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Cyclic AMP Inhibits Expression of D-Type Cyclins and cdk4 and Induces $p27^{Kip1}$ in G-CSF-Treated NFS-60 Cells

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The addition of cAMP inhibits G-CSF-mediated proliferation and suppresses pRB phosphorylation in NFS-60 cells. We show that the latter could be attributed to different effects of cAMP in these cells: (i) down-regulation of the levels of cyclins D2 and D3, and cdk4, and (ii) induction of the $p27^{Kip1}$ inhibitor of cdk4.

The cAMP-dependent protein kinase (PKA) occupies a central position in the kinase cascade induced by a variety of extracellular signals which mediate different effects on the cell (1). Increased intracellular cAMP can have both stimulatory or inhibitory effects on cell proliferation depending on the cell type (2). It stimulates the proliferation of epithelial cells, hepatocytes and Swiss 3T3 cells (3-5), while it inhibits DNA synthesis in lymphoid cells (6, 7), fibroblasts, smooth muscle cells (8) and macrophages (9, 10).

Important regulators of cell cycle progression include two sets of proteins - cyclins and their catalytic partners, cyclin dependent kinases (cdk's) - which have major roles in a wide range of cell types (11). Three D-type cyclins are known (cyclin D1, D2 and D3), and these function in the G1 phase of the cell cycle to control progression (12-15). It appears that D-type cyclins interact with the retinoblastoma gene product pRB *in vivo*, with subsequent binding of the cyclin with either cdk4 or cdk6 leading to phosphorylation of pRB (16-20). These interactions lead to the release of transcription factors, such as E2F, required for the transcription of genes essential for cell cycle progression (19). Other studies have shown cdk activity to be regulated by a series of specific inhibitors, including p15 (21), p16 (22), p18 (23), p19 (23), p21 (24), and p27 (25).

It has been shown that elevated intracellular cAMP impacts on these cell-cycle effectors. Addition of cAMP decreases D1 mRNA in M-CSF-treated bone marrow-derived macrophages (26), D-type cyclin expression in human diploid fibroblasts (27), and cyclin E, cdk2 and late D1 expression in FGF-treated astrocytes (28). In addition, reduced cyclin E- and A-dependent histone H1 kinase activity has been reported (28). However, other studies in mammalian cells have indicated that cAMP induces the $p27^{Kip1}$ inhibitor of cdk4 in M-CSF-treated macrophages, with no effect on levels of cyclin D1 or cdk4 (29).

We have previously shown that addition of 8Br-cAMP blocks cell cycle progression in G-CSF treated NFS-60 cells, which correlated with an increase in higher order E2F complexes (Ward et al., manuscript submitted). In this study, we show 8Br-cAMP also inhibits *in vivo* pRB phosphorylation, and that this could be due to both down-regulation of the levels of cyclin D2 and D3 and cdk4, and induction of the $p27^{Kip1}$ inhibitor of cdk4.

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MATERIALS AND METHODS

Cells culture conditions and reagents. NFS-60 cells are a G-CSF/IL-3-dependent cell line obtained from Dr. Judy Layton, Ludwig Institute for Cancer Research, Melbourne, Australia. These were grown in DMEM containing 2 mM glutamine, 10 % (v/v) FBS and 1 ng/ml G-CSF at 37°C in 5 % CO_2. Cells were subsequently washed and incubated in DMEM+FBS without growth factor for 14 h before use to render them quiescent. DMEM, penicillin and streptomycin were purchased from ICN-Flow Laboratories (Sydney, Australia), foetal bovine serum (FBS) from Commonwealth Serum Laboratories (Melbourne, Australia), and G-CSF from Amgen Australia Pty Ltd (Melbourne, Australia). Isobutylmethylxanthine (IBMX), prostaglandin E_2 (PGE_2), and the sodium salts of 8-bromo-adenosine 3′:5′-monophosphate (8Br-cAMP), 8-(4-chloro-)cAMP (4Chl-cAMP) and dibutyryl-cAMP (diBu-cAMP) were obtained from Sigma Chemical Company, and [methyl-^{3}H]-thymidine (70-85 Ci/mmol) was from Amersham (Sydney, Australia).

Northern blot analysis. Total RNA was prepared from NFS-60 cells using a guanidinium isothiocyanate extraction (30) and analysed by formaldehyde agarose gel electrophoresis before transfer to Hybond N^+ (Amersham) according to the manufacturer's instructions. These blots were hybridised to DNA probes, which had been ^{32}P-labelled by random priming (31). The filters were washed and then exposed to Kodak XAR film.

Western blot analysis. Cytoplasmic extracts were subjected to Western blot analysis using standard protocols. Primary antibodies were purchased from Santa Cruz Biotechnology Inc (California, USA) and detected using the ECL system (Amersham), following the manufacturer's instructions.

DNA synthesis. The incorporation of [methyl-^{3}H]-thymidine was used as a measure of DNA synthesis. Quiescent NFS-60 cells were incubated in the presence of various treatments with [methyl-^{3}H]-thymidine (2.5 μCi/ml) added at 0 hours. At the times indicated, cells were harvested onto glass filters using an Inotech harvester, and read on a Bertold Digital Autoradiograph to measure incorporated radioactivity.

RESULTS

Effect of Elevated cAMP on G-CSF Stimulated Proliferation

We have previously shown that the non-metabolisable analogue of cAMP, 8Br-cAMP, inhibited G-CSF stimulated proliferation in NFS-60 cells and targets events in mid-late G_1 to inhibit S-phase progression (Ward et al., manuscript submitted). As a further check that the effect of 8Br-cAMP was in fact due to elevated intracellular cAMP, we have examined two other cAMP analogues, diBu-cAMP and 4-Chl-cAMP, and two agents known to elevate intracellular cAMP, namely PGE_2, and the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX). As shown in Fig. 1, all of these agents inhibited G-CSF stimulated DNA synthesis.

Inhibition of pRB Phosphorylation by cAMP

Phosphorylation of pRB has been shown to be an important regulator of cell-cycle progression through G_1 and into S phase (32, 33). Since addition of 8Br-cAMP during the G_1 phase inhibits G-CSF stimulated DNA synthesis, we examined the effects of its addition on pRB phosphorylation (Fig. 2). Addition of 8Br-cAMP blocked the G-CSF-induced phosphorylation of pRB.

Effect of cAMP on Expression of cdk4 and Cyclins D2 and D3

The D-type cyclins and cdk4 have been shown to play an important role in proliferation control by mediating phosphorylation of pRB (11). Northern blot analysis of quiescent NFS-60 cells stimulated with G-CSF showed induction of the mRNAs for cdk4 and cyclins D2 and D3 within 2 h (Fig. 3). The levels then decline after 6 h through to S-phase, which commences about 9 h after G-CSF addition (Ward et al., manuscript submitted). The effects on mRNA also correlated with the levels of the corresponding protein as judged by Western blot analysis (data not shown). No cyclin D1 mRNA was observed in this cell line, either in the quiescent state or in response to G-CSF (data not shown).

Much recent work on the mechanism of action of various anti-proliferative agents has shown that prevention of cdk activity is of major importance (19, 34). Since cAMP inhibited phosphorylation of pRB in NFS-60 cells (Fig 2), we investigated whether this could be attributed to down modulation of D-type cyclins or cdk4, thereby blocking cdk activity. Fig. 3

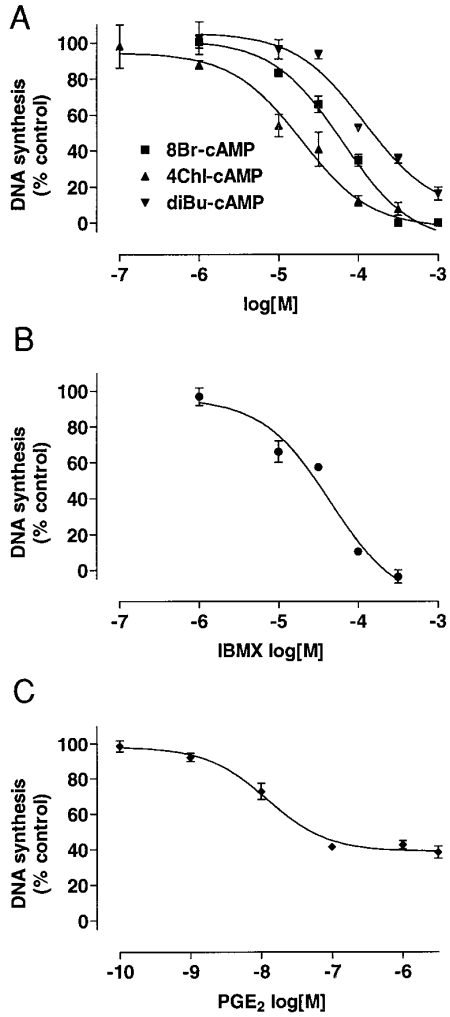

FIG. 1. Inhibition of DNA synthesis by cAMP analogues and inducers of intracellular cAMP. Quiescent NFS-60 cells were stimulated with 1 ng/ml G-CSF in the presence of tritiated thymidine ten minutes after the addition of the appropriate agents and harvested after 14 h. (A) cyclic AMP analogues: 8Br-cAMP, 4Chl-cAMP, and diBu-cAMP; (B) prostaglandin E_2; (C) phosphodiesterase inhibitor, IBMX.

shows that 8Br-cAMP suppresses the G-CSF-mediated stimulation of the mRNAs for cdk4 and cyclins D2 and D3, which was again reflected in the levels of the corresponding proteins (data not shown). This is in contrast to the effect on c-*fos* mRNA, which is superinduced by 8Br-cAMP, as described by others (35).

Since 8Br-cAMP suppressed levels of cdk4 and D-type cyclins, we next examined if this was reflected in the amounts of cyclin D/cdk4 complexes in the cell, since this is a prerequisite for pRB phosphorylation (11). Fig. 4 shows that the amount of cyclin D2/cdk4 complex increased after 2 h of G-CSF stimulation, but that this was not observed in the presence of 8Br-cAMP. Following stimulation with G-CSF for 6 h, the levels of cyclin D2/cdk4 complex had declined, but there was a detectable increase in cyclin D3/cdk 4 complex; levels of this complex were also diminished when 8Br-cAMP was included.

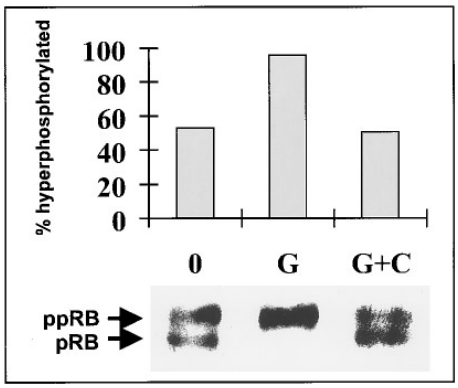

FIG. 2. Inhibition of pRB phosphorylation by cAMP. Western blot analysis of pRB in lysates prepared from quiescent NFS-60 cells (0) stimulated for 8 h with 1 ng/ml G-CSF alone (G) or in the presence of the anti-proliferative agents 8Br-cAMP (G + C). Densitometric analysis was used to determine the percentage of pRB in the hyperphosphorylated (ppRB) form.

Induction of p27^{Kip1} by cAMP

The addition of 8Br-cAMP to M-CSF stimulated macrophages has been shown to induce p27^{Kip1} (29), which is an inhibitor of cyclin dependent kinase activity. Therefore, we investigated the possible involvement of this inhibitor in NFS-60 cells. Fig. 5 shows that, while G-CSF alone has no appreciable effect on the levels of p27^{Kip1}, the inclusion of 8Br-cAMP leads to a rapid induction of p27^{Kip1}. This is transient, and declines to basal levels by 8 h (data not shown). Importantly, co-immunoprecipitation showed that levels of p27^{Kip1}/cdk4 complex parallel the changes in p27^{Kip1}, which shows that the induced p27^{Kip1} becomes associated with cdk4 and, therefore, probably inhibits cdk activity. This suggests that p27^{Kip1} induction may also contribute to the cell cycle arrest caused by 8Br-cAMP in this system.

DISCUSSION

Cyclins and their cyclin-dependent kinase partners play crucial roles in progression through the cell cycle. D-type cyclins appear vital for G1 progression, since they can bind

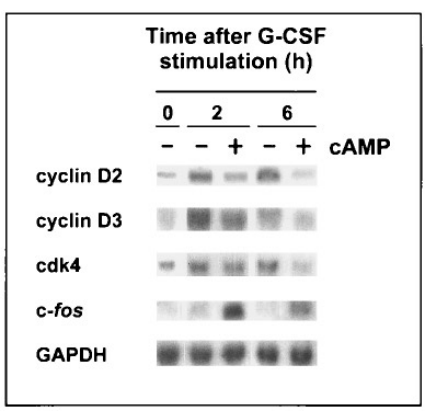

FIG. 3. Effect of cAMP on cyclin/cdk mRNA expression. Northern analysis of NFS-60 cells stimulated with 1 ng/ml G-CSF in the presence (+) or absence (−) of 1 mM 8Br-cAMP, with hybridisation to the probes indicated.

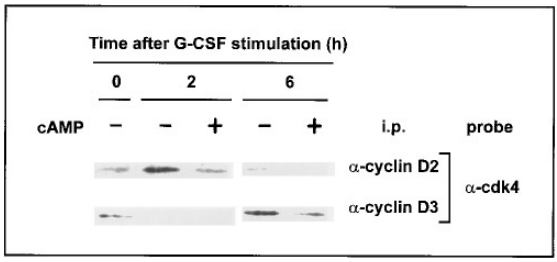

FIG. 4. Effect of cAMP on G-CSF stimulated cyclin D-cdk complex formation. Lysates were prepared from quiescent cells stimulated with 1 ng/ml G-CSF in the presence (+) or absence (−) of 1 mM 8Br-cAMP. These were immunoprecipitated with the antibodies indicated, before Western blot analysis with anti-cdk4.

to the pRB ''pocket'' region to target cdk4 to pRB, and hence lead to *in vivo* phosphorylation of pRB (11, 15). This leads to the subsequent release of transcription factors, such as E2F, required for the induction of genes required for proliferation. In this study we show that G-CSF stimulates pRB phosphorylation and expression of cdk 4 and cyclins D2 and D3, but not D1, in NFS-60 cells. This distribution of D-type cyclin expression has been reported in another granulocytic cell line in response to G-CSF (36), whereas macrophages express cyclins D1 and D2 in response to M-CSF (29). The significance of this specificity in these two myeloid systems remains to be elucidated, although it may be important in the control of differentiation (36).

We have previously described a number of agents, including 8Br-cAMP, which act in mid to late G1 phase to inhibit proliferation of G-CSF-treated NFS-60 cells (Ward et al., manuscript submitted). The addition of exogenous cAMP inhibited the G-CSF-mediated phosphorylation of pRB and, given the significance of pRB phosphorylation for cell cycle progression, it is presumably responsible for the subsequent block in DNA synthesis. The added 8Br-cAMP was also found to suppress the G-CSF-stimulated increase in expression of cdk 4 and cyclins D2 and D3, as well as the levels of the respective cyclin D-cdk4 complexes, which probably contributes to the inhibition of pRB phosphorylation by this agent. This finding is consistent with other studies showing that elevated cAMP could inhibit accumulation of cyclin D1 in macrophages (26, 37), astrocytes (28), and fibroblasts (27). Furthermore, inhibition of G1 cyclin levels by elevated cAMP is conserved through evolution, since it also occurs in *S. cerevisiae* (38, 39).

These results are different to those found in one study on M-CSF stimulated macrophages, in which there was no observed effect on the levels of cdk4 or cyclin D1 or their complex. Instead induction of $p27^{Kip1}$ was offered as the mechanism for proliferation inhibition by cAMP (29). In partial agreement with these authors, however, we show that in G-CSF-treated NFS-

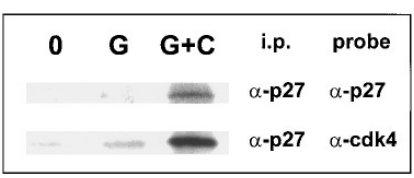

FIG. 5. Effect of cAMP on $p27^{Kip1}$ in NFS-60 cells. Lysates were prepared from quiescent cells (0), or cells stimulated with 1 ng/ml G-CSF alone (G) or in the presence of 1 mM 8Br-cAMP (G+C). These were immunoprecipitated with antibodies to $p27^{Kip1}$, then subjected to Western blot analysis with the antibodies indicated.

60 cells 8Br-cAMP transiently increased $p27^{Kip1}$ levels, and that this is associated with cdk4. We also found no induction of p21 or p16 mRNA in response to 8Br-cAMP (data not shown). We would like to suggest that elevated intracellular cAMP is capable of using different mechanisms to lead to the same biological outcome - that is, decreased pRB hyperphosphorylation and a concomitant inhibition of proliferation. Such pluralism of action may represent a general phenomenon for proliferation inhibitors, since TGFβ can affect cyclin D1/cdk4 levels in some cell types (40, 41), while in others, it can induce the p15 cdk inhibitor (21) to suppress proliferation.

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