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Regulation of Proliferation in a Murine Colony-Stimulating Factor-Dependent Myeloid Cell Line: Superinduction of C-*fos* by the Growth Inhibitor 8-Br-Cyclic Adenosine 3′:5′ Monophosphate

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We have investigated the effect of 8-Br-cyclic adenosine 3′:5′ monophosphate (cAMP), a pharmacological activator of cAMP-dependent protein kinase, on the proliferation and the nuclear proto-oncogene induction in a murine granulocyte macrophage colony-stimulating factor (GM-CSF)-dependent myeloid cell line. Cells were growth arrested by granulocyte macrophage colony-stimulating factor and serum deprivation and were allowed to proceed in the cell cycle by addition of the lymphokine in the presence or absence of 8-Br-cAMP. ^{3}H-thymidine incorporation assays showed that addition of 8-Br-cAMP inhibited the entry of cells into S phase and the subsequent proliferation. Northern analysis showed that 8-Br-cAMP had opposite effects on c-*fos* and c-*myc* mRNA induction. 8-Br-cAMP induced c-*fos* in the absence of any GM-CSF. In the presence of GM-CSF, c-*fos* mRNA was superinduced (30-fold induction compared to four- to fivefold by each signal alone). On the contrary, 8-Br-cAMP was not able to induce c-*myc* in the absence of growth factor and hardly interfered with the induction of c-*myc* by GM-CSF. Phorbol myristate acetate (PMA), a pharmacological activator of the lipid and CA^{++}-dependent protein kinase C, was shown to induce nuclear proto-oncogene mRNA in the GM-CSF-dependent cell line. We investigated the effect of 8-Br-cAMP on PMA-induced c-*fos* and c-*myc* mRNA levels. When both cAMP dependent and lipid-dependent kinase systems were co-stimulated in the absence of GM-CSF, c-*fos* message was again superinduced (60-fold induction). On the contrary, c-*myc* message induction by PMA was inhibited by 80% by coactivation of cAMP-dependent protein kinase with 8-Br-cAMP. Our data indicate that an antiproliferative signal induces or even superinduces c-*fos* message and hardly interferes with c-*myc* induction, suggesting that the intracellular pathways resulting in c-*fos* and c-*myc* induction may be distinct and that two different pathways can lead to c-*fos* induction, with synergistic effects when both are activated.

Key Words: nuclear proto-oncogenes, growth-factors, signal transduction

Proliferation and differentiation have at times been considered as two opposite and mutually exclusive biological pathways. Proliferation is induced by polypeptide hormones of the growth factor family. In general, peptide hormones stimulate two types of unique

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protein kinase activities, which are believed to mediate, in part, initial events controlling cellular metabolism [reviewed in 1]. Hormones such as adrenaline, thyroid stimulating hormone, etc. deliver their signal through the activation of a protein kinase (cyclic adenosine monophosphate [cAMP]-dependent protein kinase A) by the amplification of an internal second messenger, cAMP [2]. Hormones such as acetylcholine, serotonin, etc. activate another phospholipid Ca^{++}-dependent protein kinase, protein kinase C, in a pathway involving lipid metabolites as second messenger [3,4]. Polypeptide hormones involved in proliferation appear to utilize the lipid-dependent kinase C [4–9], whereas the activation of the cAMP-dependent kinase pathway has been associated with growth arrest [10–14].

In the hematopoietic and immune systems, cAMP is known as a growth inhibitor [15–20]. Proliferation of bone marrow hematopoietic precursors in vitro is dependent upon the presence of specific growth factors, collectively known as colony stimulating factors (CSF), which includes IL3, G-CSF, CSF1, and GM-CSF [reviewed in 21,22]. Like the other members of the growth factor family [22–28], CSFs have been shown to induce rapidly the activation of the nuclear proto-oncogenes, the cellular counterpart of the retroviral transforming genes, in CSF-dependent cell lines [29,30]. As nuclear proto-oncogene polypeptide products are located in the nucleus and as their expression is tightly regulated through the cell cycle, nuclear proto-oncogenes are thought to be part of the genetic elements involved in cell proliferation.

Using a GM-CSF-dependent cell line as a model, we have investigated the effect of 8-Br-cAMP, a stable analog of cAMP and a direct activator of the cAMP-dependent protein kinase, on proliferation and on nuclear proto-oncogenes induction by GM-CSF.

MATERIALS AND METHODS

Cell Lines, Growth Factors, and Reagents

The NSF-60.8 murine myeloid cell line is derived from NSF-60 [31] and was a kind gift of Dr. Donna Rennick (DNAX Corp., Palo Alto, CA). Recombinant murine GM-CSF (r-mu-GM-CSF) was provided by Immunex Corp. (Seattle). 8-Br cAMP and phorbol 12-myristic 13-acetate (PMA) were purchased from Sigma Chemical Company (St. Louis).

Cell Culture

The NSF-60.8 murine myeloid cell line was maintained in RPMI 1640 (Cellgro, Washington, DC) supplemented with 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycine (Gibco), and 10% fetal calf serum (FCS) (Hyclone, Logan, UT) and supplemented with 40 ng/ml of r-mu-Gm-CSF.

Proliferation Assays

Proliferation was assayed in microtiter plates (10^4 cells per well in a volume of 0.2 ml) in triplicate, in 10% FCS RPMI supplemented as indicated above, by a 6-h ^{3}H-thymidine (1 μCi/well, 120 Ci/nmol, Amersham, Arlington, IL) pulse after a 24-h period of incubation. The absence of effect of 8-Br-cAMP on cell viability was assessed using Trypan blue.

RNA Preparation

NSF-60.8 growth was arrested by extensive washing to remove the growth factor followed by a 6-h period in low serum medium (1% FCS) at 10^6 cells per ml. r-mu-

GM-CSF (100 ng/ml), 8-Br-cAMP (1 mM), or PMA (40 ng/ml) was added as indicated. Cells were harvested after the indicated period of time, washed once in phosphate-buffered saline (PBS), and resuspended in guanidium isothyocyanate followed by purification of RNA on a standard cesium chloride gradient [32].

RNA Blotting

Equal amounts of RNA (20 μg of total RNA) were denatured with 6% formaldehyde for 10 min at 55°C in 1 × MOPS (20 mM morpholine propane sulfonic acid pH 7.1, 5mM sodium acetate, 1 mM EDTA) and 50% deionized formamide (Fluka Chemical Company, Hauppage, NY), quickly chilled on ice, and size fractionated on 1% agarose gels containing 6% formaldehyde. After soaking of gels in 20 × SSC (1 × SSC = 150 mM sodium chloride, 15 mM trisodium sodium citrate, pH 7), RNA was transferred onto nitrocellulose filters (BA 85, Shleicher Schuell, Keene, NH) by capillary blotting in 20 × SSC using standard procedures [33]. Filters were then baked 2 h in vacuo at 80°C.

Probes and Probe Labeling

All probes utilized in this study were purified inserts, isolated after appropriate restriction on 1% low-melting-point agarose (Bethesda Research Laboratories, Bethesda, MD), ^{32}P labeled to 2–5 × 10^8 cpm/μg by primer extension using the Polymeraid labeling kit (PLS) and ^{32}P-dCTP (3,000 Ci/mM, Amersham Corporation, Arlington Heights, IL), and purified on NACS columns (BRL, Bethesda, MD).

The c-*myc* probe was the 1.4-kb *Eco* R1 *Cla* I fragment of the third exon of human c-*myc* [34]. The c-*fos* probe was the 1.2-kb *Eco* R1-*Nco* I fragment of mouse c-*fos* [35]. The c-*myb* probe was the 0.5-kb *Eco* R1 fragment of mouse c-*myb* [36]. Plasmids were kindly provided by Dr. John Cleveland.

Hybridization Procedures

Filters were soaked for 2–24 h in prehybridization buffer (4 × SSC, 50% formamide, 1 × Denhardt, Tris pH 7.4 20 mM, 0.2 mg/ml salmon sperm DNA, 5% dextran sulfate), and hybridized with 5–10 ng/ml of labeled probes in the same medium for 20–48 h at 42°C. Filters were washed several times in 2 × SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature, 1–3 × 15 min in 0.1 × SSC, 0.1% SDS at 50°C and exposed to Kodak XAR-5 films with Cronex lighting plus intensifying screens (E.I. Dupont De Nemours Company, Wilmington) at −70°C for 3–48 h. For relative quantities of hybridized radioactive probe, autoradiographs were scanned using a densitometer, and the relative intensities of the bands were estimated by weighing the densitometer profiles.

Hybridized radioactive probe was then removed by immersion of the nitrocellulose filters in boiling water. Filters were checked prior to use with other probes.

RESULTS

Effect of 8-Br-cAMP on NSF-60.8 Cell Proliferation

The NSF-60.8 cell is a cloned murine myeloid cell line that contains a retroviral insert at the c-*myb* locus [31] and is critically dependent on CSF for viability and cell growth. NSF-60.8 cells proliferate in response to IL3, G-CSF, and GM-CSF [30,31]. We tested whether an elevation of intracellular cAMP concentration was inhibitory to the GM-CSF-directed proliferative response. As shown in Figure 1, the addition of

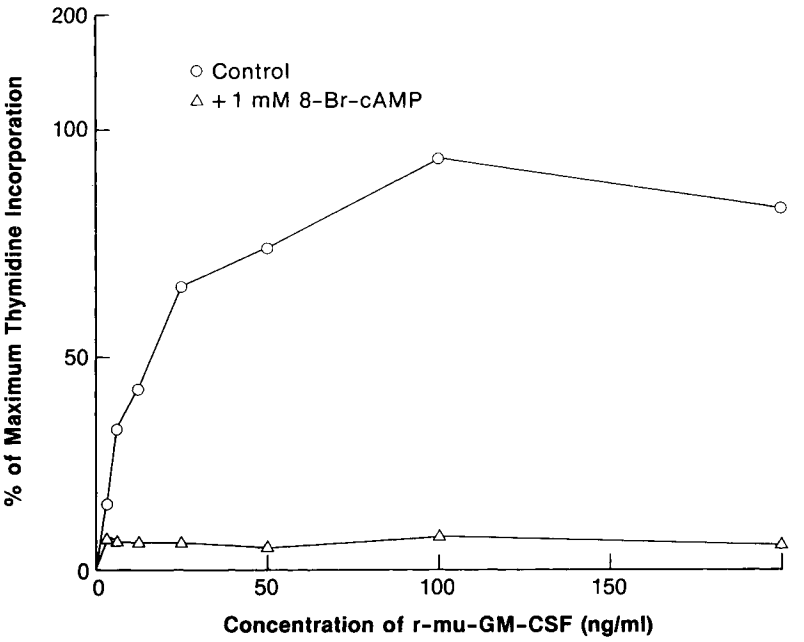

Fig. 1. Effect of 8-Br-cAMP on CSF induced myeloid cell proliferation. Proliferation of quiescent NSF-60.8 cells was induced by r-mu-GM-CSF alone or in the presence of 1 mM 8-Br-cAMP and measured by ^{3}H-thymidine incorporation.

1 mM 8-Br-cAMP had a striking inhibitory effect on GM-CSF-induced proliferation of NSF-60.8 cells as measured by thymidine incorporation. This inhibition did not reflect a toxic effect (as assessed by Trypan blue exclusion); it resulted in growth arrest and could not be overcome by increasing the amounts of growth factor. This indicates that pharmacological activation of the cAMP-dependent protein kinase inhibits the GM-CSF-induced entry of these myeloid cells into S phase.

Although the analog 8-Br-cAMP has a longer intracellular half-life than cAMP, the addition of 1 mM 8-Br-cAMP results only in a transient elevation of intracellular cAMP concentration (Suzanne Beckner, personal communication). Since such a few minutes pulse was able to prevent the entry of the cells into S phase 12–15 h later, we hypothesized that cAMP was interfering with an early event following GM-CSF activation. One of the major early transcriptional events stimulated by growth factors is the induction of nuclear proto-oncogene mRNA induction. We therefore investigated the effect of 8-Br-cAMP on c-*fos* and c-*myc* mRNA induction.

Treatment of NSF-60.8 With the cAMP Analog 8-Br-cAMP and GM CSF Superinduces c-*fos* mRNA and Hardly Interferes With c-*myc* mRNA Induction

NSF-60.8 cells were growth arrested, and GM-CSF was added in the presence or absence of 8-Br-cAMP. RNAs were purified at various time points and submitted to Northern analysis. GM-CSF alone induced a low steady-state level of c-*fos* mRNA in a very transient manner and of c-*myc* mRNA in a more stable manner (Fig. 2A). The

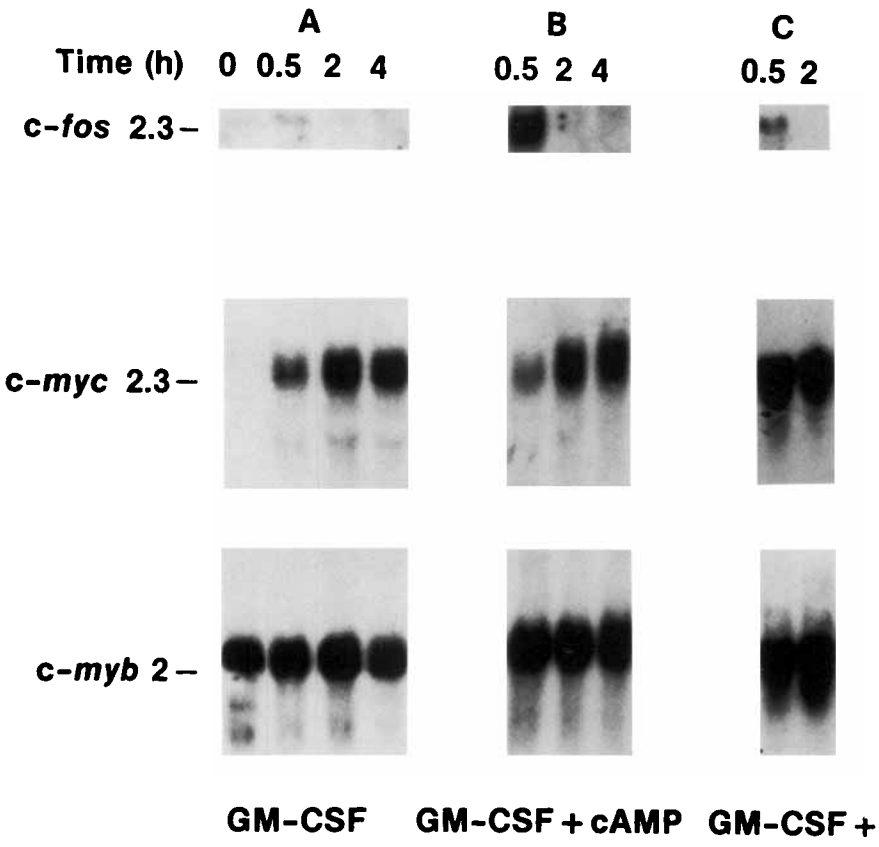

Fig. 2. Effect of 8-Br-cAMP on the induction of c-*fos* and c-*myc* by CSF. Quiescent NSF-60.8 cells were activated by 100 ng/ml of r-mu-GM-CSF, alone (**A**), or in the presence of 1 mM 8-Br-cAMP (**B**) or 40 ng/ml of PMA (**C**). Total RNAs were extracted at indicated time points and subjected to Northern analysis.

coaddition of 8-Br-cAMP resulted in a striking synergy in c-*fos* mRNA induction (32-fold induction) (Fig. 2B). On the contrary, 8-Br-cAMP had a only slight inhibitory effect, if any, on c-*myc* mRNA induction by GM-CSF (20-fold induction as compared to 30-fold by GM-CSF alone).

As PMA, a direct activator of the lipid-dependent protein kinase C, has been shown to induce c-*fos* and c-*myc* in numerous tissues, we have also studied the effect of PMA on the proto-oncogene activation. PMA had a slight enhancing effect on GM-CSF stimulation of c-*fos* and c-*myc* expression (Fig. 2C).

The steady-state level of c-*myb* mRNA did not seem to be modulated by GM-CSF in this particular myeloid cell line. This could be due to the retroviral insertion in the c-*myb* gene described for this cell line. A control probe, specific for 18S rRNA, did not show any modulation (data not shown).

These results indicate that the pharmacological activation of the cAMP-dependent protein kinase, although it is a potent antiproliferative signal, results in the superinduction of c-*fos* mRNA. We next investigated the effect of direct activation of the cAMP and

the lipid-dependent protein kinases in the absence of GM-CSF, and the interrelationship between the two kinases, on nuclear proto-oncogene mRNA induction.

Coactivation of the cAMP-Dependent Protein Kinase and of Protein Kinase C in the Absence of Growth Factor Results in Superinduction of c-*fos* mRNA and in Inhibition of c-*myc* mRNA

NSF-60.8 cells were growth arrested, and PMA, 8-Br-cAMP, or both were added. At various time points, RNAs were extracted and submitted to Northern analysis. 8-Br-cAMP, in the absence of growth factor, was able to induce c-*fos* mRNA at time and level of induction comparable to what was observed with GM-CSF (Fig. 3A; compare with Fig. 2A). On the contrary, 8-Br-cAMP was not able to induce detectable steady-state levels of c-*myc* mRNA. PMA, a pharmacological activator of the lipid-dependent protein kinase C, was able to induce significant levels of c-*fos* and c-*myc* mRNA in the absence of GM-CSF (Fig. 3B), as has been shown in numerous tissues [26,37,38]. When both kinase systems were coactivated in the absence of GM-CSF, superinduction of c-*fos* mRNA was observed (Fig. 3C) giving a 60-fold augmentation of the steady state level of mRNA. On the contrary, 8-Br-cAMP inhibited the induction of c-*myc* mRNA in response to PMA alone (Fig. 3C). A control probe, specific for the 18S rRNA, did not show any modulation (data not shown).

DISCUSSION

Using a murine myeloid cell line, we have studied the effect of an analog of the intracellular second messenger cAMP on GM-CSF-induced proliferation and nuclear proto-oncogene induction. Our results indicate that 8-Br-cAMP is a strong antiproliferative signal for myeloid cells. Therefore, it seems that pharmacological activation of the cAMP-dependent protein kinase gives a negative signal for entry into the proliferative cycle in myeloid cells, as has been shown to be the case in other cell types [13,14] and, in particular, in the immune system [15–20]. At the level of nuclear proto-oncogene mRNA induction, 8-Br-cAMP had opposite effects on c-*fos* and c-*myc* mRNA steady-state levels. 8-Br-cAMP induced and even superinduced in the presence of GM-CSF, c-*fos* mature message. A tissue-specific induction of c-*fos* message by cAMP has also been reported in macrophages [39]. Superinduction of c-*fos* was also observed in lymphoid cells costimulated by 8-Br-cAMP and interleukin 2 (IL 2) [40].

On the contrary, c-*myc* message was not induced by 8-Br-cAMP, and the cAMP analog had only minor inhibitory effect on c-*myc* induction by GM-CSF. This last result contrasts with what was observed in lymphoid cell lines induced to proliferate by IL 2, in which 8-Br-cAMP strongly inhibited the induction of c-*myc* by the growth factor [40]. This discrepancy could indicate a difference in the intracellular pathway resulting in c-*myc* induction by growth factors in lymphoid and myeloid cell lines. In fact, it has been shown that IL3 as well as IL2 induces protein kinase C translocation [6]. But, in contrast with IL2, IL3 and CSF-1 do not seem to trigger the inositol phosphate cycle in myeloid cells [41]. We also showed that PMA, the pharmacological activator of protein kinase C, is able to induce nuclear proto-oncogene in growth-arrested myeloid cells. We therefore investigated the effect of 8-Br-cAMP on PMA-induced nuclear proto-oncogene induction. 8-Br-cAMP synergized with PMA for c-*fos* mRNA induction, whereas c-*myc* mRNA was significantly inhibited. Our results indicate that the two protein kinase systems synergize in the induction of c-*fos* steady-state level of mRNA.

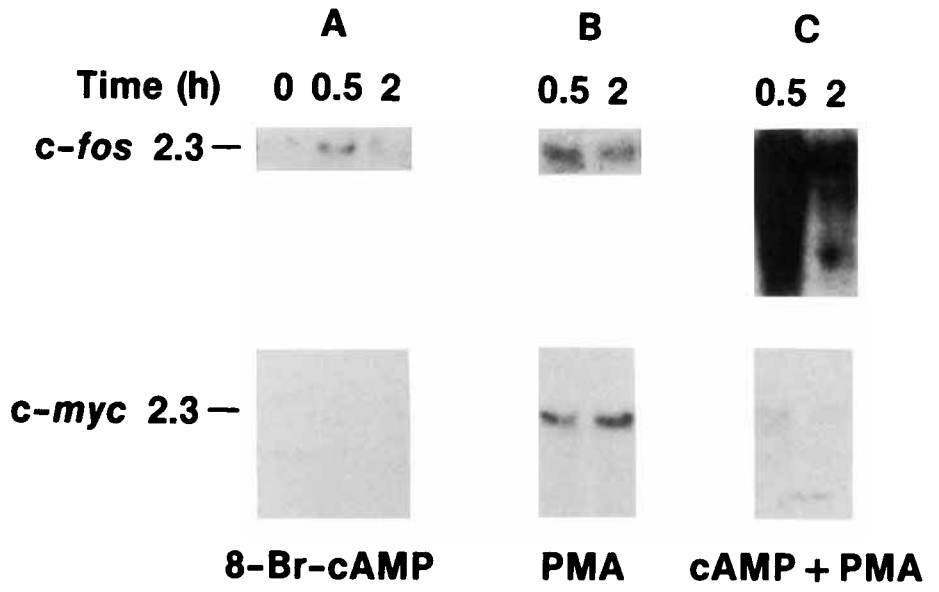

Fig. 3 Effect of activation and coactivation of the cAMP dependent and lipid-dependent protein kinases on nuclear proto-oncogene induction. Quiescent NSF-60.8 cells were activated with 1mM 8-Br-cAMP (**A**), 40 ng/ml of PMA (**B**), or both (**C**). At indicated periods of time, total RNAs were extracted from the cells and subjected to Northern analysis.

This result suggests the existence of two possible intracellular pathways for c-*fos* mRNA steady-state level accumulation in myeloid cells, as already reported for macrophages [39]. The synergy between the two kinase systems can be explained by the existence of two intracellular pathways of c-*fos* gene transcriptional activation, one involving the lipid-dependent protein kinase and the other one involving the cAMP dependent-protein kinase. Alternatively, 8-Br-cAMP could act through the stabilization of c-*fos* message, although this possibility seems remote, since no change in the time course of disappearance of c-*fos* message was observed in the presence of 8-Br-cAMP. Interestingly, growth factors, as well as PMA, have been shown to have either no effect or even to inhibit adenylate cyclase activity, the enzyme responsible for cAMP biosynthesis, in lymphoid cells [42–44]. Our results indicate that when this inhibition is bypassed by exogenous cAMP, the two kinase system can synergize in myeloid cells. This synergism is restricted to the induction of certain genes, since it is not observed for c-*myc* gene.

These results also indicate that, although c-*fos* and c-*myc* messages are always coactivated by growth factors, the internal pathways resulting in this induction of steady-state levels of messages may differ. In fact, we show here situations in which c-*fos* mRNA is superinduced, whereas c-*myc* message is inhibited. This discrepancy can again reflect a difference in the transcriptional activation process. Finally, it is of interest to note that an antiproliferative signal such as cAMP can induce or even superinduce c-*fos* message. Although c-*fos* mRNA induction is always observed upon growth factor activation and although c-*fos* gene can be transforming by itself [45], the role of c-*fos* protein in proliferation is still controversial. In fact, in numerous studies, c-*fos* expression

has been correlated with cell differentiation rather than with proliferation [46–49]. This seems to be especially true in myeloid cells [50]. A tempting speculative hypothesis would be that a faint induction of c-*fos* message through the Ca^{++}/lipid-dependent protein kinase C pathway would be associated with the proliferative cycle, whereas a quantitatively more pronounced induction through the cAMP-dependent protein kinase pathway would be associated with growth arrest and maybe a differentiation cycle. In fact, in human myeloid cells, activation of the cAMP-dependent protein kinase has been shown to induce differentiation [51]. In our system, no morphologic evidence of differentiation was observed, but this could be related to the truncation of the c-*myb* gene in those cells.

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