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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.

Keywords

cAMP signaling; histone phosphorylation; cell cycle; protein kinase A; cyclin-dependent kinase; Cyclin B

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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 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.

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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 NIH-3T3 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 2X 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 hour 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 hours in DMEM/0.25% FBS. Synchronization of cells in mitosis was achieved through treatment with nocodazole (50 ng/ml) for 18 hours. 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 hours. After two washes with PBS, cells were exposed to fresh medium without thymidine for 8 hours, followed by re-exposure to thymidine for an additional 14-16 hours. 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 minutes 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

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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/cm2) in a Stratalinker (Stratagene). Irradiated cells were allowed to incubate in medium at 37 C for 60 minutes 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 20x/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 algorithm developed using Matlab software (The Mathworks, Inc., Natick, MA). Briefly, the algorithm defined the region of interest (ROI) for each cell nucleus by auto-thresholding the DAPI image. To ensure that each ROI represented a single nucleus, the ROIs for adjacent nuclei were manually segmented using Metamorph software (Molecular Devices Corp., Downingtown, PA). Anti-phosH2AX staining in each nucleus was measured as the mean immunofluorescence intensity within each previously defined ROI. SPSS 13.0 for Windows (SPSS, Chicago, IL) was used to calculate the mean anti-phosH2AX signal and SEM for each experimental condition. Significant differences ($p < 0.05$) between experimental conditions were detected by analysis of variance and Student-Newman-Keuls post-hoc statistical tests.

For experiments on mitotic index and mitotic staging, cells were seeded onto glass coverslips as described above. After treatment with 0.1 mM 8-Br-cAMP, the cells were fixed, permeabilized and exposed to a mixture of anti-PhosH3(Ser10) polyclonal (rabbit) and anti-Aurora B monoclonal (mouse) antibodies (BD Biosciences) at dilutions recommended by the manufacturer. FITC- or Texas Red-conjugated secondary antibodies were used at a dilution of 1:250. DNA was also stained with DAPI. Images were obtained as described above. In individual images, mitotic stage was assessed manually by the combination of PhosH3, Aurora B, and DAPI staining patterns. At least 1000 cells were examined per treatment condition.

For analysis of CyclinB/Cdk1 and CyclinA/cdk2 activity, whole cell extracts were isolated according to [36]. Whole cell extract protein (250 μg) was precleared by incubation with GammaBind G Sepharose (Amersham Biosciences) in IP Buffer [20 mM Tris-HCl pH 7.5, 5 mM EDTA, 250 mM NaCl, 1 mM DTT, 25 mM β-glycerophosphate, 10 mM NaF, 0.5% NP-40 and protease inhibitors (Calbiochem)] for 1 hour at 4°C. Extracts were then incubated with fresh beads and either 5 μg anti-cyclin B1 (GNS1) (Santa Cruz Biotechnology, Inc) or 2 μg anti-cyclin A (H-432) (Santa Cruz Biotechnology, Inc) for 2 hours at 4°C. Immunoprecipitates were washed 3X with IP buffer and 2X with Kinase buffer (50 mM

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Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT). Samples were resuspended in Kinase Buffer containing 5 μg histone H1 (Upstate Biotechnology) and 10 μCi of [γ^{32}-P] ATP and incubated at 30°C for 30 minutes. For immunoprecipitates from untreated cells the cdk inhibitor, roscovitine (5 μM), was sometimes added to determine background levels of kinase activity. Kinase reactions were stopped by the addition of 5X SDS-PAGE loading buffer. Samples were resolved on 12% SDS-PAGE gels. Radiolabeled histone H1 was detected and quantitated with a phosphoimager and ImageQuant software (Molecular Dynamics).

siRNA Experiments

1470.2 or NIH/3T3 cells grown to 50 % confluency in 24-well dishes were transfected with 50 nM siRNA (SMARTpool, Dharmacon) specific for Lamin A/C, PKA Cα, or PKA Cβ using DharmaFECT reagent (Dharmacon) in serum-free OptiMEM (Invitrogen). The medium was adjusted to 10 % FBS 4 hours after transfection. At either 48 hours (NIH/3T3) or 72 hours (1470.2) after transfection, cells were treated with 1 mM 8-Br-cAMP for 60 minutes. Cell lysates were assayed by Western blotting. For analysis of siRNA transfection efficiency, cells were washed 2X in RT PBS at different timepoints after transfection, and TRIzol reagent (Invitrogen) was added. RNA was purified using Rneasy Mini kit (Qiagen) and 1 μg was reverse transcribed using an iScript cDNA Synthesis kit (Biorad). Quantitative PCR was carried out with primers specific for PKA Cα, PKA Cβ and 18 S rRNA (control) using iQ SYBRgreen Supermix and a MyIQ cycler (Biorad) according to the manufacturer's instructions.

RESULTS

cAMP signaling has distinct effects on interphase and mitotic H3 phosphorylation

We have shown that activation of cAMP signaling by β-adrenergic receptor ligands, forskolin, or 8-Br-cAMP rapidly and specifically downregulates H3 phosphorylation at serines 10 and 28 in a number of mammary-derived cell lines [37]. To further characterize this phenomenon, we continued our studies using 8-Br-cAMP to directly activate cAMP signaling, and avoid the simultaneous activation of other signaling pathways which might be induced by extracellular ligands such as epinephrine. Whereas serine 10 (Ser10) phosphorylation has been detected in both interphase and mitotic cells, threonine 3 (Thr3) phosphorylation has been observed only in mitotic cells [27]. Western blotting analysis with phospho-specific H3 antibodies shows that treatment with 8-Br-cAMP results in dramatic decreases in phosphorylation at Ser10 as well as Thr3, which are similar in time course and magnitude (Fig 1A). These results raise the possibility that cAMP signaling specifically targets mitotic H3 phosphorylation.

To measure mitotic H3 phosphorylation directly, cells stained with both propidium iodide and fluor-conjugated antibody against phosphorylated histone H3 (Ser10) were subjected to FACS analysis. Mitotic 1470.2 cells with high levels of H3 phosphorylation and 4N DNA content are boxed in Fig. 1B. The total percentage of such cells declined precipitously in the presence of 8-Br-cAMP but remained relatively constant in untreated cells (Fig. 1C, upper graph). A similar decline in mitotic H3 phosphorylation was observed in 1470.2 cells treated

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with epinephrine. We also subjected murine NIH3T3 and human U2OS cells to the same analysis and found that mitotic H3 phosphorylation was potently decreased in the presence of 8-Br-cAMP with similar kinetics (Fig. 1C, middle and lower graphs). The magnitude and rate of loss of mitotic H3 phosphorylation is similar to that of bulk H3 phosphorylation measured by Western blotting with phospho-specific H3 antibodies (Fig 1A), indicating that such blots primarily detect mitotic H3 phosphorylation in asynchronously cycling cells.

To examine the effect of cAMP signaling on non-mitotic H3 phosphorylation, we rendered 1470.2 cells quiescent through serum starvation. FACS analysis of quiescent cells showed that >85% of cells had 2N DNA content and <0.1% of cells had mitotic levels of H3 phosphorylation (data not shown). Quiescent cells were treated with 8-Br-cAMP for varying times and lysates were subjected to Western blotting to detect H3 phosphorylation at Ser10. Surprisingly, cAMP signaling induced a transient increase in H3 phosphorylation (Fig. 1D), similar to that shown by Hunzicker-Dunn and colleagues in ovarian granulosa cells [29,30].

Mitotic entry is inhibited by cAMP signaling

Cell cycle-dependent chromosomal H3 phosphorylation begins in late G2 and remains high through early anaphase [38]. Loss of mitotic H3 phosphorylation could thus occur by two basic mechanisms. cAMP signaling may cause global dephosphorylation of H3 in mitotic chromosomes or it may prevent global H3 phosphorylation through a pre-mitotic block to cell cycle progression. To test the first possibility, cells arrested in mitosis by nocodazole (>80% of the cells had 4N DNA content) were treated with or without 8-Br-cAMP for 1 hour and analyzed for H3 phosphorylation (Ser10). As expected, nocodazole-treated cells had greatly elevated levels of phosphorylated H3 relative to asynchronously-growing cells, however, treatment with 8-Br-cAMP failed to induce a loss of H3 phosphorylation (shown graphically in Fig. 2A). To directly observe H3 phosphorylation on mitotic chromosomes, indirect immunofluorescence experiments were performed in which cells were stained with both DAPI and antibody against H3 phosphorylated at Ser10. In cells treated with 8-Br-cAMP, mitotic figures representing cells in prophase, prometaphase, metaphase, and early anaphase were all brightly stained with the anti-phosH3 antibody (data not shown). These results indicate that once cells have entered mitosis, cAMP signaling does not induce aberrant dephosphorylation of mitotic chromosomes.

To determine whether cAMP signaling prevents mitotic H3 phosphorylation through a pre-mitotic cell cycle block, immunofluorescence experiments were carried out as described in Materials and Methods to calculate the mitotic index in untreated and 8-Br-cAMP-treated cells. Over a 60 minute period, the mitotic index fell dramatically in the presence of 8-Br-cAMP but remained relatively constant in untreated cells (Fig. 2B). The rate and magnitude of this decline is highly consistent with the loss of mitotic H3 phosphorylation shown in Fig. 1B. The percentage of untreated cells in each stage of mitosis did not significantly change over a 60 minute period (Fig. 2C). In contrast, cAMP signaling induced dramatic changes in the mitotic profile (Fig. 2D). Only 15 minutes of treatment with 8-Br-cAMP resulted in a significant loss of cells in prophase. By 30 minutes of treatment there were very few cells in prophase and prometaphase. By 60 minutes of treatment all mitotic figures observed were in telophase. In fact, the percentage of mitotic figures in telophase increased steadily over the

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course of treatment. The cells were not, however, accumulating in telophase as only a very small percentage of cells were mitotic after 1 hour of treatment (Fig. 2B). These results clearly show that cells stop entering prophase very quickly after exposure to 8-Br-cAMP, suggesting a rapid block to G2 progression.

cAMP signaling inhibits G2 progression.

We previously reported that cAMP signaling significantly inhibits growth of 1470.2 cells and results in arrest of cell cycle progression in both G1 and G2/M 48 hours after treatment with 8-Br-cAMP [37]. The rapid effects on mitotic entry (Fig. 2) suggest that the G2/M block occurs quickly in response to activation of cAMP signaling. The relative timing of the inhibition to progression of G1 and G2/M was examined by analyzing cell cycle distribution in asynchronously growing cells for up to 8 hours (Fig. 3A). Untreated cells showed little change in G2/M content over the entire time course. Cells treated with 8-Br-cAMP, however, steadily accumulated in G2/M, reaching about 40% in 6-8 hours. In addition, the percentage of cells in G1 declined over the treatment period in cells exposed to 8-Br-cAMP, while in the untreated cells it steadily increased. Since we find that cAMP signaling does not block transit through M phase (Fig. 2), we conclude that cells accumulate initially in G2 and the block to G1 progression occurs later. Interestingly, cells in which cAMP signaling was activated were slightly slower to exit S phase, especially at the early time points. This is likely to reflect the transient, cAMP-induced delay in S phase reported by Blomhoff and colleagues. Thus, the accumulation of cells in G2 phase at 8 h post-treatment could be a combination of a block to mitotic entry and delayed exit from S phase.

To analyze the cAMP-induced accumulation of cells in G2 in more detail, 1470.2 cells were synchronized in early S phase by double thymidine block. Between 4-5 hours after release a significant fraction of cells moved from S into G2 as measured by FACS analysis (Fig. 3B). Thus, at 5 hours post-release cells were treated with 8-Br-cAMP to monitor effects on G2 progression. In the absence of cAMP, the cells rapidly accumulated in G2/M, reaching a maximum 7 hours after release (Fig. 3B, solid line). In contrast, after a brief pause cAMP-treated cells accumulated in G2/M at a similar pace but were delayed in exiting for 3-5 hours as evidenced by a flattening of the G2/M peak between 8 and 12 hours post-release (Fig. 3B, dashed line). At 14 hours post-release the majority of untreated cells had exited G2/M but in the 8-Br-cAMP-treated population a significant fraction (twice the amount relative to control cells) remained in G2/M. These results indicate that the effects of cAMP signaling on S phase progression are very transient, since cells were only delayed for an hour in accumulating in G2. In contrast, the inhibitory effect of cAMP signaling on G2 progression is much more prolonged.

In the same experiments we measured mitotic H3 phosphorylation by FACS to monitor entry into M phase. As shown in Fig. 3C, untreated cells reached a sharp peak in levels of mitotic H3 phosphorylation at 8 hours post-release, just 1 hour after the peak of cells in G2/M. As observed in asynchronously cycling cells (Fig. 1B), 8-Br-cAMP treatment caused an abrupt cessation of entry into M phase as evidenced by a near-complete loss of mitotic H3 phosphorylation 60 minutes after treatment (6 hours post-release). Cells began to recover by 2 hours of treatment and reached a level of mitotic H3 phosphorylation equivalent to that

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at the beginning of cAMP treatment by 8 hours post-release. However, the percentage of cells in early M phase formed a flattened peak, similar to that formed by G2/M cells (Fig. 3D). Unlike untreated cells, cAMP-treated cells exit G2 and enter mitosis at a very slow rate for up to 14 hours post-release.

cAMP signaling prevents activation of Cyclin B/Cdk1.

The critical event in the cellular transition from G2 to M phase is the activation of the Cyclin B/Cdk1 kinase. DNA damage or expression of the HIV-encoded Vpr protein induce G2 arrest by preventing the normal activation of this complex by various mechanisms [39-42]. To determine whether cAMP signaling modulates the activity of this complex, we generated whole cell extracts from cells treated with 8-Br-cAMP and measured both the levels of Cyclin B1 and Cdk1 and the activity of the complex. Fig. 4A shows that cAMP signaling did not result in a significant change in cellular levels of either Cyclin B1 or Cdk1 over the course of treatment.

The Cyclin B/Cdk1 complex was immunoprecipitated from 8-Br-cAMP-treated cells and tested for kinase activity using histone H1 as a substrate (Fig. 4B). The results show that by 60 minutes of treatment Cyclin B/Cdk1 activity was significantly reduced to a level close to background as assessed in samples treated with the Cdk inhibitor, roscovitine. Thus, consistent with its negative effects on mitotic entry (Fig. 2), cAMP signaling leads to downregulation of total cellular Cyclin B/Cdk1 kinase activity.

The activity of the Cyclin B/Cdk1 complex is highest in early M phase. Thus, the cAMP-mediated downregulation of Cyclin B/Cdk1 activity may result from the loss of mitotic cells. Alternatively, cAMP signaling may prevent the activation of the complex that normally triggers the transition from G2 to M, as do known inducers of the G2 checkpoint. One key event in the activation of the complex is the dephosphorylation of Cdk1 at threonine 14 and tyrosine 15 (Y15) [43]. We used synchronized 1470.2 cells to determine the effect of cAMP signaling on phosphorylation of Cdk1 at Y15 as shown in Fig. 4C,D. Most untreated cells pass through mitosis between 6 and 11 hours post-release (Fig. 3C). Over this same time period the amount of Cdk1 phosphorylated at Y15 [pCdk1(Y15)] relative to total Cdk1 decreases, consistent with the synchronized entry of cells into mitosis (Fig 4C,D). In contrast, cells in which cAMP signaling was activated do not show this coordinated decrease. The ratio of pCdk1(Y15) to total Cdk remains relatively constant, consistent with the inhibition to mitotic entry. Thus, cAMP signaling prevents activation of the CyclinB/Cdk1 complex. The cAMP-induced G2 delay also affects levels of total cellular Cdk1. In untreated cells total Cdk1 levels increase as the cells pass from mitosis into G1 7-10 hours after release. In cells treated with 8-Br-cAMP, this increase does not occur. Rather, levels of total Cdk1 gradually decrease as the cells linger in G2/M. This is not a direct effect since Cdk1 levels do not change within the first hour after treatment with cAMP (Fig. 4A,C) but is likely the result of the prolonged cell cycle delay.

CyclinA/cdk2 activity is highest during the G2 phase of the cell cycle [44,45] and several reports indicate that it plays an important role in mitotic entry and early mitosis in part through upstream effects on CyclinB/Cdk1 [46-51]. RNAi-mediated depletion of CyclinA/cdk2 causes a delay in mitotic entry and inhibits the ability of CyclinB/Cdk1 to promote

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mitosis by various mechanisms [48,50,51]. cAMP signaling may thus prevent activation of CyclinB/Cdk1 though negative effects on CyclinA/Cdk2. Therefore, we examined the effect of cAMP signaling on CyclinA/cdk2 levels and activity. Fig. 5A shows that activation of cAMP signaling did not have a significant effect on levels of either Cyclin A or cdk2 in 1470.2 cells over the time frame in which mitotic entry is drastically inhibited. Immunoprecipitation-kinase assays revealed that cAMP signaling has an inhibitory effect on Cyclin A-dependent kinase activity but it is modest and significantly slower than the inhibition of Cyclin B activity (Fig. 4B). Thus, we conclude that cAMP effects on Cyclin A/cdk2 activity are probably not the cause of Cyclin B inhibition.

cAMP-induced loss of H3 phosphorylation requires expression of PKA catalytic subunits

PKA and the recently discovered cAMP-regulated guanine nucleotide exchange factors, Epac 1 and 2 [52,53], are thought to mediate most of the effects of cAMP signaling in non-neuronal mammalian cells. The cell lines used in our experiments express PKA catalytic subunits, α and β (Fig. 6A), as well as Epac1 (data not shown). To test the possible involvement of Epac, we treated cells with a cAMP analog, 8-CPT-2'-O-Me-cAMP, which specifically activates Epac but not PKA [54]. Over a period of 2 hours, H3 phosphorylation was unchanged (Fig. 6). In addition, a dose response study covering a wide range of 8-CPT-2'-O-Me-cAMP concentrations showed no effects on H3 phosphorylation in both 1470.2 and NIH3T3 cells (data not shown). To support the lack of Epac involvement we also treated cells with N^6-phenyl-cAMP, which activates PKA but not Epac [55], and found that it efficiently induces the loss of H3 phosphorylation.

G2 arrest in meiotic Xenopus oocytes is dependent on PKA activity [56] but the PKA dependence of cAMP-induced G2 delay in the mammalian mitotic cell cycle has never been thoroughly examined with the specialized exception of one cell mouse embryos [57]. To assess the role of PKA in mediating cAMP effects on G2 progression, we used siRNAs to knockdown expression of the PKA catalytic subunits, α and β, in 1470.2 and NIH3T3 cells (Fig. 7A and data not shown). Lamin A/C siRNA was used as a control for non-specific effects of siRNA and expression of HDAC1 was monitored for potential off-target effects. The VASP protein is phosphorylated at two serines in response to cAMP signaling in a PKA-dependent fashion [58], thus, we monitored VASP phosphorylation at Ser239 as a marker of PKA activity. The phosphorylation of H3 and VASP was assayed by immunoblotting in both 1470.2 (shown in Fig. 7B) and NIH3T3 cells treated with various combinations of siRNAs in the presence or absence of 8-Br-cAMP for 1 hour. The results for cAMP-induced VASP phosphorylation are shown graphically in Fig. 7C. Knockdown of the Cα subunit alone almost eliminates VASP phosphorylation in NIH3T3 cells and in 1470.2 cells is more effective in blocking it than knockdown of the Cβ subunit. In contrast, knockdown of either PKA subunit alone has little effect on the ability of cAMP to induce loss of H3 phosphorylation (Fig. 7D). However, knockdown of both subunits almost completely blocks it. These results indicate that expression of the PKA catalytic subunits is required for cAMP-induced inhibition of G2 progression.

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Sensitivity of G2 Progression to cAMP Concentration

Studies have shown that intracellular cAMP levels fall prior to the onset of mitosis in the absence of extracellular stimuli that induce or inhibit cAMP synthesis [57,59,60]. To determine the sensitivity of G2 progression to cAMP concentration, we carried out a dose response analysis with 8-Br-cAMP (Fig. 8A). Surprisingly, very little 8-Br-cAMP (0.1 mM) was required to induce maximal loss of bulk H3 phosphorylation; the EC50 for this effect was 8.9 μM (Fig. 8C). This low dose was also sufficient for virtually complete loss of mitotic H3 phosphorylation (Fig. 1A,B) and delay of G2 progression (Fig. 3B,C). cAMP signaling also mediates a decrease in phosphorylation of p70 S6 kinase (p70S6K) at serine and threonine residues (Thr421/Ser424) in its pseudosubstrate region [61]. As in the case of H3, loss of p70S6K phosphorylation is very sensitive to 8-Br-cAMP concentration, having an EC50 of 21.5 μM (Fig. 8A,C).

Increased CREB phosphorylation is typically used as a measure of intracellular PKA activity in the presence of extracellular stimuli which induce increased cellular cAMP levels. The dose response curve for CREB phosphorylation was very different from that observed for H3 phosphorylation (Fig. 8B). 8-Br-cAMP-induced CREB phosphorylation was half-maximal at 144 μM and required concentrations as high as 1 mM for full induction (Fig. 8B,C). Similarly, the cAMP-induced increase in VASP phosphorylation also requires higher doses of cAMP, having an EC50 of 481 μM (Fig. 8B,C). Thus, the difference in exogenously-applied cAMP concentrations required to elicit PKA-mediated events can be greater than 10-fold.

The cAMP-induced block to G2 progression is independent of DNA damage and MAP kinase signaling pathways

To determine how cAMP signaling may disrupt G2 progression we assessed the possible involvement of signaling machinery from two pathways known to induce G2 checkpoints. In mammalian somatic cells a G2 checkpoint can be activated through DNA damage [42]. Disruption of the normal progression of chromatin modification in preparation for mitosis may result in abnormal chromatin structures which make DNA more vulnerable to damage from endogenous agents. To check for DNA damage we assayed H2AX phosphorylation by immunofluorescence using cells treated with 8-Br-cAMP (Fig. 9A). UV-irradiated cells showed a robust increase in H2AX phosphorylation. In contrast, cAMP signaling does not induce any significant changes over the time course in which G2 progression is disrupted. Even in the absence of DNA damage, cAMP signaling may target downstream components of the DNA damage signaling machinery to inhibit G2 progression. Chk kinases are activated by phosphorylation in response to DNA damage and function to inactivate cdc25 phosphatases that control the activity of the CyclinB/Cdk1 complex [62]. To test the possibility that cAMP signaling targets Chk1, we used a phospho-specific antibody to monitor its activation. Total Chk1 levels were also examined. While Chk1 phosphorylation is significantly induced by UV exposure, it is not affected by cAMP signaling (Fig. 9B).

MAP kinases are also important in regulating G2 progression. A G2 checkpoint can be activated through stress signaling via p38 MAP kinase [63] while the MEK/ERK signaling pathway can promote the G2/M transition [64]. We used specific inhibitors of MEK1/2

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(U0126) and p38 (SB203580) to inhibit these MAP kinase signaling pathways. As shown in Fig. 10A, the effects of U0126 can be demonstrated through a rapid loss of ERK1/2 phosphorylation which persists for up to 2 hours. Treatment with vehicle or U0126 alone had little effect on H3 phosphorylation (Fig. 10C), thus inhibition of the MEK/ERK pathway does not mimic the effects of cAMP signaling. The p38 activator anisomycin increased phosphorylation of CREB/ATF1, downstream targets in the p38 signaling pathway, however this increase was completely inhibited in the presence of SB203580 (Fig. 10B). In the presence of cAMP, neither SB203580 or U0126 blocked the activation of the G2 checkpoint as evidenced by lack effect on loss of H3 phosphorylation (Fig. 10D). Therefore, cAMP signaling regulates G2 progression independently of MAP kinase activity.

DISCUSSION

In Xenopus and mouse oocytes cAMP signaling maintains G2 arrest during the meiotic cell cycle. In this study we show that cAMP signaling causes a rapid and dramatic loss of mitotic H3 phosphorylation due to the inhibition of G2 progression in several mammalian somatic cell lines, thus, regulation of G2 progression via cAMP extends to the mitotic cell cycle. Furthermore, we report that the cAMP-mediated effect on G2 progression is dependent on expression of PKA catalytic subunits and is highly sensitive to cAMP concentration relative to other PKA-mediated events. Finally, we have shown that the mechanism by which cAMP signaling disrupts G2 progression is distinct in that it occurs independently of other pathways known to inhibit G2 progression, such as DNA damage and MAP kinase signaling. We suggest that cAMP signaling activates a G2 checkpoint in mammalian somatic cells through a unique pathway.

We show that regulation of histone H3 phosphorylation by cAMP signaling is dependent on whether cells are quiescent or actively cycling. The dramatic and global cAMP-induced loss of phosphorylation at Ser10 and Thr3 in cycling cells suggested a specific effect on mitotic H3 phosphorylation. Global phosphorylation of histone H3 at these sites is a hallmark of the onset of mitosis and is catalyzed by distinct kinases [25-27]. FACS analysis confirmed that cAMP signaling caused a drastic reduction of mitotic H3 phosphorylation in both mouse and human cell lines. Remarkably, in quiescent cells treated with 8-Br-cAMP, H3 phosphorylation was transiently but significantly increased. This may represent targeted, promoter-specific changes in H3 phosphorylation, much like that observed in quiescent cells in which MAP kinase signaling is activated [65,66] and in ovarian granulosa cells treated with follicle stimulating hormone [29,30].

Loss of mitotic H3 phosphorylation could be achieved through aberrant dephosphorylation of mitotic chromosomes or an inhibition of G2 progression prior to the point at which global H3 phosphorylation begins. Our results support the latter; cAMP-treated cells stop entering mitosis within 15 minutes (Fig. 2D), but progression of cells already in M phase is not disrupted, thereby resulting in a dramatic decrease in the mitotic index (Fig. 2B). cAMP-mediated inhibition of mitotic entry is further supported by the simultaneous decrease in cellular activity of the Cyclin B/Cdk1 kinase, which is essential for transition from G2 into mitosis. We show that cAMP signaling prevents activation of CyclinB/Cdk1 as evidenced by persistence of inhibitory Cdk1(Y15) phosphorylation in synchronized cells treated with

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8-Br-cAMP, a behavior consistent with several agents which inhibit G2 progression [42,63,67]. Taken together, our results suggest that cAMP signaling rapidly activates a G2 checkpoint, perhaps through direct targeting of proteins which control Cyclin B/Cdk1 activity. Experiments with synchronized cells reveal that the cAMP-induced block to G2 progression can be reversed based on the partial recovery of mitotic H3 phosphorylation (Fig. 3C). However, the persistence of a significantly-elevated percentage of cells in G2/M for up to 9 hours after treatment and an incomplete recovery of mitotic H3 phosphorylation over the same time period (Figs. 3B,C) indicates that there is variability in the rate at which cells recover from the putative checkpoint. The cells remaining in G2/M after prolonged 8-Br-cAMP treatment [37] may be arrested. The ability of these somatic cells to at least partially recover from the cAMP-induced inhibition of G2 progression differentiates them from germ line oocytes undergoing meiosis, in which the cells remain arrested in G2 until they receive a signal to divide [17].

Our previous study showed that prolonged cAMP signaling results in a block to both G1 and G2 progression and significantly inhibits cell growth [37]. Here we show that the disruption of G2 progression occurs prior to the block to G1 progression. The mechanism of cAMP-induced G1 arrest has been demonstrated by several groups to involve changes in gene expression [8,10,12] which is consistent with the delayed onset of the G1 arrest we observed. Based on the fact that changes in the cellular expression profile are required to achieve the G1 block, it is possible that the function of the reversible cAMP-mediated disruption to G2 progression is to allow time for these changes to occur so that the cells efficiently arrest in G1 after mitosis. This is supported by our previous results showing that asynchronously-cycling cells treated with 8-Br-cAMP for 48 hours undergo only one division while untreated cells increase in number by a factor of 5 [37].

The cAMP-binding proteins, Epac and the PKA holoenzyme, are thought to mediate the majority of effects of cAMP signaling in non-neuronal cells. We ruled out the involvement of Epac in the cAMP-mediated effect on G2 progression using a specific agonist and a cAMP analog that does not activate Epac. The sensitivity of H3 phosphorylation to cAMP concentration (Fig. 8) also supports this conclusion. Epac has a significantly lower affinity for cAMP than PKA [68], making it less likely that Epac initiates this pathway. Experiments with siRNA directed against the catalytic subunits of PKA showed that their expression is necessary for the disruption of G2 progression using the cAMP-induced loss of H3 phosphorylation as an indicator. This is similar to the G2 arrest observed in Xenopus oocytes, which is maintained by the catalytic activity of PKA [17,69]. Also in accordance with our findings, PKA activity fluctuates throughout the cell cycle in mammalian cells and mouse embryos [57,70], falling prior to mitosis and rising upon mitotic exit. These results show that cAMP and PKA are important regulators of G2 progression and mitotic entry in mammalian somatic cells as well as embryos and oocytes.

It has been proposed that intracellular cAMP levels are important for maintaining the interphase state [71]. In Xenopus and mammalian systems, cAMP levels fluctuate like those of PKA activity during the cell cycle, falling prior to mitosis and rising again upon transition to G1 [57,59,60] without any exogenous inhibition or stimulation of cAMP signaling. Our experiments show that G2 progession is highly sensitive to small increases in cAMP

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concentration, which were achieved by the treatment of cells with a membrane-permeable form of cAMP, 8-Br-cAMP. The loss of H3 phosphorylation and disruption of G2 progression were maximally-induced by 0.1 mM 8-Br-cAMP, a concentration at which CREB phosphorylation is only slightly induced and VASP phosphorylation is undetectable (Fig. 7A,B). In fact, we demonstrate that phosphorylation of two proteins, histone H3 and pp70S6 kinase, is modulated with exogenous 8-Br-cAMP concentrations which are an order of magnitude lower than those required to modulate phosphorylation of CREB and VASP (Fig. 7C). This is likely explained by the fact that intracellular concentrations of cAMP are not uniform throughout the cell [72-74] due to variation in localized synthesis and breakdown of cAMP. Through basal activity of adenylate cyclases and low PDE activity [75] cells may maintain threshold levels of cAMP and PKA activity at particular intracellular locations where proteins which promote mitotic entry are concentrated during interphase, keeping them inactivated. In late G2 cells may selectively downregulate cAMP levels in these areas, perhaps through local activation of PDEs, thereby reducing PKA activity and allowing these proteins to be activated. Because of low PDE activity prior to mitosis, these local areas may be sites of rapid accumulation of exogenously-applied 8-Br-cAMP. The PKA activity inhibitory to mitotic progression is thus maintained until the cells are able to reduce it. Other cellular compartments containing proteins, such as CREB or VASP, which respond to extracellular signals may have higher levels of localized PDE activity and thus require higher levels of exogenous cAMP to trigger PKA activity. These local and global effects of intracellular cAMP are reminiscent of signaling through calcium [76].

To investigate the mechanism by which cAMP signaling disrupts G2 progression we considered the possibility of crosstalk with other pathways known to bring about checkpoint activation in mammalian cells, including DNA damage and MAP kinase signaling [42,63,64]. We found that cAMP signaling had no effect on either H2AX phosphorylation, which is a hallmark of DNA damage [77,78] or Chk1 phosphorylation, which occurs as part of DNA damage signaling cascade but also functions in normal cell cycle progression [79]. Likewise, inhibition of the p38 or MEK/ERK signaling pathways does not mimic or block the cAMP-induced loss of H3 phosphorylation, making it unlikely that these pathways are activated or inhibited by cAMP signaling to disrupt G2 progression (Fig. 10). Thus, we conclude that the mechanism by which cAMP signaling inhibits progression through G2 is distinct because it occurs independently of signaling pathways known to activate mammalian G2 checkpoints.

What target of PKA prevents the activation of CyclinB/Cdk1 to inhibit mitotic entry? CyclinA/Cdk2 is an excellent candidate because the effects of its inhibition on cell cycle events are similar to those of cAMP signaling. However, we did not observe rapid and dramatic changes in cellular Cyclin A-dependent kinase activity upon activation of cAMP signaling (Fig. 5). Thus, changes in Cyclin A/cdk2 activity do not appear to be responsible although this does not rule out a regulatory mechanism such as localization or association with proteins essential for its G2 function. Xenopus Cdc25C, which dephosphorylates and activates CyclinB/cdk1, was shown to be phosphorylated by PKA *in vitro* at a site which inactivates it [56]. However, it is unlikely that PKA phosphorylates the analogous site in the

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human homolog because its sequence context does not resemble a PKA consensus site and mouse Cdc25C does not contain the corresponding serine residue. In addition, two recent studies suggest that Cdc25C may not be the key regulator of mitotic entry in mammalian cells; Cdc25A and B seem to have more important roles in this process [80,81]. Another recent study showed that the mouse oocyte-specific kinase, Wee1B, which inactivates the Cyclin B/Cdk1 complex by phosphorylation of Thr14 and Tyr15, can be phosphorylated by PKA *in vitro* and suggested that PKA targets this kinase to maintain meiotic arrest in mouse oocytes [18]. It is possible that PKA also targets somatic forms of Wee1 in mammalian cells.

In summary, our investigation of cAMP effects on mitotic H3 phosphorylation and G2 progression have provided strong evidence of a conserved role for cAMP in regulation of mitotic entry. This pathway may be initiated from local areas of intracellular cAMP synthesis which are regulated during cell cycle progression. Tumor cells deficient in activation of G1 checkpoints are heavily dependent on the G2 checkpoint to repair DNA damage prior to entering mitosis and this has led to an effort to identify drugs which abrogate G2 checkpoint activation to aid in selective killing of tumor cells [82]. Inhibitors of PKA could be candidates for this class of anti-tumor drugs.

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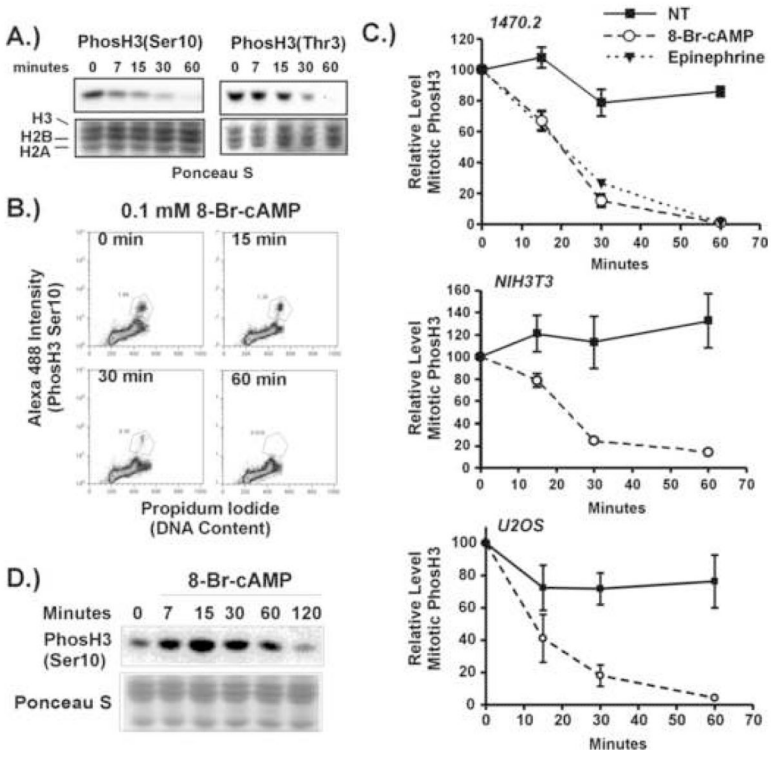

Figure 1. cAMP signaling targets mitotic H3 phosphorylation

(A) Western blot analysis of lysates from cells treated for various times with 0.1 mM 8-Br-cAMP using antibodies against histone H3 phosphorylated at Ser10 or Thr3. (B) FACS analysis of mitotic H3 phosphorylation at Ser10. 1470.2 cells were treated with 0.1 mM 8-Br-cAMP for up to 60 minutes. Along with matched untreated controls, cell were harvested and processed for staining with antibody to phosphorylated H3 and propidium iodide. Dot plots from the 8-Br-cAMP-treated samples are shown. The boxed areas contain cells with 4N DNA content which are highly stained with the phosphorylated H3 antibody and represent cells in the early stages of mitosis. (C) Statistical analysis of the results from 3 independent FACS experiments in 1470.2, NIH3T3, and U2OS cells. 8-Br-cAMP was used at 0.1 mM and epinephrine at 1 μM. In each experiment, the percentage of cells containing high levels of H3 phosphorylation without treatment was set to 100 and the percentages of like cells at various times of 8-Br-cAMP or epinephrine treatment were expressed relative to 100. (D) Analysis of the effects of cAMP signaling on H3 phosphorylation in quiescent, non-mitotic cells. 1470.2 cells were synchronized in G0 by serum starvation and treated for various times with 1 mM 8-Br-cAMP. Acid-extracted histones were subjected to Western blotting with antibody against H3 phosphorylated at Ser10.

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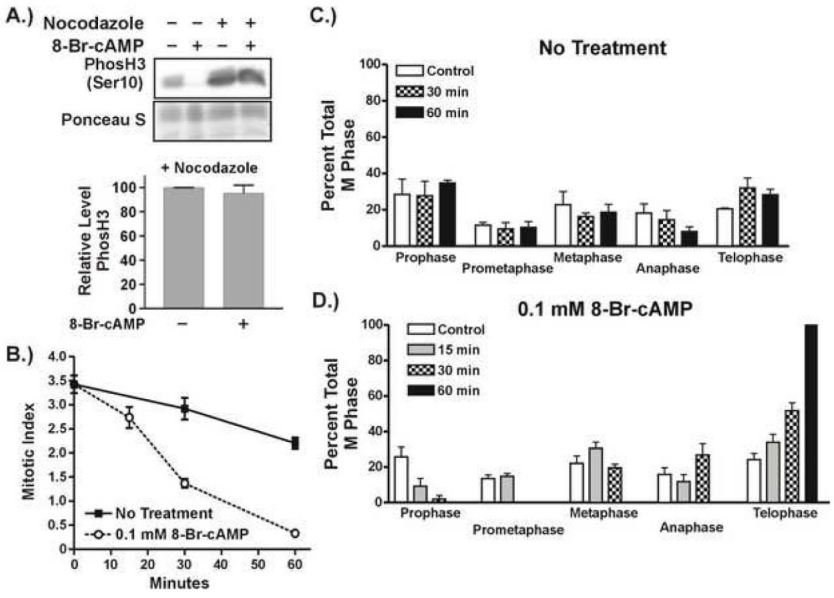

Figure 2. cAMP signaling inhibits entry of cells into mitosis

(A) 1470.2 cells were untreated or treated with nocodazole for 18 hours followed by incubation in the presence or absence of 0.1 mM 8-Br-cAMP for 1 hour. Cell lysates were subjected to Western blotting with antibody against H3 phosphorylated at Ser10. Nocodazole-treated cells were also processed for FACS analysis of mitotic H3 phosphorylation. The results from 3 independent FACS experiments are shown graphically. In each experiment the percentage of untreated cells containing mitotic levels of H3 phosphorylation was set to 100 and the value from 8-Br-cAMP-treated cells was expressed relative to 100. (B-D) cAMP signaling reduces the mitotic index and causes dramatic changes in the mitotic profile. 1470.2 cells were treated with 0.1 mM 8-Br-cAMP for up to 60 minutes. Along with matched, untreated controls, cells were processed for indirect immunofluorescence. Mitotic figures were counted and staged using staining from DAPI, phosphorylated H3, and Aurora B as benchmarks. In each experiment 1000-2000 cells were examined per treatment condition. B.) The mitotic index from untreated and 8-Br-cAMP-treated cells over time. C,D.) The percentage of cells in each stage of mitosis at the various times sampled in untreated (C) and cells treated with 0.1 mM 8-Br-cAMP (D). Graphs represent statistical analysis of results from 3 independent experiments. Error bars represent SEM.

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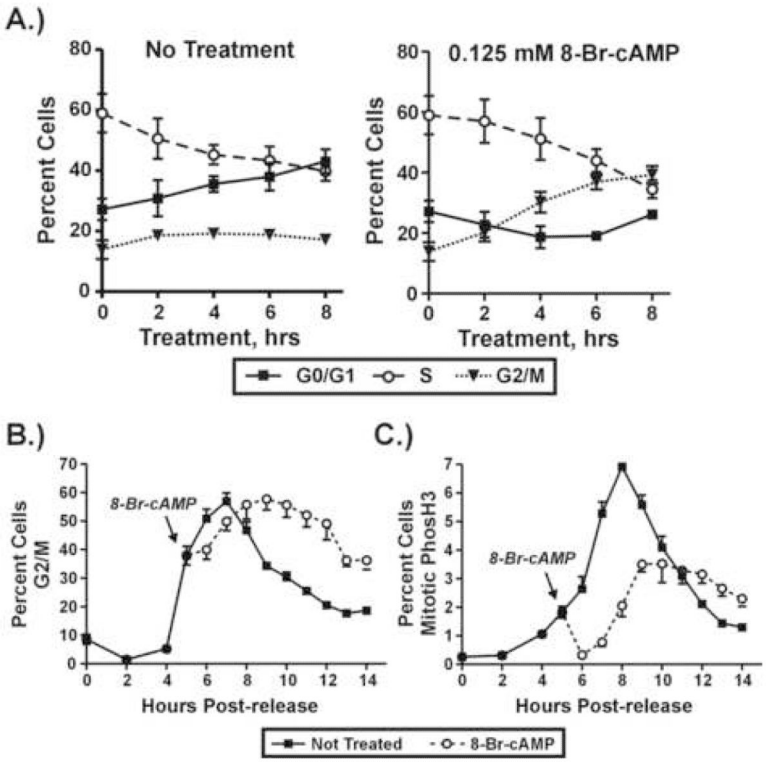

Figure 3. Effects of cAMP signaling on cell cycle progression

A.) 1470.2 cells were treated with or without 0.125 mM 8-Br-cAMP. Every 2 hours cells were harvested and processed for cell cycle analysis. Results from 4 independent experiments are shown graphically. B,C.) 1470.2 cells were synchronized at G1/S by double thymidine block. Five hours after release, half the cells were treated with 0.1 mM 8-Br-cAMP. Samples were collected every hour and processed for cell cycle analysis (B) and measurement of mitotic H3 phosphorylation by FACS (C). The graphs represent results from 3-4 independent experiments. Error bars represent SEM.

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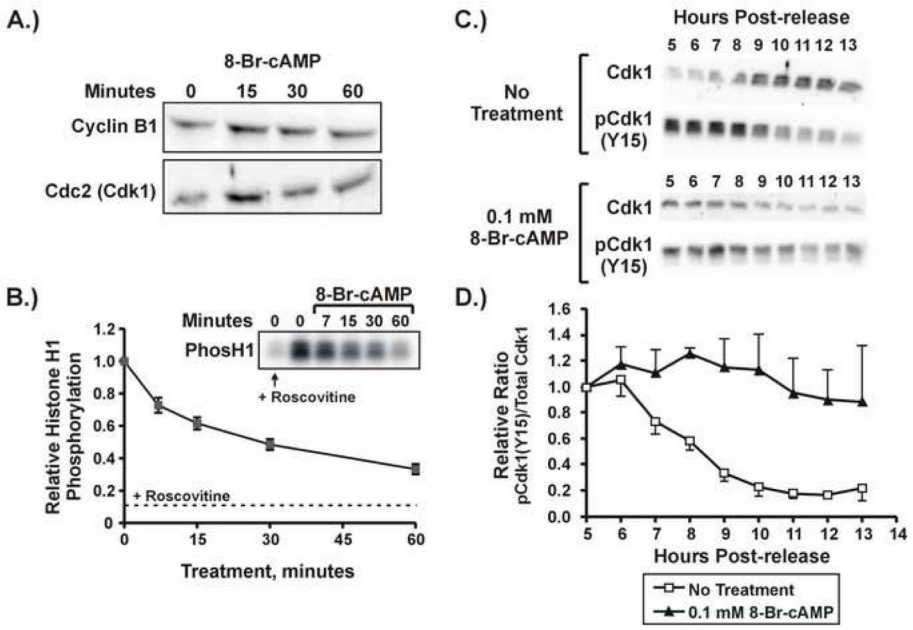

Figure 4. cAMP signaling prevents activation of Cyclin B/Cdk1

A.) 1470.2 cells were treated with 0.1 mM 8-Br-cAMP for up to 60 minutes and harvested for preparation of whole cell lysates. Equal amounts of whole cell extract protein were separated by SDS-PAGE and subjected to Western blotting with either anti-Cyclin B or anti-cdc2 antibodies. B.) Immunoprecipitation from whole cell extracts was carried out with Cyclin B antibody. Immunoprecipitates were assayed for Cyclin B/Cdk1 kinase activity using histone H1 as a substrate. Samples were separated by SDS-PAGE and autoradiographed (inset). Statistical analysis of results from 3-4 independent experiments is shown graphically. The relative level of histone H1 phosphorylation in untreated cell extracts in the presence of the cdk inhibitor roscovitine is indicated as a dashed line. Error bars represent SEM. C,D.) Western blot analysis of lysates from cells synchronized by double thymidine block and treated with or without 0.1 mM 8-Br-cAMP at 5 hours post-release. Immunoblotting was carried out to detect total cellular Cdk1 or pCdk1(Y15) in lysates collected from 5 to 13 hours post-release (C). Bands on the western blots were detected by chemiluminescence imaging and quantitated. For each time point the ratio of pCdk1(Y15) to total Cdk1 was calculated. In each experiment the ratio at the 5 hour time point was set to 1 and all other time points were expressed relative to that. Graphic representation of the data from 3 independent experiments is shown (D). Error bars represent SEM.

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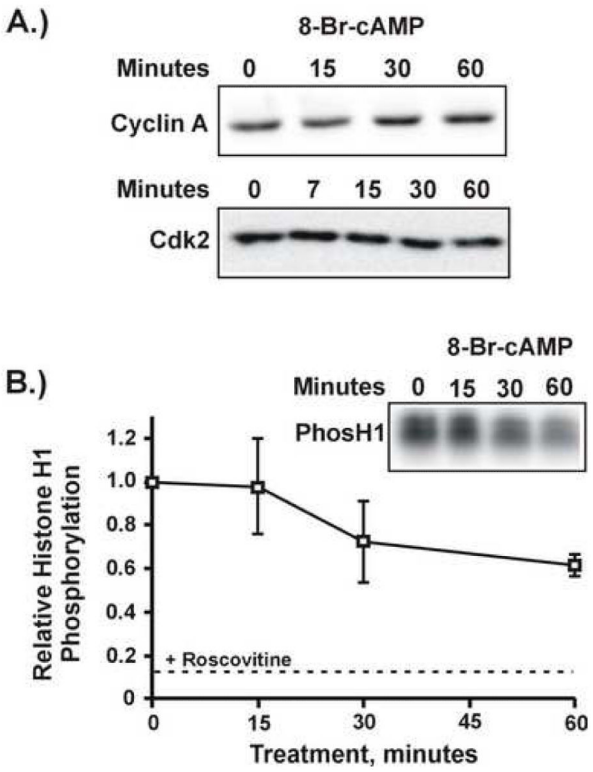

Figure 5. Effects of cAMP signaling on CyclinA/cdk2 levels and activity

1470.2 cells were treated with various times with 0.1 mM 8-Br-cAMP. Cells were harvested and cell lysates or whole cell extracts were generated. A.) Whole cell extracts (Cyclin A) or cell lysates (cdk2) were subjected to Western blotting with antibodies against Cyclin A or cdk2. The results shown are representative of 3-4 experiments. B.) Immunoprecipitation from whole cell extracts was carried out with Cyclin A antibody. Immunoprecipitates were assayed for Cyclin A-dependent kinase activity using histone H1 as a substrate. Samples were separated by SDS-PAGE and autoradiographed (inset). Statistical analysis of results from 3-4 independent experiments is shown graphically.

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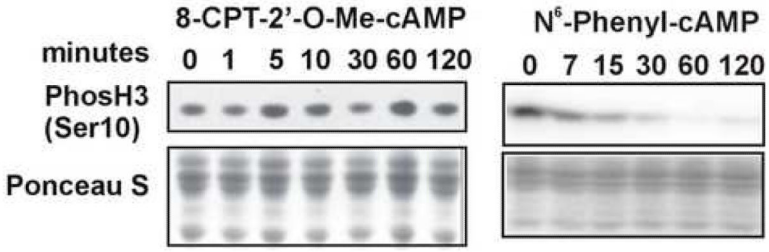

Figure 6. cAMP-induced loss of H3 phosphorylation is independent of Epac

1470.2 cells were treated for the times indicated with 30 μM 8-CPT-2'-O-Me-cAMP or 0.1 mM N^6-Phenyl-cAMP. Cell lysates were immunoblotted with antibody to H3 phosphorylated at serine 10. The data shown is representative of at least three independent experiments.

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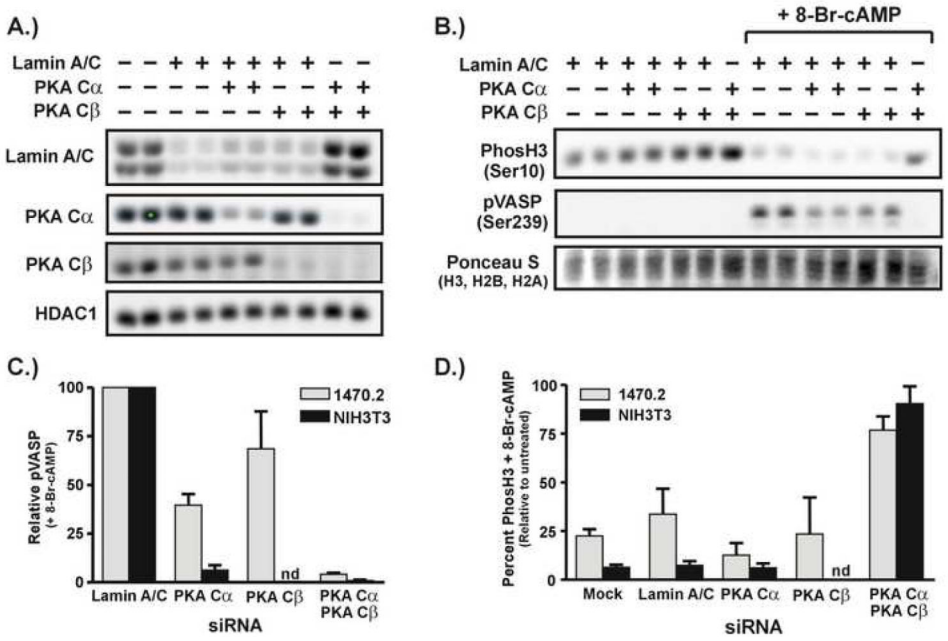

Figure 7. cAMP signaling regulates G2 progression and mitotic H3 phosphorylation in a PKA-dependent fashion

1470.2 cells were transfected with siRNAs specific for Lamin A/C, and the α and β isoforms of the PKA catalytic subunit in various combinations as listed above the panels in A and B. A.) Transfected cells were assayed by Western blot for Lamin A/C, PKA Cα, PKA Cβ, and HDAC1. B.) Transfected 1470.2 cells were treated with 1 mM 8-Br-cAMP or untreated. Phosphorylation of histone H3 (Ser10) and VASP (Ser239) were assayed by Western blotting with phosphorylation site-specific antibodies. Membranes were also stained with ponceau S to assess sample loading. C.) The effects of various siRNAs on VASP phosphorylation in the presence of cAMP in 1470.2 and NIH3T3 cells. In each experiment the level of cAMP-induced VASP phosphorylation measured in the presence of Lamin A/C siRNA was set to 100 and VASP phosphorylation levels in other conditions were expressed as a relative percentage. Data from three independent experiments is presented graphically. D.) Effects of siRNAs on cAMP-induced loss of H3 phosphorylation. For each experiment the level of H3 phosphorylation in the absence of cAMP treatment was set to 100 for each transfection condition and the level of H3 phosphorylation in the presence of cAMP treatment was expressed as a relative percentage. The graph represents the results from 3 independent experiments. Error bars represent SEM. nd – not determined.

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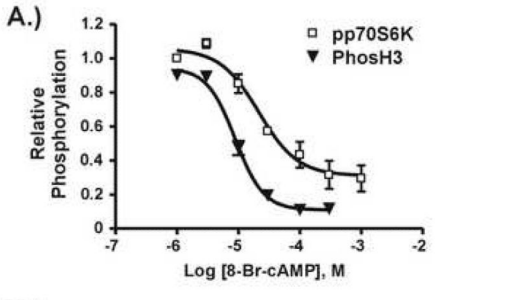

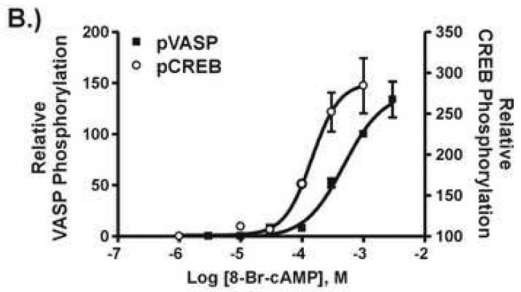

C.)

	EC50 (μM)
pVASP	481
pCREB	144
pp70S6K	21.5
phosH3	8.9

Figure 8. Dose dependency of cAMP-regulated protein phosphorylation

1470.2 cells were treated with various concentrations of 8-Br-cAMP for 60 minutes (A) or 15 minutes (B). These treatment times coincide with the maximal effect of cAMP signaling on phosphorylation of the proteins tested. Phosphorylation of histone H3 (Ser10) and p70S6K (Thr421/Ser424) as assayed by Western blotting is shown in (A), while phosphorylation of CREB (Ser133) and VASP (Ser239) are shown in (B). The data was analyzed using GraphPad Prism software, including curve fitting and calculation of EC50 as shown in (C). The graphs include data from at least three independent experiments. Error bars represent SEM.

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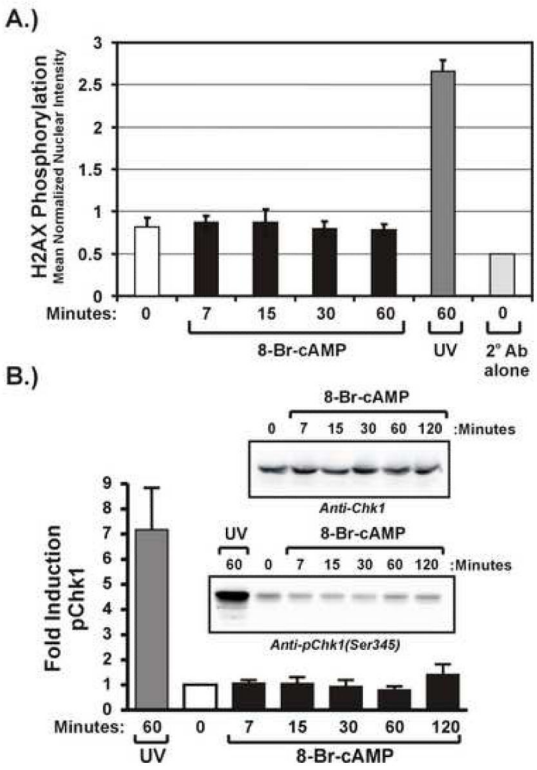

Figure 9. cAMP induced G2 arrest occurs independently of DNA damage signaling

(A) 1470.2 cells were analyzed for H2AX phosphorylation by immunofluorscence after UV irradiation or treatment with 0.1 mM 8-Br-cAMP for various times. Cells in images taken randomly were analyzed for mean nuclear intensity of staining with fluor-conjugated anti-phosH2AX(Ser139) or secondary antibody alone (background) as described in Materials and Methods. For each condition, the mean normalized nuclear intensities in each experiment were averaged and the graph represents the averages of three independent experiments. Error bars represent SEM. (B) 1470.2 cells were either UV-irradiated or treated with 0.1 mM 8-Br-cAMP for various times. Phosphorylation of total Chk1 or phosphoChk1 (Ser345) was examined by Western blotting. The inset shows representative blots and the graph represents the results of at least 3 independent experiments measuring Chk1 phosphorylation. Error bars represent SEM.

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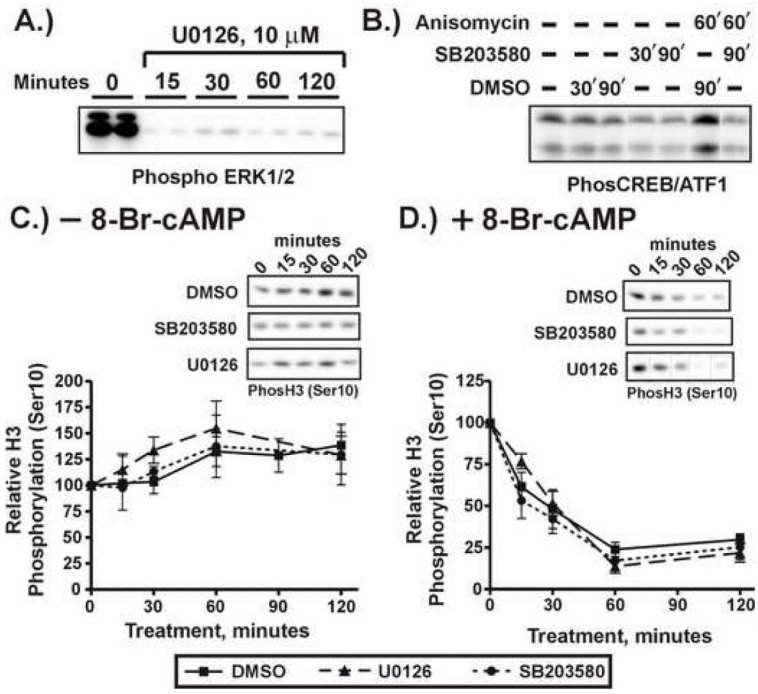

Figure 10. Effects of MAP kinase inhibitors on cAMP-induced loss of H3 phosphorylation

A.) Cells were treated with 10 μM U0126 for the times indicated. Cell lysates were subjected to Western blotting with antibodies against phosphorylated ERK1/2. B.) Cells were treated for the times indicated with vehicle (DMSO) alone, SB203580 (10 μM) alone, or anisomycin (50 ng/ml) in combination with either DMSO or SB230580. Cell extracts were subjected to Western blotting with an antibody against phosphorylated CREB/ATF1. C, D) Cells were treated for varying times with DMSO, 10 μM SB203580, or 10 μM U0126 alone (C) or were pretreated with these reagents for 30 minutes prior to the addition of 8-Br-cAMP (1 mM) (D). Cell extracts were subjected to Western blotting with an antibody against phosphorylated H3(Ser10). The results of at least three independent experiments were subjected to statistical analysis and are shown graphically. Error bars represent SEM.