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An antisense oligodeoxynucleotide targeted against the type II_β regulatory subunit mRNA of protein kinase inhibits cAMP-induced differentiation in HL-60 leukemia cells without affecting phorbol ester effects

(cAMP kinase/regulatory-subunit antisense)

GIAMPAOLO TORTORA, TIMOTHY CLAIR, AND YOON SANG CHO-CHUNG*

Cellular Biochemistry Section, Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **The type II_β regulatory subunit of cAMP-dependent protein kinase (RII_β) has been hypothesized to play an important role in the growth inhibition and differentiation induced by site-selective cAMP analogs in human cancer cells, but direct proof of this function has been lacking. To address this issue, HL-60 human promyelocytic leukemia cells were exposed to RII_β antisense synthetic oligodeoxynucleotide, and the effects on cAMP-induced growth regulation were examined. Exposure of these cells to RII_β antisense oligodeoxynucleotide resulted in a decrease in cAMP analog-induced growth inhibition and differentiation without apparent effect on differentiation induced by phorbol esters. This loss in cAMP growth regulatory function correlated with a decrease in basal and induced levels of RII_β protein. Exposure to RII_β sense, RI_α and RII_α antisense, or irrelevant oligodeoxynucleotides had no such effect. These results show that the RII_β regulatory subunit of protein kinase plays a critical role in the cAMP-induced growth regulation of HL-60 leukemia cells.**

Control mechanisms for cell growth and differentiation are disrupted in neoplastic cells (1–3). cAMP, an intracellular regulatory agent, has been considered to have a role in the control of cell proliferation and differentiation (4–7). Either inhibitory or stimulatory effects of cAMP on cell growth have been reported previously in studies in which cAMP analogs such as N^6-$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate or agents that raise intracellular cAMP to abnormal and continuously high levels were used, and available data are interpreted very differently (8–11).

Recently, we discovered that site-selective cAMP analogs, which show a preference for binding to purified preparations of type II rather than type I cAMP-dependent protein kinase *in vitro* (12, 13), provoke potent growth inhibition, differentiation, and reverse transformation in a broad spectrum of human and rodent cancer cell lines (14–16). The type I and type II protein kinases are distinguished by their regulatory subunits (RI and RII, respectively) (17, 18). Four different regulatory subunits [RI_α (previously designated RI) (19), RI_β (20), RII_α (RII_{54}) (21), and RII_β (RII_{51}) (22)] have now been identified at the gene/mRNA level (see ref. 23 for nomenclature). Two different catalytic subunits [C_α (24) and C_β (25, 26)] have also been identified; however, preferential coexpression of either one of these catalytic subunits with either the type I or type II protein kinase regulatory subunit has not been found (26).

The growth inhibition by site-selective cAMP analogs parallels reduction in RI_α with an increase in RII_β, resulting in an increase of the RII_β/RI_α ratio in cancer cells (27, 28). Such selective modulation of RI_α versus RII_β is not mimicked by treatment with N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate, a previously studied cAMP analog (27). The growth inhibition further correlates with a rapid translocation of RII_β to the nucleus and an increase in the transcription of the RII_β gene (27). These results support the hypothesis that RII_β plays an important role in the cAMP growth regulatory function (28).

To directly determine whether RII_β has a role in the cAMP analog-induced growth control of cancer cells, at least *in vitro*, we used the antisense strategy (29). This approach to the study of specific gene function has been used successfully to study the effects of c-*myc* (30, 31) and proliferating cell nuclear antigen (cyclin) (32) on cell proliferation and differentiation. We used 21-base-pair (bp) oligodeoxynucleotides complementary to either the sense or the antisense strands of the human RII_β mRNA transcript starting from the first codon (33, 34). As additional controls, we also used RI_α and RII_α antisense and irrelevant oligodeoxynucleotides of identical size.

The effect of exposing HL-60 cells to different oligodeoxynucleotides was assessed by two criteria: (*i*) we monitored the biological response—growth and differentiation—to cAMP analog treatment and (*ii*) we determined the ability of these cells to synthesize RII_β.

MATERIALS AND METHODS

Oligodeoxynucleotides. The 21-bp oligodeoxynucleotides used in the present studies were synthesized at Midland Certified Reagent (Midland, TX) and had the sequences of the following: human RII_β (35) antisense, 5′-CGC-CGG-GAT-CTC-GAT-GCT-CAT-3′; human RII_α (36) antisense, 5′-CGG-GAT-CTG-GAT-GTG-GCT-CAT-3′; human RI_α (37) antisense, 5′-GGC-GGT-ACT-GCC-AGA-CTC-CAT-3′; and the irrelevant oligodeoxynucleotide was a mixture of all four nucleotides at every position.

Cell Growth Experiment. Cells grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), streptomycin (500 μg/ml), and 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 4–7 days were reseeded (day 0) at 5×10^5 cells per dish, and cells preexposed to the oligodeoxynucle-

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; RI and RII, type I and type II regulatory subunits of cAMP-dependent protein kinase; 8-N_3cAMP, 8-azidoadenosine 3′,5′-cyclic monophosphate; 8-Cl-cAMP, 8-chloroadenosine 3′,5′-cyclic monophosphate; N^6-benzyl-cAMP, N^6-benzyladenosine 3′,5′-cyclic monophosphate.

*To whom reprint requests should be addressed.

otide were further treated with the oligomer at day 0 and day 2. cAMP analogs (kindly 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 Mono[^{32}P]phosphate (8-N_3-[^{32}P]cAMP). Cell extracts were prepared at 0–4°C. The cell pellets (2×10^6 cells), after two washes with phosphate-buffered saline (PBS), 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/2 kallikrein inhibitor units of bovine aprotinin per ml/20 mM Tris·HCl, pH 7.4) containing proteolysis inhibitors (0.1 mM pepstatin, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, and 0.5 mg/ml soybean trypsin inhibitor), mixed in a Vortex, 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 8-N_3-[^{32}P]cAMP (60.0 Ci/mmol; 1 Ci = 37 GBq) was performed as described (38) at cAMP exchange conditions of 23°C for 45 min, using 25 μg of sample proteins (lysates) in a total volume of 50 μl in buffer Ten. After ultraviolet irradiation for 30 s, 5 μl of 10 mM unlabeled cAMP was added to the reaction mixture, and immunoprecipitation was performed using the anti-RII_β antiserum [described in ref. 39 as rat skeletal muscle RII antibody that specifically cross-reacts with RII_α and RII_β of rat brain but selectively cross-reacts with only RII_β of LS-174T human colon carcinoma cells (27), and this antibody also cross-reacts with RII_β of HL-60 leukemia cells] and protein A-Sepharose by the method described in ref. 39. The pellets of antigen–antibody complex, after three washes with PBS (500 μl each wash), were solubilized, subjected to SDS/PAGE (40), and transferred to nitrocellulose sheets (41). The nitrocellulose sheets were air dried and exposed to Kodak XAR film for 12–36 hr at −20°C.

RESULTS

Effect of Oligodeoxynucleotides on Cell Growth and Response to cAMP Analog Treatment. Exposure of HL-60 cells to RII_β antisense oligodeoxynucleotide (15 μM) for 7 days did not alter the basal rate of cell growth. Cells either exposed or unexposed to RII_β antisense oligomer grew at the same rate (Fig. 1*A*). The RII_β antisense oligodeoxynucleotide had an immediate effect on the response of these cells to cAMP analog treatment. By day 5, cells unexposed to RII_β antisense oligodeoxynucleotide demonstrated 60–70% growth inhibition in response to 8-Cl-cAMP treatment and 80% growth inhibition by 8-Cl-cAMP in combination with N^6-benzyl-cAMP (Fig. 1*B*) (15). In contrast, cells exposed to RII_β antisense oligodeoxynucleotide exhibited only 20% growth inhibition after 8-Cl-cAMP treatment, and this growth inhibition was not enhanced by the treatment with 8-Cl-cAMP plus N^6-benzyl-cAMP (Fig. 1*B*). RII_β sense, RII_α antisense, or irrelevant oligodeoxynucleotides had no such interfering effect on the growth inhibitory effect of these cAMP analogs.

RII_β antisense oligodeoxynucleotide, however, did not interfere with the effect of phorbol esters. Cells either exposed or unexposed to RII_β antisense oligomer exhibited the same 70% growth inhibition after treatment with PMA (Fig. 1*B*).

We examined whether the loss in the ability of HL-60 cells to respond to cAMP analogs correlated with the concentration of oligodeoxynucleotide. Cells were exposed for 4 days to 0.3, 1.4, 7, and 15 μM RII_β antisense oligodeoxynucleotide and then exposed to 8-Cl-cAMP plus N^6-benzyl-cAMP in the continued presence of the oligomer at respective concentrations. There was a clear dose-dependent response between increasing amounts of RII_β antisense oligodeoxynucleotide and the degree of loss in the growth inhibitory response. Exposure to RII_β antisense oligomer at the concentrations mentioned above led to growth inhibition of 80% ± 7%, 75% ± 5%, 65% ± 5%, and 20% ± 2%, respectively.

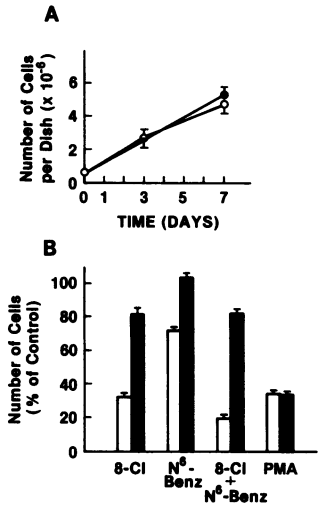

FIG. 1. Effect of RII_β antisense oligodeoxynucleotide on the basal rate of growth of HL-60 leukemia cells (*A*) and the growth of these cells when treated with cAMP analogs or PMA (*B*). (*A*) Cells grown in suspension culture were seeded at 5×10^5 cells per dish and exposed to oligodeoxynucleotides as described in the text. Cells were grown in the absence (○) or presence (●) of RII_β antisense oligodeoxynucleotide (15 μM). Data represent the average values ± SD of four experiments. (*B*) On day 7 of the experiment in *A*, cells exposed and unexposed to RII_β oligodeoxynucleotide were reseeded (day 0) at 5×10^5 cells per dish, and cells preexposed to RII_β antisense oligodeoxynucleotide were further treated with the oligomer at day 0 and day 2. cAMP analogs and PMA were added one time at day 0. Cell counts were performed on a Coulter Counter on day 4. 8-Cl, 8-chloroadenosine 3',5'-cyclic monophosphate (8-Cl-cAMP), 10 μM; N^6-Benz, N^6-benzyladenosine 3',5'-cyclic monophosphate (N^6-benzyl-cAMP), 10 μM; 8-Cl + N^6-Benz, 8-Cl-cAMP 5 μM plus N^6-benzyl-cAMP, 5 μM; PMA, 0.01 μM. Cells were grown in the absence (□) or presence (■) of RII_β antisense oligodeoxynucleotide (15 μM). Data are expressed as the percentage of the number of cells in control cells that received no cAMP analog or PMA treatment and represent the average values ± SD of four experiments.

We also determined the time dependence of the effect of RII_β antisense oligodeoxynucleotide. Cells were preexposed to RII_β antisense oligomer for 2, 4, and 7 days and treated for 3 days with 8-Cl-cAMP plus N^6-benzyl-cAMP in the continued presence of the oligomer. Exposure to RII_β antisense oligodeoxynucleotide for the preexposure times mentioned above led to growth inhibition of 53% ± 5%, 30% ± 4%, and 24% ± 3%, respectively, demonstrating the time-dependent effect of RII_β antisense oligomer.

Effect of Oligodeoxynucleotides on cAMP- and PMA-Induced Morphologic Change. HL-60 cells undergo a monocytic morphologic transformation upon treatment with site-selective cAMP analogs (15). Cells either exposed or unexposed to RII_β antisense oligodeoxynucleotide were treated with cAMP analogs and examined for their morphology. As shown in Fig. 2, cells unexposed to RII_β antisense oligomer, 8-Cl-cAMP, especially in combination with 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. In contrast, cells exposed to RII_β antisense oligodeoxynucleotide exhibited no morphologic change upon treatment with cAMP analogs (Fig. 2). Exposure to RII_β sense, RII_α antisense, or irrelevant oligodeoxynucleotides did not interfere with the monocytic differentiation of these cells. PMA, however, induced the monocytic morphologic transforma-

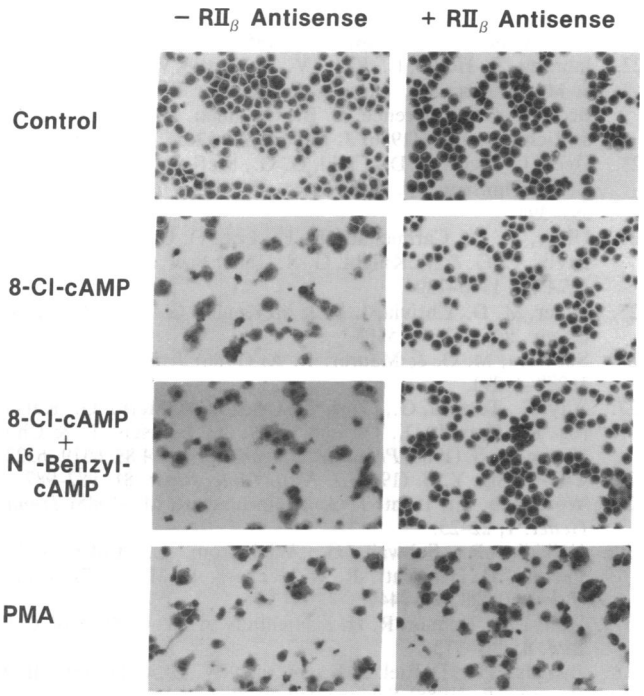

FIG. 2. Effect of RII_β antisense oligodeoxynucleotide on the monocytic morphologic transformation of HL-60 cells. Cells either exposed or unexposed to RII_β antisense oligodeoxynucleotide were treated with cAMP analogs or PMA as described in the legend to Fig. 1*B*. On day 4 (see Fig. 1*B*), cells were washed twice in Dulbecco's PBS and were pelleted onto a glass slide by cytocentrifuge. The resulting cytopreparations were fixed and stained by Wright's stain. (×30.) Data present one of two identical experiments.

tion equally in cells either exposed or unexposed to the RII_β antisense oligodeoxynucleotide (Fig. 2). Thus, RII_β antisense oligodeoxynucleotide specifically inhibited the differentiation induced by cAMP without affecting the ability of these cells to respond to phorbol esters.

Effect of Oligodeoxynucleotides on RII_β cAMP Receptor Synthesis. We have previously shown that during growth inhibition by site-selective cAMP analogs, there is an increase in the rate of transcription of RII_β gene (27) and an increase in the amounts of RII_β mRNA and protein (27, 42). To provide more evidence that the inability of these leukemia cells to respond to cAMP analog treatment described above was due to an intracellular effect of RII_β antisense oligodeoxynucleotide, we determined the basal and induced levels of RII_β in these cells when either unexposed or exposed to the RII_β antisense oligodeoxynucleotide.

The levels of RII_β cAMP receptor protein were determined by immunoprecipitation of RII_β with anti-RII_β antiserum (39) after photoaffinity labeling (38) of the cellular cAMP receptor proteins with 8-N_3-[^{32}P]cAMP. As shown in Fig. 3, in cells unexposed to RII_β antisense oligodeoxynucleotide a single major band of RII_β was detected (lane 1), and the band intensity markedly increased after treatment with 8-Cl-cAMP (lane 2) or 8-Cl-cAMP plus N^6-benzyl-cAMP (lane 3). In contrast, cells exposed to RII_β antisense oligodeoxynucleotide contained no detectable levels of RII_β (lane 9) and cAMP analogs could not induce RII_β (lanes 10 and 11). RII_β sense (lanes 5–7), RI_α and RII_α antisense, or irrelevant oligodeoxynucleotides (data not shown) had no such interfering effect on either the basal or the induced levels of RII_β. As expected, PMA had no effect on the RII_β levels (lanes 4, 8, and 12).

DISCUSSION

In the present work, we have provided direct evidence that the RII_β regulatory subunit of cAMP-dependent protein

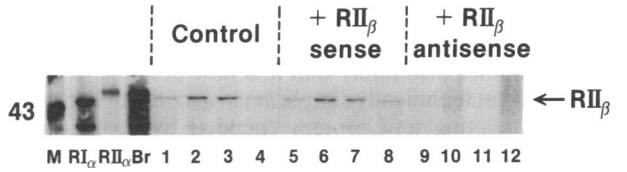

FIG. 3. Effect of RII_β sense or antisense oligodeoxynucleotide on the basal and induced levels of RII_β cAMP receptor in HL-60 leukemia cells. Cells were exposed to RII_β sense or antisense oligodeoxynucleotide (15 μM) and treated with cAMP analogs or PMA as described in the legend to Fig. 1*B*. Cell extracts were prepared and the photoaffinity labeling with 8-N_3-[^{32}P]cAMP and immunoprecipitation with the anti-RII_β antiserum and protein A-Sepharose were performed. The pellets of antigen–antibody complex, after three washes with PBS (500 μl each wash), were solubilized, subjected to SDS/PAGE (40), and transferred to nitrocellulose sheets (41). The nitrocellulose sheets were air dried and exposed to Kodak XAR film for 12–36 hr at −20°C. Preimmune serum controls were carried out simultaneously and detected no immunoprecipitated band. M, ^{14}C-labeled marker proteins of known molecular weight (shown as $M_r \times 10^{-3}$); RI_α, the M_r 48,000 RI (Sigma); RII_α, the M_r 56,000 RII (Sigma); Br, extracts of rat brain. Lanes 1, 5, and 9, untreated with either cAMP analog or PMA; lanes 2, 6, and 10, treatment with 8-Cl-cAMP (10 μM); lanes 3, 7, and 11, treatment with 8-Cl-cAMP (5 μM) plus N^6-benzyl-cAMP (5 μM); lanes 4, 8, and 12, treatment with PMA (0.01 μM). Lanes RI_α, RII_β, and Br are from photoaffinity labeling with 8-N_3-[^{32}P]cAMP only; lanes 1–12, photoaffinity labeling with 8-N_3-[^{32}P]cAMP followed by immunoprecipitation with anti-RII_β antiserum (39). Data present one of two identical experiments.

kinase is required for the growth inhibition and differentiation induced by cAMP analogs in human HL-60 leukemia cells *in vitro*. These studies suggest an important *in vivo* role for the RII_β cAMP receptor in the growth regulatory function of cAMP as well.

The role of RII_β cAMP receptor was specific toward cAMP growth regulatory function, since RII_β antisense oligodeoxynucleotide inhibited cAMP-induced differentiation only, without affecting differentiation of these cells by other means, such as that by phorbol esters.

The specific effect of the RII_β among the isoforms of the regulatory subunits of cAMP-dependent protein kinase was provided by the observation that RI_α or RII_α antisense oligodeoxynucleotide could not mimic the effect of RII_β antisense oligodeoxynucleotide. In fact, RI_α antisense oligodeoxynucleotide produced an effect opposite to that of RII_β antisense oligodeoxynucleotide. Exposure to RI_α antisense oligodeoxynucleotide led to growth inhibition and differentiation of the leukemia cells, bypassing the effect of exogenous cAMP analogs (unpublished observations). These results suggest that RII_β cAMP receptor, but not RI_α or RII_α, is the mediator of cAMP growth regulatory function in HL-60 leukemia cells. Determination of whether the RII_β cAMP plays such a pivotal role in the growth regulation of other cancer cells as well as normal cells awaits further investigation.

The function served by RII_β cAMP receptor remains unclear. Based on our previous observation (27) that RII_β rapidly translocates from the cytoplasm to the nucleus during cAMP analog-induced growth inhibition, we hypothesize that RII_β may be the modulator of gene transcription involved in the regulation of cell proliferation and differentiation. Such function of RII_β is indirectly supported by the previous findings (43) that the somatostatin gene, which contains the cAMP-responsive element, does not respond to cAMP in mutant PC12 cells (44), which lack cAMP-dependent protein kinase type II activity but contain the type I protein kinase activity. We cannot exclude, however, a more subtle effect of RII_β. It may merely regulate phosphorylation of proteins involved in cell proliferation by way of modulating the

activation or intracellular localization of the catalytic subunit of protein kinase.

These studies also imply that it is now possible to employ a powerful yet technically simple *in vitro* model to explore the effect of deleting any protein encoded by specific genes involved in the control of cell proliferation and differentiation.

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