1 mM antipain/0.1 mM chymostatin/0.2 mM leupeptin/aprotinin at 0.4 mg/ml/soybean trypsin inhibitor at 0.5 mg/ml, using 100 strokes of a Dounce homogenizer. After centrifugation (Eppendorf 5412) for 5 min, the supernatants were adjusted to 0.7 mg of protein per ml and assayed (21) immediately. Assays (40 μl, total volume) were performed for 10 min at 30°C and contained 200 μM ATP, 2.7×10^6 cpm [γ-^{32}P]ATP, 20 mM $MgCl_2$, 100 μM kemptide (Sigma K-1127) (22), 40 mM Tris (pH 7.5), ± 100 μM protein kinase inhibitor (Sigma P-3294) (23), ± 8 μM cAMP and 7 μg of cell extract. The phosphorylation of kemptide was determined by spotting 20 μl of incubation mixture on phosphocellulose filters (Whatman P81) and washing them in phosphoric acid as described (24). Radioactivity was measured by liquid scintillation with Econofluor-2 (NEN Research Products NEF-969).

Isolation of Total RNA and Northern (RNA) Blot Analysis. The cells (10^8 washed twice with phosphate-buffered saline) were lysed in 4.2 M guanidine isothiocyanate/25 mM sodium citrate, pH 7.0/0.5% sarcosyl (*N*-lauroylsarcosine Na^+)/0.1 M 2-mercaptoethanol, and the lysates were homogenized; total cellular RNA was sedimented through a CsCl cushion (5.7 M CsCl/10 mM EDTA) as described by Chirgwin *et al.* (25). Total cellular RNA in 20 mM 3-(*N*-morpholine)propanesulfonic acid, pH 7.0/50% (vol/vol) formamide/6% (vol/vol) formaldehyde was denatured at 65°C for 10 min and electrophoresed through a denaturing 1.2% agarose/2.2 M formaldehyde gel. The gels were then transferred to Biotrans nylon membranes (ICN) by the method of Thomas (26) and hybridized to the following two ^{32}P-labeled nick-translated cDNA probes: 1.5-kilobase (kb) cDNA clone containing the entire coding region for the human cAMP-dependent protein kinase type I R, RI_α (14) (provided by T. Jahnsen, Institute of Pathology, Rikshospitalet, Oslo, Norway) and human β actin (Oncor p7000 β actin).

RESULTS AND DISCUSSION

The RI_α antisense oligodeoxynucleotide at 15 μM concentration (17) had immediate effects on the rate of proliferation of HL-60 cells. By 4–5 days in culture, cells unexposed to RI_α antisense oligomer demonstrated an exponential rate of growth, whereas cells exposed to the RI_α antisense oligomer exhibited a reduced growth rate and eventually stopped replicating (Fig. 1*A*). This inhibitory effect on cell proliferation persisted throughout the culture period. The growth inhibition was not due to cell killing; cells were >90% viable after exposure to RI_α antisense oligomer (15 μM) for 7 days as assessed by flow cytometry by using forward and side scatter. RI_α, sense, RII_α, RII_β antisense, or a random-sequence oligodeoxynucleotide had no such growth inhibitory effect.

Cells unexposed or exposed to RI_α antisense oligodeoxynucleotide for 4 days in culture were reseeded and examined for their response to treatment with cAMP analogs or PMA. In cells unexposed to RI_α antisense oligodeoxynucleotide, 8-chloradenosine 3′,5′-cyclic monophosphate (8-Cl-cAMP) (10 μM) produced 60% growth inhibition, and 80% growth inhibition was achieved by 8-Cl-cAMP (5 μM) plus N^6-benzyl-cAMP (5 μM) (Fig. 1*B*) (27); PMA (10^{-8} M) exhibited 60% growth inhibition (Fig. 1*B*). In contrast, cells exposed to RI_α antisense oligodeoxynucleotide exhibited retarded growth (≈25% the rate of growth of cells unexposed to the

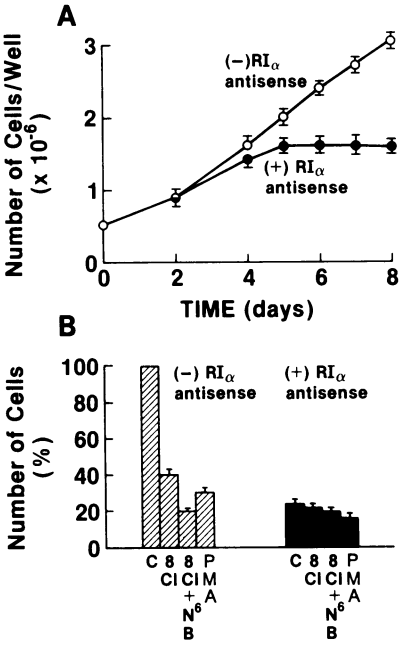


FIG. 1. Effect of RI_α antisense oligodeoxynucleotide on the basal rate of growth of HL-60 leukemic cells (*A*) and the growth of these cells when treated with cAMP analogs or PMA (*B*). (*A*) Cells were grown without (○) or with (●) RI_α antisense oligodeoxynucleotide (15 μM). At indicated times, cell counts in duplicate were performed. Data represent the average values ± SDs of four experiments. (*B*) On day 4 of experiment *A*, cells exposed or unexposed to RI_α antisense oligodeoxynucleotide were reseeded (day 0) at 5×10^5 cells per dish, and cells preexposed to RI_α antisense oligodeoxynucleotide were further treated with the oligomer at day 0 and 2. cAMP analogs and PMA were added once at day 0. Cells were counted on a Coulter Counter on day 4. 8-Cl, 8-Cl-cAMP (10 μM); 8-Cl + N^6-B, 8-Cl-cAMP (5 μM) + N^6-benzyl-cAMP (5 μM); PMA (10^{-8} M). Data represent the average values ± SDs of four experiments.

RI_α antisense oligomer), and neither cAMP analogs nor PMA further retarded growth (Fig. 1*B*).

HL-60 cells undergo a monocytic differentiation upon treatment with site-selective cAMP analogs (27). Cells either unexposed or exposed to RI_α antisense oligodeoxynucleotide were examined for their morphology before and after treatment with cAMP analogs. As shown in Fig. 2, in cells unexposed to RI_α antisense oligomer, 8-Cl-cAMP plus N^6-benzyl-cAMP induced a monocytic morphologic change characterized by a decrease in nuclear-to-cytoplasm ratio, abundant ruffled and vacuolated cytoplasm, and loss of nucleoli (27). Strikingly, the same morphologic change was induced when cells were exposed to RI_α antisense oligodeoxynucleotide (Fig. 2). Moreover, the morphologic changes induced by RI_α antisense oligomer were indistinguishable from that induced by PMA (Fig. 2).

To provide more evidence that the growth inhibition and monocytic differentiation induced in HL-60 cells exposed to the RI_α antisense oligodeoxynucleotide were due to an intracellular effect of the oligomer, we determined the RI_α mRNA level. As shown in Fig. 3, 3.0-kb RI_α mRNA (14) was virtually undetectable in cells exposed for 8 hr to RI_α antisense oligodeoxynucleotide (Fig. 3*B*, lane 2), and the decrease in RI_α mRNA was not due to a lower amount of total RNA as shown by the ethidium bromide staining (compare lane 2 with lane 1 of Fig. 3*A*). Conversely, an enhanced level of actin mRNA was detected in cells exposed to RI_α antisense oligomer (Fig. 3*B*). Whether the increase in actin mRNA level represents changes in cytoskeletal structure is not known.

We next determined the levels of cAMP receptor proteins in these cells by immunoprecipitation with anti-RI_α and

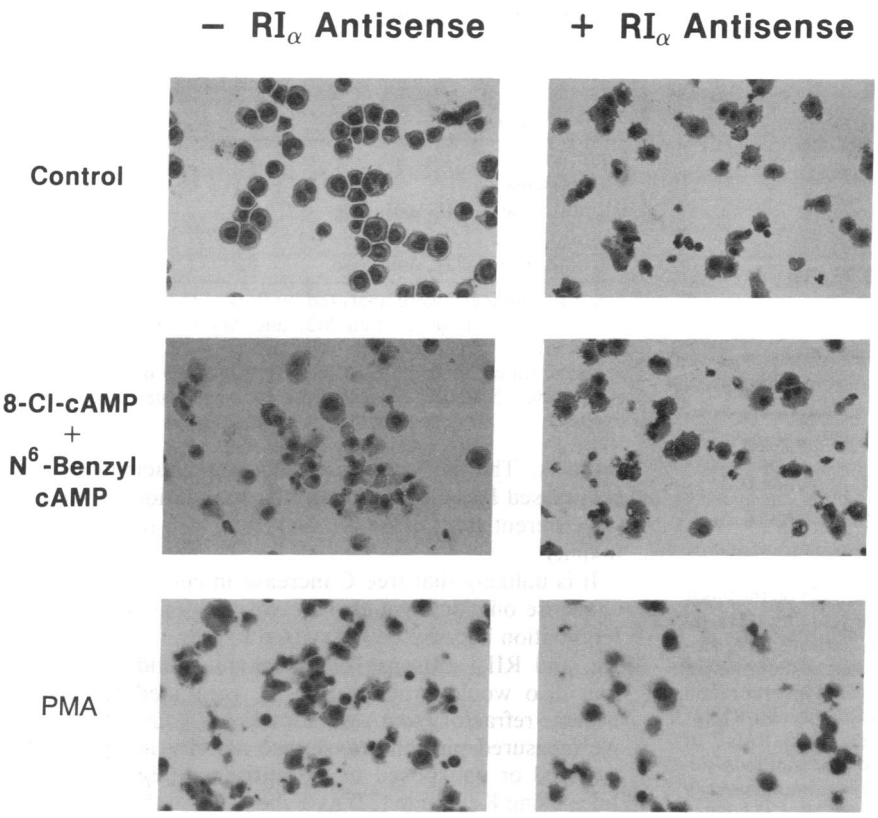


FIG. 2. Effect of RI_α antisense oligodeoxynucleotide on the morphologic transformation of HL-60 cells. Cells either exposed or unexposed to RI_α antisense oligodeoxynucleotide were treated with cAMP analogs or PMA as described in Fig. 1*B*. On day 4 (see Fig. 1*B*), cells were washed twice in Dulbecco's phosphate-buffered saline and were pelleted onto a glass slide by cytocentrifuge. The resulting cytopreparations were fixed and stained by Wright's stain. (×120.)

anti-RII_β antisera (17, 20) after photoaffinity labeling of these receptor proteins with [^{32}P]8-N_3-cAMP. In control cells, treatment with 8-Cl-cAMP plus N^6-benzyl-cAMP reduced RI_α ≈70% and increased RII_β 3-fold, resulting in a 10-fold increase in the ratio of RII_β/RI_α (Fig. 4) (28). Exposure of these cells to RI_α antisense oligodeoxynucleotide for 4 days brought about marked changes in both RI_α and RII_β levels; an 80% reduction in RI_α with a 5-fold increase in RII_β resulted in a 25-fold increase in the ratio of RII_β/RI_α compared with that in control cells (Fig. 4). Because growth inhibition and differentiation were appreciable after 3- to 4-day exposure to RI_α antisense oligomer, the changing levels of RI_α and RII_β proteins appear an early event necessary for commitment to differentiation.

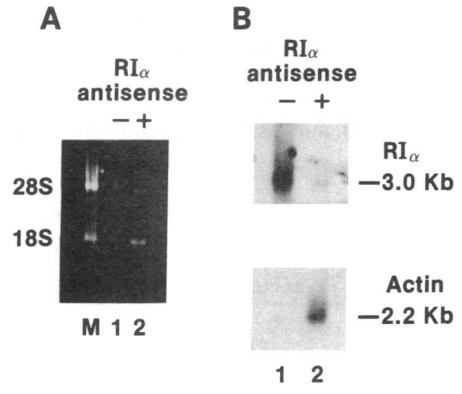
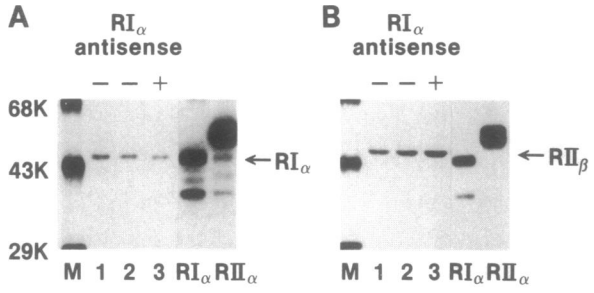


FIG. 3. Decreased RI_α mRNA expression in HL-60 leukemic cells exposed to RI_α antisense oligodeoxynucleotide. Cells were either exposed or unexposed to RI_α antisense oligodeoxynucleotide (15 μM) for 8 hr. Isolation of total RNA and Northern blot analysis followed the described methods. (*A*) Ethidium bromide staining of RNA. Lanes: M, markers of ribosomal RNAs; 1, cells unexposed to RI_α antisense oligomer; 2, cells exposed to RI_α antisense oligomer. (*B*) Northern blot analysis; the same nitrocellulose filter was hybridized to both RI_α and actin probes in sequential manner. Lanes: 1, cells unexposed to RI_α antisense oligomer; 2, cells exposed to RI_α antisense oligomer.

Data in Fig. 4 showed that suppression of RI_α by the antisense oligodeoxynucleotide brought about a compensatory increase in RII_β level. Such coordinated expression of RI and RII without changes in the amount of C has been shown (29, 30). The increase in RII_β may be responsible for the differentiation induced in these cells after exposure to RI_α antisense oligodeoxynucleotide. The increase in RII_β mRNA or RII_β protein level has been correlated with cAMP analog-induced differentiation in K-562 chronic myelocytic leukemic cells (31) and in erythroid differentiation of Friend erythrocytic leukemic cells (32). In a recent report (17), we have provided direct evidence that RII_β is essential for the cAMP-induced differentiation in HL-60 cells. HL-60 cells that were exposed to RII_β antisense oligodeoxynucleotide became refractory to treatment with cAMP analogs and continued to grow.

The essential role of RII_β in differentiation of HL-60 cells was further demonstrated when these cells were exposed to both RI_α and RII_β antisense oligodeoxynucleotides simultaneously. As shown in Table 1, RI_α antisense oligodeoxynucleotide markedly increased the expression of monocytic surface antigens [Leu 15 (33) and Leu M3 (34)] and decreased markers related to the immature myelogenous cells [My9 (35, 36)]. These changes in surface marker expression were abolished when cells were exposed simultaneously to both RI_α and RII_β antisense oligodeoxynucleotides (Table 1). RII_α cAMP receptor was not detected in HL-60 cells (37), and RII_α antisense oligodeoxynucleotide showed no interference with the effects of RI_α antisense oligomer (Table 1).

Cells exposed to both RI_α and RII_β antisense oligodeoxynucleotides were neither growth inhibited nor differentiated regardless of cAMP analog treatment. We interpret these results to reflect the blockage of cAMP-dependent growth regulatory pathway. Cells under these conditions are no longer cAMP-dependent but survive and proliferate probably through an alternate pathway. Thus, suppression of both RI_α



Lane	Treatment	Relative Levels RIα	RIIβ
1	Control	1.0±0.1	1.0±0.1
2	8-Cl + N^6-Benzyl	0.3±0.04	3.0±0.3
3	RI_α Antisense	0.2±0.03	5.0±0.4

FIG. 4. Effect of RI_α antisense oligodeoxynucleotide on the basal and induced levels of RI_α and RII_β cAMP receptor proteins in HL-60 leukemic cells. Cells were either exposed to RI_α antisense oligodeoxynucleotide (15 μM) or treated with cAMP analogs as described in Fig. 1. Preparation of cell extracts, the photoactivated incorporation of [^{32}P]8-N_3-cAMP and immunoprecipitation with the anti-RI_α or anti-RII_β antiserum and protein A–Sepharose, and SDS/PAGE of solubilized antigen–antibody complex followed the methods described. Preimmune serum controls were carried out simultaneously and showed no immunoprecipitated band. Lanes: M, ^{14}C-labeled marker proteins of known M_r; RI_α, 48,000 M_r RI (Sigma); RII_α, 56,000 M_r RII (Sigma). Lanes RI_α and RII_β are from photoaffinity labeling with [^{32}P]8-N_3-cAMP only; lanes 1–3, photoaffinity labeling with [^{32}P]8-N_3-cAMP followed by immunoprecipitation with anti-RI_α or anti-RII_β antiserum. 8-Cl, 8-Cl-cAMP (5 μM); N^6-benzyl, N^6-benzyl-cAMP (5 μM). Data in the table represent quantification by densitometric scanning of autoradiograms. Data are expressed relative to levels in control cells unexposed to RI_α antisense oligomer and untreated with cAMP analog, which are set equal to one arbitrary unit. Data represent an average ± SD of three experiments. Immunoprecipitation was with anti-RI_α (*A*) or anti-RII_β (*B*) antisera.

and RII_β gene expression led to an abnormal cellular growth regulation similar to that in those mutant cell lines (38) that contain either deficient or defective regulatory subunits of cAMP-dependent protein kinase and are no longer sensitive to cAMP stimulus.

Our results demonstrated that cAMP transduces signals for dual, either positive or negative, controls on cell proliferation, depending on the availability of RI_α or RII_β receptor proteins. The RI_α antisense oligodeoxynucleotide, which suppressed RI_α and enhanced RII_β expression, led to terminal differentiation of HL-60 leukemia with no sign of cytotoxicity.

Table 1. Modulation of differentiation markers in HL-60 cells by RI_α antisense oligodeoxynucleotide

Treatment	Surface markers, % positive Leu 15	Leu M3	My9
Control	10	2	100
RI_α antisense	80	98	80
RI_α antisense + RII_β antisense	11	2	100
RII_β antisense	13	3	100
RI_α antisense + RII_α antisense	85	100	80

Surface-antigens were analyzed by flow cytometry and monoclonal antibodies (Leu 15, Leu M3, and My9) reactive with either monocytic or myeloid cells. Approximately 2 × 10^4 cells were analyzed for each sample, and cells were gated by using forward and side scatter. Numbers represent the average values of three experiments.

It is unlikely that free C increase in cells exposed to RI_α antisense oligodeoxynucleotide was responsible for the differentiation because cells exposed to RII_β antisense or both RI_α and RII_β antisense oligodeoxynucleotides, conditions that also would produce free C, continued to grow and became refractory to cAMP stimulus. To directly verify this we measured phosphotransferase activity in cells that are exposed or unexposed to the antisense oligodeoxynucleotides using Kemptide (22) as a substrate in the presence and absence of a saturating concentration of cAMP and in the presence and absence of the heat-stable protein kinase inhibitor (23). This method of assay gives accurate determination of the relative levels of dissociated C and total C activity. Cell extracts from untreated HL-60 cells exhibited a very low level of dissociated C and were stimulated 36-fold by cAMP (Table 2). This cAMP-stimulated activity was almost completely inhibited by the heat-stable protein kinase inhibitor (Table 2), indicating that the total C activity measured was cAMP-dependent protein kinase. In cells exposed to RI_α antisense, RII_β antisense, or RI_α and RII_β antisense oligodeoxynucleotides, the free C activity was not increased as compared with unexposed control cells, although the total cAMP-stimulated activity differed slightly (Table 2). These results provide direct evidence that free C is not responsible for the differentiation seen in HL-60 cells.

Overexpression of RI_α cAMP receptor protein has also been found in most human breast and colon primary carcinomas examined (39), suggesting an important *in vivo* role of

Table 2. Protein kinase activity in HL-60 cells

Treatment	−cAMP Activity*	Relative to control	+cAMP Activity*	Relative to control	Stimulation, -fold
−PKI					
Control	23.0 ± 6.6	1.0	837 ± 87	1.0	36
RI_α antisense	22.9 ± 5.4	1.0	944 ± 18	1.1	41
RII_β antisense	22.8 ± 8.1	1.0	1028 ± 154	1.2	45
RI_α and RII_β antisense	24.3 ± 7.0	1.1	802 ± 36	1.0	33
+PKI					
Control	17.5 ± 8.7	1.0	37.0 ± 8.4	1.0	2.1
RI_α antisense	25.0 ± 8.8	1.4	22.6 ± 8.8	0.6	0.9
RII_β antisense	24.0 ± 2.6	1.4	24.8 ± 3.9	0.7	1.0
RI_α and RII_β antisense	19.0 ± 5.9	1.1	19.1 ± 8.2	0.5	1.0

Cells were exposed to 15 μM concentrations of RI_α, RII_β, or to 15 μM each of both antisense oligodeoxynucleotide for 4 days (see Fig. 1*A*). Data represent an average ± SD of duplicate determinations from three identical experiments. PKI, protein kinase inhibitor.

*pmol of phosphate transferred to kemptide per min/mg of protein.

RI_α cAMP receptor in tumor growth as well. However, the precise role of RI_α in cell proliferation is not yet known. RI_α may suppress RII_β production by titrating out C, or it may be a transducer of mitogenic signals leading to cell proliferation. Our results show that RI_α antisense oligodeoxynucleotide provides a useful genetic tool for studies on the role of cAMP receptor proteins in cell proliferation and differentiation and contribute to another approach in the control of malignancy.

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