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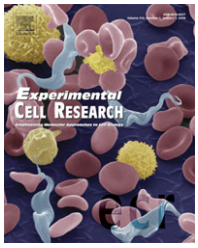


Research Article

# cAMP signaling regulates histone H3 phosphorylation and mitotic entry through a disruption of G2 progression

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ABSTRACT

cAMP signaling is known to have significant effects on cell growth, either inhibitory or stimulatory depending on the cell type. Study of cAMP-induced growth inhibition in mammalian somatic cells has focused mainly on the combined role of protein kinase A (PKA) and mitogen-activated protein (MAP) kinases in regulation of progression through the G1 phase of the cell cycle. Here we show that cAMP signaling regulates histone H3 phosphorylation in a cell cycle-dependent fashion, increasing it in quiescent cells but dramatically reducing it in cycling cells. The latter is due to a rapid and dramatic loss of mitotic histone H3 phosphorylation caused by a disruption in G2 progression, as evidenced by the inhibition of mitotic entry and decreased activity of the CyclinB/Cdk1 kinase. The inhibition of G2 progression induced through cAMP signaling is dependent on expression of the catalytic subunit of PKA and is highly sensitive to intracellular cAMP concentration. The mechanism by which G2 progression is inhibited is independent of both DNA damage and MAP kinase signaling. Our results suggest that cAMP signaling activates a G2 checkpoint by a unique mechanism and provide new insight into normal cellular regulation of G2 progression.



## Introduction

A remarkable variety of hormones, growth factors, and neurotransmitters use cAMP as a second messenger to impact cellular metabolism. Interestingly, cAMP signaling can either inhibit or stimulate cell proliferation depending on the cell type [1,2], however, inhibition of cell growth is most frequently observed [3]. In blocking cell cycle progression, cAMP signaling serves to promote cell differentiation, induce apoptosis, or restore cellular homeostasis after normal proliferative signals [4–7]. Crosstalk with other signaling pathways is often required for cAMP effects on cell growth. In particular, cAMP signaling targets the MAP kinase or Akt/PKB pathways to mediate its growth effects by cell-type specific mechanisms [1–3].

cAMP signaling pathways can inhibit progression through G1, S, and G2/M [8–11]. The most well-characterized of these effects is cAMP-induced arrest in G1, which is mediated through modulation of G1 regulatory proteins such as cyclins D1 and D3 and the cyclin-dependent kinase (cdk) inhibitors $p21^{cip1}$ and $p27^{kip1}$ [8,10,12–14]. Inducers of cAMP synthesis can also delay S phase progression in a

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variety of cell lines, an effect dependent on retinoblastoma protein (Rb) and $p21^{cip1}$ [15]. Effects of cAMP signaling on G2 progression in mammalian somatic cells were first reported in 1976 [11] and have since been observed by several different groups [9,10,16], but its molecular basis has not been examined in detail. Studies of G2 arrest in meiosis I of *Xenopus* and mouse oocytes have shown, however, that cAMP signaling maintains this arrest in a PKA-dependent fashion [17,18].

One hallmark of entry into mitosis is the global phosphorylation of histone H3 in chromosomes [19]. Histone H3 can be phosphorylated at serines 10 and 28 [19,20] as well as threonines 3 and 11 [21,21,22], and all 4 sites have been shown to be phosphorylated in mitotic chromatin. Histone H3 phosphorylation during mitosis is highly conserved from yeast to humans but its function is unclear [23,24]. Three different kinases are involved in phosphorylation of H3 during G2/M: Aurora B kinase (serines 10 and 28) [25,26], haspin kinase (threonine 3) [27], and the death-associated protein-like kinase (threonine 11) [22]. In addition to mitosis, histone H3 can be phosphorylated at serine 10 during interphase in response to the activation of signaling pathways involving MAP kinases, PKA, or NF-κB [28–33]. Signaling-induced H3 phosphorylation occurs in a gene-specific fashion and is thought to serve a transcriptional regulatory role.

In the current study we find that activation of cAMP signaling induces a rapid loss of mitotic H3 phosphorylation in several different cell lines. This loss correlates kinetically with an inhibition of mitotic entry and a rapid decrease in CyclinB/Cdk1 kinase activity. Consistent with this observation, cAMP signaling also causes delayed progression of synchronized cells through G2. The effect on G2 progression requires expression of the catalytic subunits of PKA and is highly sensitive to intracellular cAMP concentration. However, it occurs independently of other pathways which are known to block G2 progression. Our results suggest that cAMP signaling rapidly activates a G2 checkpoint in the mitotic cell division cycle by a distinct mechanism.

## Materials and methods

### *Cell culture and reagents*

Cell line 1470.2 is derived from C127 mouse mammary adenocarcinoma cells and has been described previously [34]. 1470.2, U2OS, and NIH3T3 cells were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals). 8-Br-cAMP and nocodazole were purchased from Sigma. Epinephrine, anisomycin, and SB203580 were purchased from Calbiochem. $N^6$-Phenyl-cAMP and 8-CPT-2′-*O*-Me-cAMP were purchased from Biolog/Axxora. U0126 was purchased from Cell Signaling Technology.

### *Gel electrophoresis, Western transfer, and immunoblotting*

Cells were washed twice with DMEM after treatment and lysates were generated by direct addition of 2× reducing sample buffer (80 mM Tris–HCl pH 6.8, 100 mM DTT, 20% glycerol and 4% SDS). Equal volumes of cellular lysates were resolved by SDS-PAGE. Transfer of proteins onto nitrocellulose membranes was carried out for 1–2 h at 400 mA. Proteins were visualized by staining of membranes with Ponceau S. Immunoblotting was performed with antibodies against H3 phosphorylated at Ser10 or Thr3, HDAC1, and phosphorylated CREB/ATF1 (Upstate Biotechnology), phosphorylated VASP, phosphorylated p70S6K, cdc2 (Cdk1), pCdk1(Y15), and Cyclin B1 (Cell Signaling Technology), phosphorylated ERK1/2 (Promega), Lamin A/C, and PKA catalytic subunits α and β (Santa Cruz Biotechnology) according to manufacturer's instructions. Secondary antibodies (Jackson Immunoresearch) and chemiluminescence reagents (Super Signal, Pierce) were used to detect bound antibodies using chemiluminescence imaging (Alpha Innotech Corp.). Quantitation of signals was carried out with Fluorchem software (Alpha Innotech Corp.).

### *Cell growth, synchronization, and FACS analysis*

Synchronization of cells in G0 was carried out by maintaining 1470.2 cells for 96 h in DMEM/0.25% FBS. Synchronization of cells in mitosis was achieved through treatment with nocodazole (50 ng/ml) for 18 h. Double thymidine block was used to synchronize cells at the G1/S border. 1470.2 cells were treated with 2 mM thymidine for 14–16 h. After two washes with PBS, cells were exposed to fresh medium without thymidine for 8 h, followed by re-exposure to thymidine for an additional 14–16 h. Cells were released from thymidine block by washing and exposure to fresh medium without thymidine.

Cell cycle analysis and monitoring of mitotic H3 phosphorylation was carried out as follows. Cells harvested by trypsinization were washed and fixed for 10 min at 37 °C in PBS/1% methanol-free formaldehyde. After cooling the cells were permeabilized by addition of methanol to a final concentration of 90%. Cells were stained with Alexa 488-conjugated anti-Phospho(Ser10)H3 (Cell Signaling Technology) according to the manufacturer's instructions. Cells were then washed with PBS/0.5% BSA and treated with RNase A and propidium iodide. Both cellular DNA content and H3 phosphorylation were analyzed using a FACS Calibur (BD Biosciences). Determination of cell cycle distribution was carried out using Modfit software BD (Verity Software House, Inc.). FlowJo software (Treestar, Inc.) was used to quantitate levels of mitotic H3 phosphorylation.

### *Immunofluorescence, immunoprecipitation and kinase assay*

For immunofluorescence assay, cells seeded onto glass coverslips were treated with 8-Br-cAMP, left untreated, or UV-irradiated (50 J/$cm^2$) in a Stratalinker (Stratagene). Irradiated cells were allowed to incubate in medium at 37 °C for 60 min prior to fixation. Cells were fixed and stained with antibodies as described in [35]. For experiments on DNA damage, cells were exposed to anti-phosH2AX(Ser139) polyclonal antibody (Cell Signaling Technology) according to manufacturer's instructions.

Digital images of the fixed cells were captured using a DeltaVision RT integrated epifluorescence microscopy system (Applied Precision, Issaquah, WA) fitted with a 20×/0.75NA objective lens (Olympus America Inc., PA). Multiple, random, non-overlapping fields from each coverslip were selected for imaging based only on the DAPI signal. In each experiment at least 1000 cells were imaged per treatment condition. Anti-phosH2AX staining was quantified by a custom automated image analysis