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Cyclic AMP induces inhibition of cyclin A expression and growth arrest in human hepatoma cells

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Abstract

Classical cytotoxic therapy has been minimally useful in the treatment of hepatocellular carcinoma. In an effort to develop a new approach to the treatment of this neoplasm, we have investigated the signal transduction pathways regulating the growth of human hepatoma cells. In the data reported here, cyclic AMP (cAMP), a negative growth regulator for many cells of epithelial origin, induced G1 synchronization and apoptosis in the HepG2 human hepatoma cell line. The effects of cAMP on the components of the G1/S transition were analyzed. There was no detectable effect of two different cAMP analogs, 8-bromo cAMP or dibutyryl cAMP on the level of the D-type cyclins, cyclin E, cyclin-dependent kinase 2, cyclin-dependent kinase 4, p53, or the cyclin-dependent kinase inhibitors p21 or p27. In contrast, the cAMP analogs induced a dramatic downregulation of cyclin A protein, cyclin A messenger RNA, and cyclin A-dependent kinase activity. Cyclin A-dependent kinase has been shown to be required for the G1–S transition. Furthermore, cyclin A deregulation has been implicated in the pathogenesis of hepatocellular carcinoma. The data reported here suggest a novel signal transduction-based approach to hepatoma therapy.

Keywords: Cyclic AMP; Apoptosis; Cell cycle arrest; Cyclin A; HepG2; Hepatocellular carcinoma

1. Introduction

Primary hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [1], and HCC may be the most common fatal cancer [2]. In cases of localized HCC, surgical resection is potentially curative. However, many patients with HCC have metastatic disease at the time of initial diagnosis, and even in patients with HCC confined to the liver, curative surgical resection is frequently not an option due to cirrhosis and other pathologic changes in the liver parenchyma [3]. In patients with advanced disease, the average survival is 1 year or less. Thus, the development of a new therapeutic approach to HCC remains one of the most challenging areas in cancer research.

Uncontrolled proliferation is a universal property of tumor cells. Recent studies in HCC using various animal models, hepatoma cell lines, or primary human tumors have identified quantitative or qualitative activation of growth-regulatory genes including c-myc, ras, and cyclin A [4–8]. However, the func-

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tional roles of these gene products in HCC development have yet to be defined.

HCC is an intrinsically chemoresistant tumor [9]. Therefore, in an effort to identify a new approach to HCC treatment, we have examined the impact on HCC growth of signal transduction pathway modulators. In this study, we investigated the effect of cyclic AMP (cAMP) on cell cycle progression in the human HepG2 hepatoma cell line. cAMP, which acts as a second messenger for transmitting the signals of a diverse group of hormones and neurotransmitters serves as either a positive or a negative growth regulator, depending on the cell context [10,11]. cAMP has been shown to induce G1 synchronization, growth arrest, and terminal differentiation in certain cells [12–14]. The data presented here demonstrate that cyclic AMP induces G1 synchronization and subsequent cell death in HepG2 cells. Furthermore, examination of the components of the G1–S phase transition in control and cAMP-treated cells demonstrated that cAMP induces a selective downregulation of cyclin A protein, downregulation of cyclin A messenger RNA expression and a selective block in the activity of cyclin A-dependent kinase.

2. Materials and methods

2.1. Cells

The human hepatoma cell line HepG2 [5] was obtained from the American Type Culture Collection (ATCC) and cultured as a monolayer in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum, penicillin and streptomycin at 37°C in 5% CO_2.

2.2. cAMP analogs and antibodies

8-Bromo-cAMP (8-br-cAMP, 8-bromo-adenosine-3′:5′-monophosphate, cyclic monosodium salt) and dibutyryl-cAMP (db-cAMP, N^6,2′-*O*-dibutyryl-adenosine-3′:5′-monophosphate, cyclic monosodium salt) were obtained from Boehringer Mannheim. Monoclonal anti-cyclin A antibody, polyclonal anti-p27, -cdk2, -cdk4, -cyclin D1, -cyclin D2, and -cyclin D3 antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-p21 antibody was purchased from PharMingen. Monoclonal anti-p53 antibody was purchased from Oncogene Research Products.

2.3. Cell cycle analysis

Exponentially growing cells were compared to cells treated with 1 mM db-cAMP and 1 mM 8-br-cAMP for 24, 48, and 72 h. Cells were harvested, fixed in 50% ethanol and incubated with RNAse A and the DNA intercalating dye propidium iodide. Cell cycle phase analysis was performed by flow cytometry using a Becton Dickinson FACStar flow cytometer and Becton Dickinson Cell Fit software.

2.4. DAPI staining

Cells were washed two times with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Fixed cells were washed two times with PBS and 4,6-diamidino-2-phenylindole (DAPI, Sigma) was added and allowed to incubate for 10 min at room temperature. The cells were washed two more times with PBS. Coverslips were mounted on glass slides and analyzed by fluorescence microscopy using a Zeiss Axiophot microscope.

2.5. Western blot analysis

Cell lysates were subjected to electrophoresis in SDS-polyacrylamide gels. Western blot analysis was performed as described previously [14]. Proteins were detected using an enhanced chemiluminesence system (ECL, Amersham).

2.6. Northern blot analysis

Total RNA was isolated from cells lysed in guanidium thiocyanate solution and Northern blot analysis was performed as described previously [15] using a ^{32}P (ICN)-labeled 2.7-kb cyclin A cDNA probe and 30 μg total RNA per lane.

2.7. Immune complex kinase assay

The immune complex kinase assay was performed as described previously [16]. Briefly, cell lysates were

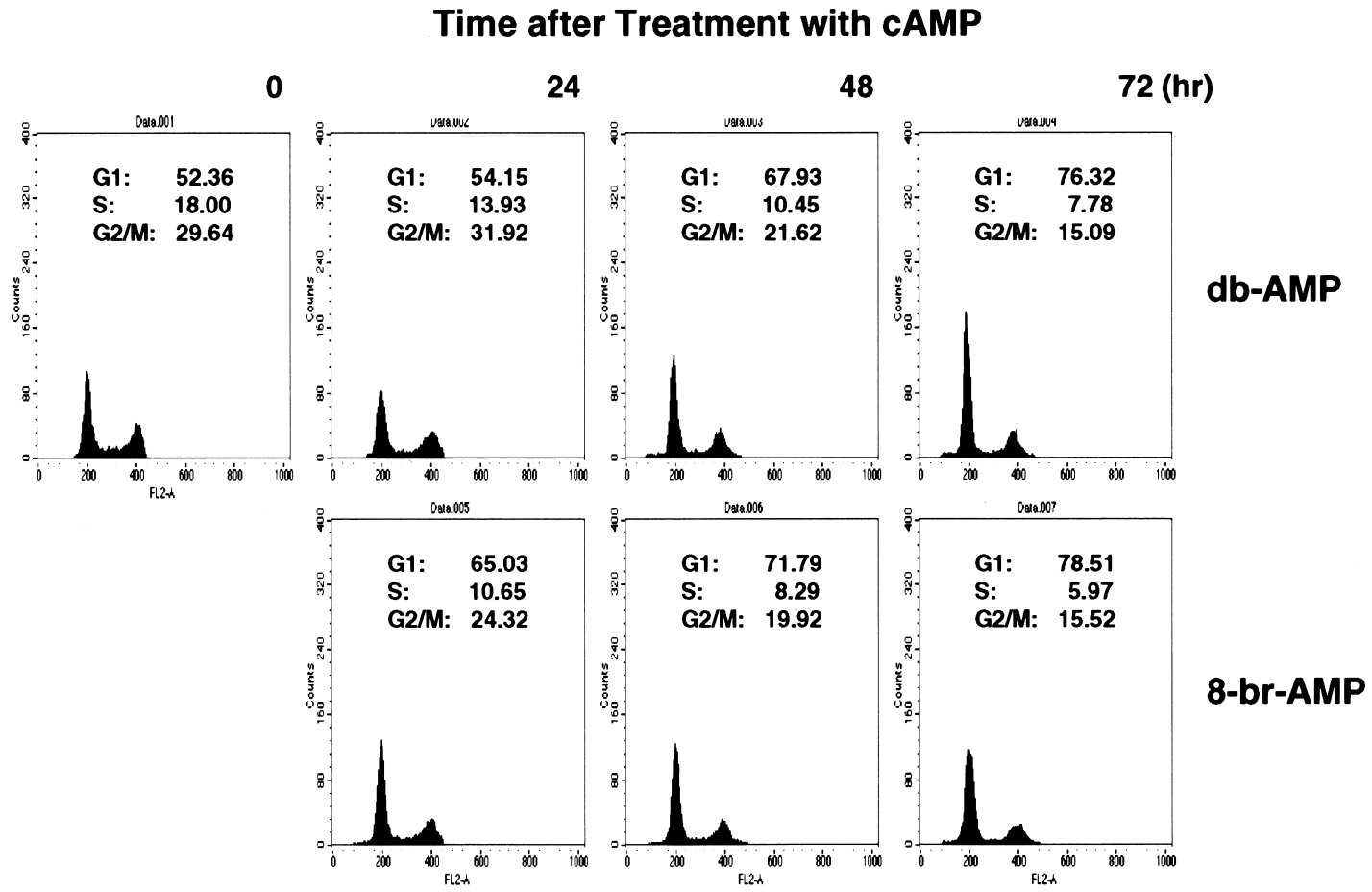

Fig. 1. Effect of cAMP on cell cycle phase distribution in HepG2 cells. Exponentially growing cells and cells treated with 1 mM db-cAMP and 1 mM 8-br-cAMP for 24, 48, and 72 h were harvested and the cell cycle phase distribution was determined by flow cytometric analysis of DNA content.

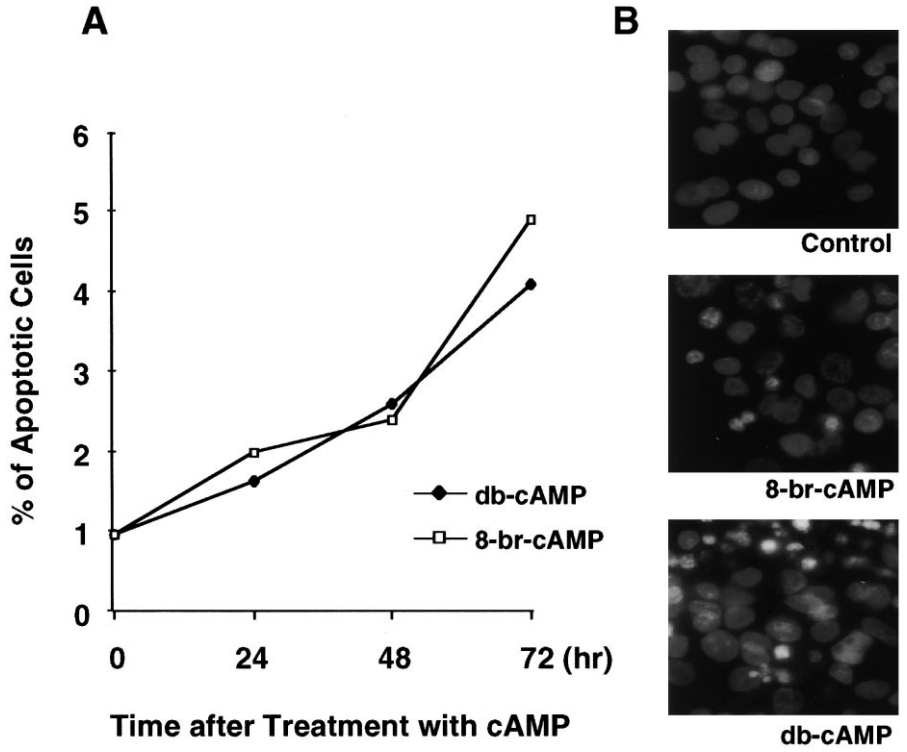

Fig. 2. cAMP analogs induce apoptosis in HepG2 cells. (A) Cells were treated with cAMP analogs for the times indicated. The percent of apoptotic hypodiploid cells was determined by flow cytometric analysis. (B) Cells were cultured in the absence (control) or presence of either 1 mM 8-br-cAMP or 1 mM db-cAMP for 72 h. The cells were fixed with formaldehyde, stained with the nuclear stain DAPI, and examined by fluorescence microscopy.

incubated with primary antibody, immune complexes were collected on protein A-Sepharose beads (Sigma), the beads were washed extensively, resuspended in kinase assay reaction mixture containing [γ-^{32}P]ATP (ICN) and histone H1 as substrate and incubated for 30 min at 37°C. The reaction was stopped by adding 2×SDS sample buffer and boiling. Proteins were separated on 10% SDS-PAGE, dried and autoradiographed using Kodak XAR-5 film.

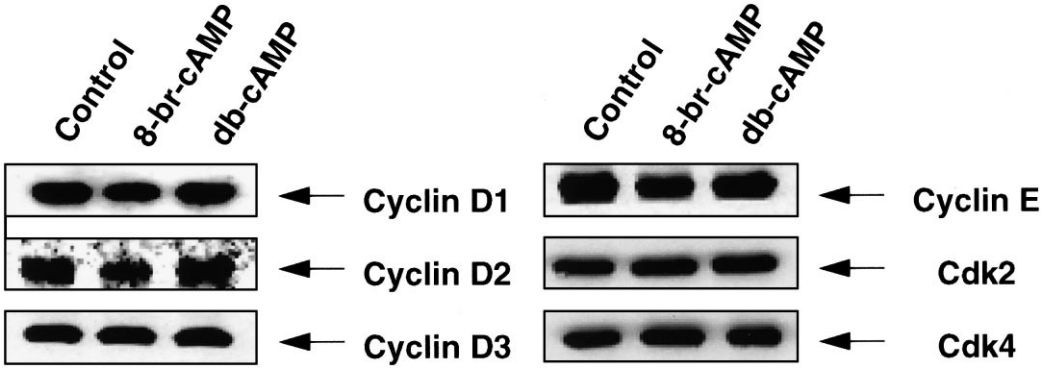

Fig. 3. Western blot analysis of D-type cyclins, cyclin E, cdk2, and cdk4 in control and cAMP-treated cells. Cells were cultured in the absence (control) or presence of either 1 mM 8-br-cAMP or 1 mM db-cAMP for 72 h. Equal amounts of total protein (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with specific antibodies. Proteins were detected by enhanced chemiluminescence.

3. Results

3.1. cAMP induces cell cycle arrest

The cell cycle phase distribution of exponentially growing HepG2 cells was compared to cells treated with the cell-permeant cAMP analogs, db-cAMP and 8-bromo-cAMP (Fig. 1). Twenty-four hours after cAMP addition there was a marked increase in the percent of cells in G1. By 72 h following db-cAMP and 8-br-cAMP addition, 76 and 79% of the HepG2 cells were arrested in G1, respectively. Growth curves demonstrated that cells failed to grow in the presence of cAMP, and that by 72 h of continuous exposure the cells were dying, as evidenced by a decrease in the viable cell count to below the initial number of cells plated (data not shown). Untreated control cells, and cells treated with either of two membrane-permeant cAMP analogs were grown on coverslips, stained with DAPI, and analyzed for changes in nuclear morphology characteristic of apoptosis. In the cultures treated with cAMP, there was an increase in the percent of apoptotic cells, as detected by DNA condensation and fragmentation in DAPI-stained cells, and by the flow cytometric detection of cells with a hypodiploid DNA content (Fig. 2).

3.2. Effect of cAMP on G1 cyclins, cdk2 and cdk4

The first stages of the G1/S transition are regulated by D-type cyclins which bind to cdk4 in mid-G1 prior to the restriction point, and by cyclin E which binds and activates cdk2 later in G1 [17–19]. The data in Fig. 1 demonstrated that cAMP induced G1 phase-specific arrest. Therefore, we examined whether cAMP-induced growth arrest in HepG2 cells was associated with decreased protein levels of the D-type cyclins, cyclin E, cdk2, or cdk4. Western blot analyses showed that cAMP had no detectable effect on the level of these proteins for up to 72 h of treatment (Fig. 3).

3.3. Levels of cdk inhibitors, $p21^{waf1/cip1}$ and $p27^{kip1}$ after treatment with cAMP

Cdk inhibitors are critical regulators of cdk activity. It has been reported that cAMP can increase the level of the cdk inhibitor $p27^{Kip1}$ [20] and increase binding of $p27^{Kip1}$ with the cyclin–cdk2 complex. Thus, cAMP-induced cell cycle arrest in HepG2 cells might be mediated by upregulation of the cdk inhibitors $p27^{Kip1}$ or $p21^{Waf1/Cip1}$, and/or upregulation of p53, a tumor suppressor protein that strongly transactivates the $p21^{Waf1/Cip1}$ promoter [21]. As shown in Fig. 4, cAMP had no detectable effect on the level of p53 or the cdk inhibitors, as determined by Western blot analysis.

3.4. Downregulation of expression of cyclin A by cAMP

Because there was no detectable change in the protein levels of the D-type cyclins, cyclin E, cdk2, cdk4, $p21^{Waf1/Cip1}$ or $p27^{Kip1}$, we examined the protein level

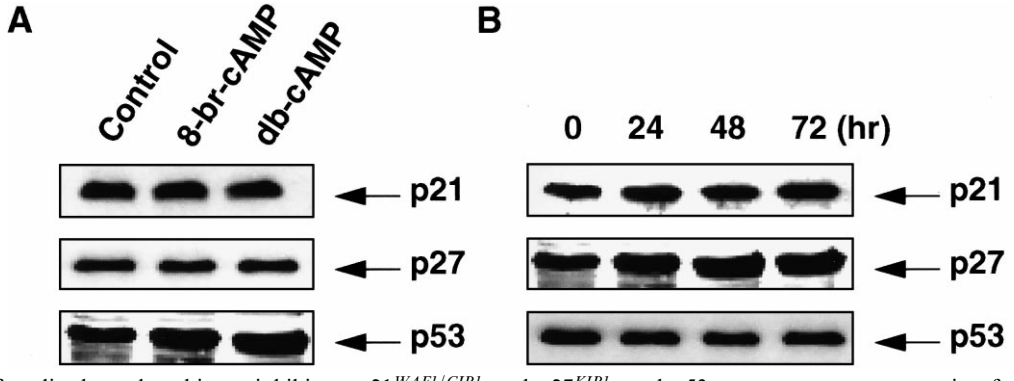

Fig. 4. Levels of cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, and p53 tumor suppressor protein after treatment with cAMP analogs. (A) Cells were treated with 1 mM db-cAMP or 1 mM 8-br-cAMP for 72 h. (B) Cells were incubated with 1 mM 8-br-cAMP for the time indicated. Total cell lysates were subjected to Western blot analysis using anti-p21, -p27 and-p53 antibodies, and detected by enhanced chemiluminescence.

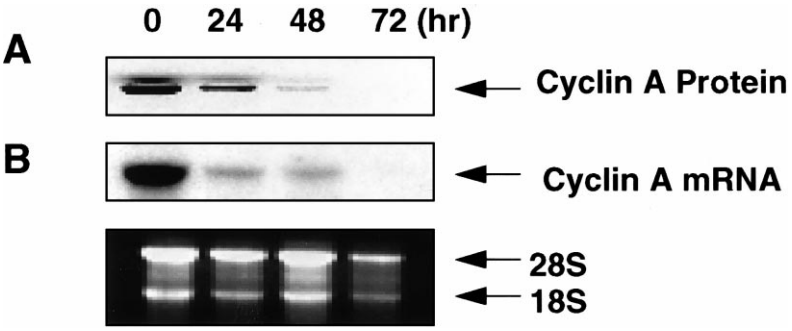

Fig. 5. Down-regulation of cyclin A by cAMP. Untreated control cells were compared to cells treated with 1 mM 8-br-cAMP for the time indicated. (A) Western blot analysis of cyclin A protein using monoclonal anti-cyclin A antibody and 60 μg of protein per lane. (B) Northern blot analysis. Total RNA was prepared, equal amounts (30 μg) were loaded per lane, and Northern blot analysis was performed using cDNA probes for cyclin A and for the control gene GAPDH [15].

of cyclin A by Western blot analysis. Until recently cyclin A was thought to function only in S phase and in mitosis. Reznitsky and colleagues, however, demonstrated that cyclin A can be rate-limiting for the G1–S phase transition [22]. In marked contrast to the other G1-regulatory components studied, the level of cyclin A protein was decreased after 24 h treatment with cAMP, and cyclin A protein was almost undetectable by 72 h following cAMP addition (Fig. 5).

3.5. Downregulation of cyclin A mRNA by cAMP

A principal mechanism of cAMP action is through the regulation of gene expression. Therefore, we tested if cAMP could downregulate cyclin A mRNA. Kinetic analysis demonstrated progressive loss of cyclin A mRNA in cAMP-treated cells. The time course of mRNA downregulation corresponded with the time-course of cyclin A protein downregulation. These data are consistent with inhibition of cyclin A transcription by cAMP (Fig. 5).

3.6. Inhibition of cyclin A–cdk2 associated kinase activity

Because cyclin A/cdk2-associated kinase activity can be essential to the late G1/S transition and S phase progression, we examined whether decreased expression of cyclin A was associated with an inhibition of cdk2 activity. As shown in Fig. 6 cyclin A-associated cdk2 activity was inhibited after incubation of HepG2 cells with cAMP. The protein levels of p21 and cdk2 were not changed and thus the block in cyclin A–cdk2 activity would appear to be attributable to the cAMP-induced downregulation of cyclin A.

4. Discussion

Cell cycle progression is regulated by the orchestrated activation and inactivation of a family of cdks [17,18,22,23]. Derangements in the cell cycle machinery are pivotal to the uncontrolled cell growth char-

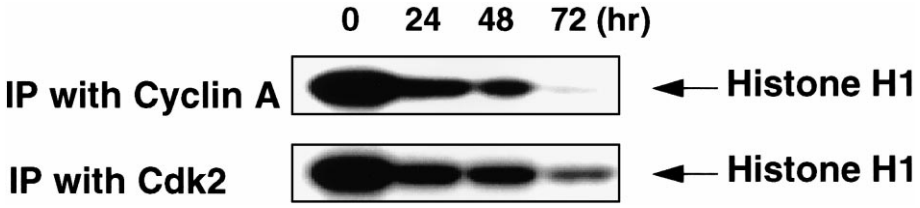

Fig. 6. Inhibition of cyclin A-associated and cdk2 kinase activity by cAMP. Cells were incubated with 1 mM 8-br-cAMP for the time indicated. Cell lysates were prepared, immunoprecipitations were performed with either anti-cyclin A antibody or anti-cdk2 antibody, and kinase activity was assayed using histone H1 (H1) as substrate.

acteristic of malignant neoplasms [24]. In the data presented here, elevation of intracellular cAMP with hydrolysis-resistant cAMP analogs induced growth arrest and apoptosis in human HCC cells. Analysis of the impact of cAMP on HCC cell cycle-regulatory proteins demonstrated that cAMP induced specific downregulation of cyclin A protein, downregulation of cyclin A mRNA, and marked inhibition of cyclin A-dependent cdk2 activity.

Cyclin A forms a complex with cdc2 kinase in G2 and M phase, and with cdk2 kinase in S phase and in G1. Cyclin A–cdk2 complexes participate in the control of DNA synthesis [25–27], and the abolition of cyclin A function by microinjection of anti-cyclin A antibody or by plasmids encoding cyclin A antisense abrogates the synthesis of cellular DNA [28]. Deregulation and overexpression of cyclin A have been linked to several forms of cancer, including HCC [8,29,30]. In view of the fact that there is a clear association between hepatitis B virus and HCC, it is provocative that the cyclin A gene was originally identified as the target of hepatitis B virus integration in a human primary liver cancer, and that in that tumor, cyclin A transcription was under the control of the HBV promoter [31,32]. Consistent with a role for cyclin A deregulation in HCC pathogenesis, an analysis of patients with primary liver cancer demonstrated that there was a very significant ($P < 0.0001$) positive correlation between the cyclin A messenger RNA level and the percentage of tumor cells in S+G2/M [8]. In addition, a recent study of HCC patients showed that when cyclin A was overexpressed by the tumor cells, the median survival was 6 months. In contrast, when cyclin A was not overexpressed, the median survival was 29 months [33].

In the experiments reported here, the loss of cyclin A protein was accompanied by a loss of cyclin A mRNA. These data suggest that cAMP may be acting at the transcriptional level to block cyclin A synthesis. This interpretation would be consistent with previous studies showing cAMP-induced inhibition of the cyclin A promoter [34,35]. However, similarly to the effect of cAMP on cell growth, this effect is context-specific, because in other reports, cAMP has been shown to stimulate the cyclin A promoter [36,37].

A critical substrate of the cyclin A–cdk2 complex is the transcription factor heterodimer E2F–DP, which activates the promoter of genes required for DNA synthesis [38,39]. It has been demonstrated that phosphorylation of DP by cyclin A–cdk2 results in release of the E2F–DP complex from its DNA binding site [40]. Furthermore, when cyclin A–cdk2 catalyzed E2F–DP phosphorylation was blocked, the cells died via apoptosis [40]. As demonstrated in the data presented here, cAMP induced loss of cyclin A, loss of cyclin A–cdk2 activity, and apoptosis. This raises the possibility that a mechanism of apoptosis in the cAMP-treated HCC cells may be loss of cyclin A/cdk2-catalyzed phosphorylation of the E2F–DP heterodimer.

In response to the inherent chemoresistance of many of the most prevalent carcinomas of adult life, including HCC, there has been a surge of interest in signal transduction-based approaches to anticancer therapy. A variety of approaches are being developed to employ cAMP or cAMP-dependent kinase (protein kinase A) as a therapeutic target. Phosphodiesterase inhibitors, such as pentoxyphylline, may be useful in combination therapy or in chemoprevention protocols [41,42]. Protein kinase A is found in distinct isoforms [43], and recent efforts have been directed at developing site-selective analogs and site-selective oligonucleotide antisense sequences that may have antiproliferative activity [44–46]. The results presented here demonstrate that cAMP induces G1 synchronization in HepG2 cells, and that this growth arrest is associated with suppression of cyclin A expression and function. These data suggest that the cAMP/PKA pathway may be a useful target in the development of new signal transduction-based approaches to HCC chemoprevention and therapy.

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