

Induction of megakaryocytic differentiation and modulation of protein kinase gene expression by site-selective cAMP analogs in K-562 human leukemic cells

(cAMP receptor mRNAs/*c-myc* mRNA/glycoprotein II_b-III_a complex)

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ABSTRACT Two classes (site 1- and site 2-selective) of cAMP analogs, which either alone or in combination demonstrate a preference for binding to type II rather than type I cAMP-dependent protein kinase isozyme, potently inhibit growth in a spectrum of human cancer cell lines in culture. Treatment of K-562 human leukemic cells for 3 days with 30 and 10 μ M 8-chloroadenosine 3',5'-cyclic monophosphate (8-Cl-cAMP) (site 1-selective) resulted in 60% and 20% growth inhibition, respectively (with over 90% viability). *N*⁶-Benzyl-cAMP (site 2-selective) (30 μ M) treatment resulted in 20% growth inhibition by day 3. When 8-Cl-cAMP (10 μ M) and *N*⁶-benzyl-cAMP (30 μ M) were both added, growth was almost completely arrested. The growth inhibition was accompanied by megakaryocytic differentiation in K-562 cells. The untreated control cells expressed little or no detectable levels of glycoprotein II_b-III_a surface antigen complex. 8-Cl-cAMP (30 μ M) treatment for 3 days substantially increased the antigen expression, while *N*⁶-benzyl-cAMP caused little or no change in the antigen expression. When cells were treated with 8-Cl-cAMP in combination with *N*⁶-benzyl-cAMP, antigen expression was synergistically enhanced, and cells demonstrated megakaryocyte morphology. By Northern blotting, we examined the mRNA levels of the type I and type II protein kinase regulatory subunits (RI _{α} and RII _{β}), the catalytic subunit, and *c-myc* during 8-Cl-cAMP treatment. The steady-state level of RII _{β} cAMP receptor mRNA sharply increased within 1 hr of treatment and remained elevated for 3 days, while that of the RI _{α} receptor markedly decreased to below control level within 6 hr and remained low during treatment. However, 8-Cl-cAMP did not affect the mRNA level of the catalytic subunit. 8-Cl-cAMP treatment also brought about a rapid decrease in *c-myc* mRNA. Thus, differential regulation of cAMP receptor genes is an early event in cAMP-induced differentiation and growth control of K-562 leukemia cells.

A major defect of cancer cells is their inability to differentiate normally (1–4). This defect may be partly reversible, and study of such models may help to elucidate the mechanism responsible for differentiation. The K-562 human leukemic cell line, which carries a Philadelphia chromosome and was established from a patient with chronic myelogenous leukemia in blast crisis (5), is such a model. This cell line is considered to consist of a multipotent hematopoietic stem cell, since it responds to inducers of erythroid differentiation (6–8), megakaryocytic differentiation (9, 10), and granulo-

cytic differentiation (11, 12). The cellular mechanisms responsible for these processes, however, are not known.

cAMP, an intracellular regulatory agent, has long been considered to have a role in the control of cell proliferation and differentiation in a variety of cell types (13–16). cAMP exerts its effect in eukaryotes by binding to the regulatory subunits of either type I or type II cAMP-dependent protein kinase (17, 18). Differential expression of these protein kinase isozymes has been observed during cell development, differentiation, and neoplastic transformation (19–22).

Recently, we discovered that site-selective cAMP analogs, which show a preference for binding to type II rather than type I protein kinase of purified preparations *in vitro* (23, 24), provoke potent growth inhibition, differentiation, and reverse transformation in a broad spectrum of human and rodent cancer cell lines (25–27). Furthermore, these effects were synergistically enhanced when two classes of cAMP analog (site 1- and site 2-selective) were used in combination (25–27). 8-Chloroadenosine 3',5'-cyclic monophosphate (8-Cl-cAMP), the most potent site-selective cAMP analog, is now in preclinical phase I studies at the National Cancer Institute.

There have been no previous studies that have demonstrated the potential for cAMP or cAMP analogs to induce differentiation in K-562 stem cell leukemia. In this study, we correlated the growth inhibitory effect of site-selective cAMP analogs in K-562 cells with their effect on the induction of differentiation as well as the expression of the regulatory and catalytic genes of protein kinase isozymes and the growth-associated oncogene *c-myc*.

MATERIALS AND METHODS

Cell Culture and Morphological Studies. K-562 cells (American Type Culture Collection) were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), streptomycin (500 μ g/ml), and 1 mM glutamine (GIBCO) at 5×10^5 cells per ml in a humidified 5% CO₂ atmosphere. For cell growth experiments, cells were treated with cAMP analogs (28) once at 3 hr after seeding, and cell counts were performed in duplicate on a Coulter Counter at indicated times. Cell viability was determined by the trypan-blue exclusion method. Cell morphology studies were performed on cytocentrifuged slides stained with Wright-Giemsa stain.

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Abbreviations: 8-Cl-cAMP, 8-chloroadenosine 3',5'-cyclic monophosphate; RI _{α} and RII _{β} , type I and II protein kinase regulatory subunits; C _{α} , protein kinase catalytic subunit.

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Cell Surface Antigen Analysis. Cell surface antigens were analyzed by indirect immunofluorescence, using a FACSTAR flow cytometer (Becton Dickinson FACS Systems) (29). The following antibodies were used: B4, My4, My7, My9 (Coulter Immunology); 10E5 (kindly provided by Barry Collier, University of New York, Stony Brook); and anti-glycophorin A (Amac, Westbrook, ME). After incubation at 4°C with the primary antibody, cells (10^4 cells per sample) were washed and allowed to react with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at 4°C. A nonimmune mouse ascites preparation was used as a control for nonspecific reactivity of the primary antibody. After several washes, cells were analyzed on a FACSTAR flow cytometer.

Northern Blot Analysis. Total cellular RNA was obtained from cells (10^8 cells washed twice with Dulbecco's phosphate-buffered saline) lysed with 4 M guanidium isothiocyanate by centrifugation through a CsCl cushion as described by Chirgwin *et al.* (30). Total cellular RNA (20 μ g per lane) was denatured in 50% formamide/6% formaldehyde (vol/vol) by heating for 15 min at 50°C followed by 15 min on ice, fractionated by electrophoresis through a 1.5% agarose gel containing 6.7% formaldehyde in 20 mM sodium phosphate, pH 7.0, and blotted by the method of Thomas onto nitrocellulose filters (31). Hybridization was performed at 37°C over 16 hr in 50% formamide/1 \times Denhardt's solution/4 \times SSC (Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone; SSC = 0.15 M NaCl/0.015 M sodium citrate). After hybridization, filters were washed in 0.2 \times SSC/0.1% NaDodSO₄ at 65°C three times per hr and once per hr at 65°C with 1 \times SSC. Autoradiography was performed at -70°C with Kodak X-Omat AR film. The nick-translated probes were obtained from Lofstrand Laboratories (Gaithersburg, MD). The type I protein kinase regulatory subunit RI α (see ref. 32 for nomenclature) probe was a 0.6-kilobase (kb) *Pst* I fragment (mouse), entirely from inside the open reading frame (33), and the catalytic subunit (C α) probe was a 0.6-kb *Eco*RI fragment (mouse) containing a 3' coding region and about 160 base pairs (bp) of 3' nontranslating sequence (34) (kindly provided by S. McKnight, University of Washington, Seattle). The human RII β probe was a 1.6-kb *Bam*HI fragment derived from a cDNA clone (35), spanning the 3' half of the open reading frame and part of the nontranslating region. The *c-myc* probe was a recombinant plasmid pRyc 7.4 (36), containing a portion of *c-myc* exon II and exon III (kindly provided by C. M. Croce, Wistar Institute, Philadelphia).

RESULTS

Effect of cAMP Analogs on Cell Growth. Site-selective cAMP analogs inhibit the growth of several human leukemic cell lines in a concentration-dependent manner (26). The growth curves of the K-562 leukemic cell line in the absence and presence of cAMP analogs are shown in Fig. 1A. 8-Cl-cAMP (site 1-selective) at 30 μ M produced 60% growth inhibition by 72 hr. *N*⁶-Benzyl-cAMP (site 2-selective) at 30 μ M produced a 20% growth inhibition. When 8-Cl-cAMP (10 μ M), which alone gave a 20% growth inhibition (26), was added in combination with *N*⁶-benzyl-cAMP (30 μ M), cell growth was almost completely stopped. Thus, the growth of K-562 leukemic cells was strongly inhibited by site 1-selective 8-Cl-cAMP alone and also synergistically inhibited by site 1- and site 2-selective cAMP analogs in combination, as was previously shown (25) in human colon and breast cancer cell lines.

Effect of cAMP Analogs on Differentiation. We examined the effect of site-selective cAMP analogs on the expression of differentiation markers in K-562 cells to determine whether

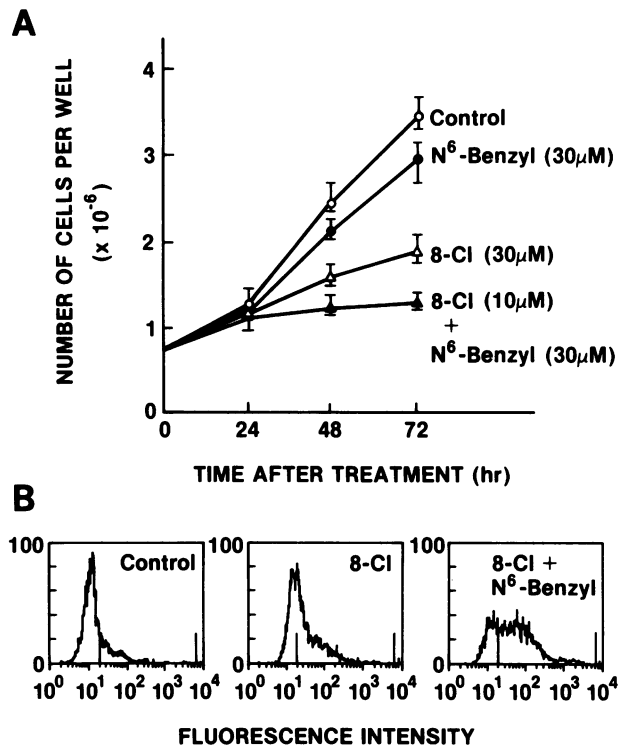


FIG. 1. Effect of cAMP analogs on the growth and differentiation of K-562 cells. (A) Growth curves in the absence and presence of the cAMP analogs *N*⁶-benzyl-cAMP (*N*⁶-Benzyl) and 8-Cl-cAMP (8-Cl) at given concentrations. Points represent the means of triplicate determinations; bars, SD. (B) Flow cytometric analysis of glycoprotein II_b-III_a surface marker. Cells were treated with cAMP analogs (30 μ M) as in A once at 3 hr after seeding, were harvested after 3 days, and were allowed to react with the monoclonal antibody 10E5 (specific for the glycoprotein II_b-III_a complex of megakaryocytes). The indirect immunofluorescence was measured by FACSTAR flow cytometry.

growth-inhibited cells are more differentiated than the untreated cells. Megakaryocytic differentiation was assessed by flow cytometric analysis with the monoclonal antibody 10E5, which recognizes the glycoprotein II_b-III_a complex, a specific megakaryocytic marker expressed in the differentiation of this lineage (37, 38). Treatment for 3 days with 8-Cl-cAMP (30 μ M) induced a marked increase in the fraction of cells positive for the glycoprotein II_b-III_a antigen (Fig. 1B, Table 1). Untreated cells expressed little or no detectable level of this antigen. *N*⁶-Benzyl-cAMP (30 μ M) treatment alone did not induce an appreciable increase of this antigen expression (data not shown), but when *N*⁶-benzyl-cAMP (30 μ M) was added with 8-Cl-cAMP (10–30 μ M), the majority of cells expressed this antigen (Fig. 1B). This agrees with the

Table 1. Modulation of differentiation markers in K-562 cells by 8-Cl-cAMP

Cell lineage	Surface marker	No. of positive cells	
		Control	8-Cl-cAMP
Megakaryocytic	Glycoprotein II _b -III _a	-	++
Erythrocytic	Glycophorin A	++	+
Lymphocytic	B4	-	+
Myelomonocytic	My4	+	+
Myelocytic	My7	++	+
Myelocytic	My9	++	+

Cells were treated with 8-Cl-cAMP (30 μ M) once at 3 hr after seeding, were harvested at 3 days, and were allowed to react with monoclonal antibodies to the indicated surface markers. Indirect immunofluorescence was determined by flow cytometry. Number of positive cells: -, <15%; +, \approx 30%; ++, \approx 50%.

synergistic effect of 8-Cl-cAMP and *N*⁶-benzyl-cAMP on growth inhibition (Fig. 1A). Moreover, glycoprotein II_b-III_a expression was accompanied by morphological changes typical of megakaryocytes (data not shown). 8-Cl-cAMP also induced B4 lymphocytic marker antigen, but it either decreased or produced no change in the expression of myelocytic and monocytic differentiation antigens (Table 1). Thus, 8-Cl-cAMP induced megakaryocytic differentiation in K-562 leukemic cells in a lineage-specific manner, as was shown previously with phorbol esters (39).

mRNA Levels of Protein Kinase Subunits. mRNA levels for the regulatory and catalytic subunits of cAMP-dependent protein kinase isozymes were examined in K-562 cells after treatment with 8-Cl-cAMP. The RNA filters prepared were probed with the radioactive cDNAs of protein kinase subunits; the Northern blot analysis is shown in Fig. 2. The mouse RI_α (regulatory subunit of protein kinase type I) cDNA probe detected 3.0- and 1.7-kb mRNA bands in the untreated control K-562 cells. 8-Cl-cAMP treatment resulted in a decrease in the intensity of both bands to 50% of that of untreated control cells by 6 hr, after a 50% transient increase at 1 hr (see densitometry data in Fig. 2). With the human RII_β (the *M_r* 51,000 regulatory subunit of protein kinase type II) cDNA probe, a single mRNA band at 3.2 kb was detected. Within 1 hr of 8-Cl-cAMP treatment, the band intensity increased 4-fold over the control, and the mRNA remained elevated during treatment (see densitometry data in Fig. 2). Thus, the RI_α and RII_β mRNA levels changed in an inverse manner in response to 8-Cl-cAMP treatment. Using the

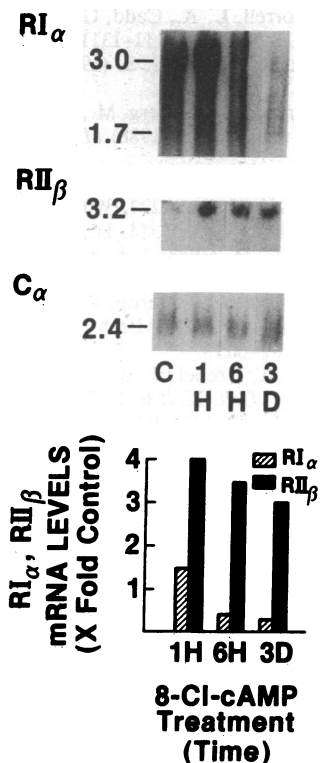
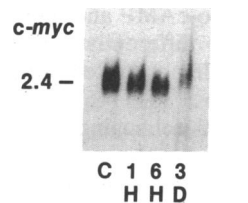


FIG. 2. Northern blot analysis of the regulatory (RI_α and RII_β) and catalytic (C_α) mRNAs in K-562 cells during 8-Cl-cAMP treatment. Cells were treated with 8-Cl-cAMP (30 μM) once at 3 hr after seeding and harvested at given times after treatment, and total RNA was extracted by the guanidinium isothiocyanate method. Twenty micrograms of total RNA per lane were run on 1.5% agarose/formaldehyde gels and blotted on nitrocellulose. The bar graph represents densitometric scanning of the autoradiograms; the data are expressed as X-fold of the levels in treated control cells. (Both 3.0- and 1.7-kb bands were counted for RI_α mRNA quantification.) Similar data were obtained by scintillation counting of bands. C, untreated control cells grown for 3 days; H, hour; D, days.



LANE	c-myc mRNA (Relative Level)
C	1.0 ± 0.15
1H	0.1 ± 0.02
6H	0.1 ± 0.02
3D	0.02 ± 0.005

FIG. 3. Northern blot analysis of *c-myc* mRNA in K-562 cells during 8-Cl-cAMP (30 μM) treatment. Cells were treated and harvested at the given times after treatment, and the total RNA fractionated on agarose gels was blotted on nitrocellulose as described in the legend to Fig. 2. The data in the table were from densitometry of autoradiograms, where the untreated control cell level (C) was set as 1.0 arbitrary unit; the values represent mean ± SEM of six separate experiments.

mouse cDNA probe for C_α, we detected a 2.4-kb mRNA. The band intensity showed no appreciable change during 8-Cl-cAMP treatment (Fig. 2).

c-myc mRNA Level. Nuclei-associated oncogenes are regulated by proliferative signals (40), and a decrease in *c-myc* mRNA has been shown to be a critical event in differentiation of leukemic cells (41). 8-Cl-cAMP treatment of K-562 cells brought about a sharp decrease in the *c-myc* mRNA: within 1 hr of treatment, the mRNA decreased to 0.1 of the control, and the level was almost undetectable (<0.02 control) by 3 days (Fig. 3).

DISCUSSION

We have shown here that site-selective cAMP analogs induce growth inhibition and differentiation in the K-562 chronic myelocytic leukemic cell line. Two classes of cAMP analogs, the site 1-selective 8-Cl-cAMP and the site 2-selective *N*⁶-benzyl-cAMP, demonstrated a synergistic effect when combined. This synergism has previously been found in differentiation of HL60 and MOLT 4 leukemia cell lines (26) and in the inhibition of various types of human cancer cell lines (25). This analog effect has been correlated with the selective increase of type II protein kinase holoenzyme and its RII regulatory subunit, and the decrease of type I protein kinase and the RI regulatory subunit in the cancer cells (25-27); this parallels the analogs' preferential binding to protein kinase type II rather than type I *in vitro* (42-44).

The results of this study demonstrate that the effect of 8-Cl-cAMP on growth inhibition and differentiation correlates with differential expression of the genes for the regulatory subunits (RI_α and RII_β) of protein kinase isozymes. The analog treatment induced an increase in the RII_β mRNA along with a decrease in the RI_α mRNA. These changes occurred within a few hours of analog treatment, indicating that the changes are early events rather than the consequences of growth inhibition and differentiation.

The occurrence of a selective increase in the expression of the RII_β gene but not in that of the RI_α gene during differentiation of K-562 cells is in agreement with the results of other studies. In Friend erythroleukemic cells, two species of the regulatory subunits of type II protein kinase, RII_α (*M_r* = 54,000) and RII_β (*M_r* = 51,000-52,000), were expressed, but only the concentration of RII_β increased in response to

treatment with 8-bromo-cAMP and methylisobutylxanthine or when stimulated to differentiate (45). Increase of RII β mRNA has also been demonstrated during hormonal differentiation of rat granulosa cells (46).

The analog effect on the changing levels of the RII β and RI α mRNAs may be due to changes in gene transcription, stabilization of these mRNAs, or both. In LS-174T human colon cancer cells, the 8-Cl-cAMP-induced growth inhibition accompanied an increased transcription of the RII β gene and decreased transcription of the RI α gene (44). However, these changes in the rates of transcription of RI α and RII β were preceded by nuclear translocation of RII β protein, which occurred within 10 min of 8-Cl-cAMP treatment, while the changes in the transcription of the cAMP receptor genes were not detected (44). Nuclear translocation of cAMP receptor proteins, which has been previously observed during cell development (19), tumor regression (22), and reverse transformation (47), suggests the possible role of these proteins in gene regulation.

Both cAMP-dependent protein kinase and protein kinase C have been shown to play a role in the transcription regulation of the cAMP-responsive element (48). We showed here that site-selective cAMP analogs induced megakaryocytic differentiation in K-562 cells in a lineage-specific manner, as was shown previously by phorbol esters (39). The fact that both cAMP analogs and phorbol esters induce megakaryocytic differentiation of leukemic cells suggests either separate or coordinated roles of cAMP-dependent protein kinase and protein kinase C in cell differentiation. During 8-Cl-cAMP-induced differentiation of K-562 cells, a marked decrease in *c-myc* mRNA also occurred, indicating that 8-Cl-cAMP antagonized proliferative signal(s). The results reported here suggest that cAMP receptor proteins may have a major role in regulating the cascade of events that affect expression of the differentiation program in leukemic cells.

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