

Cyclic AMP Delays G2 Progression and Prevents Efficient Accumulation of Cyclin B1 Proteins in Mouse Macrophage Cells

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Key words: cAMP/macrophage/cyclin B/cell cycle/G2 progression

ABSTRACT. In mouse macrophage cells, the increase of the intracellular cAMP level activates protein kinase A (PKA) and results in inhibition of cell cycle progression in both G1 and G2/M phases. G1 arrest is mediated by a cdk inhibitor, p27^{Kip1}, which prevents G1 cyclin/cdk complexes from being activated in response to colony stimulating factor-1, whereas inhibition of G2/M progression has not been fully elucidated. In this report we analyzed the effect of cAMP on G2/M progression in a mouse macrophage cell line, BAC1.2F5A. Flow cytometric analysis and mitotic index measurement using both synchronized and asynchronized cells revealed that addition of cAMP-elevating agents (8-bromoadenosine 3':5'-cyclic monophosphate and 3-isobutyl-methylxanthine), although they did not affect S phase progression or M/G1 transition, temporarily arrested cells in G2 but eventually the cells proceeded to M phase, resulting in about 4 hours delay of G2 progression. Timing of cyclin B1/Cdc2 kinase activation was also retarded by about 4 hours, which was accompanied by inhibition of efficient accumulation of cyclin B1 proteins. Initial induction and accumulation of cyclin B1 mRNA were not hampered, but the half life of cyclin B1 proteins was significantly shorter during G2 phase in the presence of cAMP-elevating agents compared with that of the cells blocked from progressing through M phase by nocodazole. These results imply that the cAMP/PKA pathway regulates G2 phase progression by altering the stability of a crucial cell cycle regulator.

Antiproliferative signals exert their effects in G1 and G2/M phases of the mammalian cell cycle. The factors that induce terminal differentiation or cell senescence, and reagents such as TGF- β and rapamycin primarily arrest cells in G1 (1–4), while DNA-damaging or cAMP-elevating agents, p53, and p19^{ARF}, block both G1 and G2/M progression (5–8). G1 arrest is now shown to be mediated by inhibition of G1 cyclin/Cdk activities either by prevention of expression of cyclin or Cdk subunits or by induction of various Cdk inhibitors (9, 10). G2/M progression is regulated by the cyclin B/Cdc2 complex (11), and antiproliferative signals directly or indirectly target this kinase complex.

cAMP functions as a second messenger in the signal transduction pathway initiated by a variety of hormones (12). Elevation of intracellular cAMP levels induces activation of PKA (cAMP-dependent protein kinase) and all the biological effects of cAMP known so far occur through the activation of PKA (13). Cellular responses mediated by cAMP differ from one cell type to another. For example, cAMP induces glycogenolysis of liver cells, absorption of calcium from bone by osteocyte cells, proliferation of Schwann cells, and growth-inhibition of bone marrow-derived granulocyte/macrophage progenitors (12).

Mouse macrophages have provided a useful model system for investigations into the mechanism involved in both positive and negative growth control including cAMP-mediated growth inhibition (14). cAMP-elevating agents including prostaglandin E₂, 3-isobutylmethylxanthine (IBMX), and cholera toxin, as well as cAMP analogs such as dibutyryladenosine 3':5'-cyclic monophosphate and 8-bromoadenosine 3':5'-cyclic monophosphate (8Br-cAMP), inhibit proliferation of murine bone marrow-derived macrophages (BMM) (15) and a colony stimulating factor-1 (CSF-1)-dependent murine macrophage cell line, BAC1.2F5 (6, 16). cAMP-mediated signals seem to affect both G1/S and

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Abbreviations: TGF- β , transforming growth factor- β ; Cdk, cyclin-dependent kinase; PKA, cAMP-dependent protein kinase; IBMX, 3-isobutyl-methylxanthine; 8Br-cAMP, 8-bromoadenosine 3':5'-cyclic monophosphate; BMM, murine bone marrow-derived macrophages; CSF-1, colony stimulating factor-1; CAK, Cdk activating kinase; APC, anaphase promoting complex; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LCM, L cell-conditioned medium; RT-PCR, reverse transcription and polymerase chain reaction; AMV, avian myeloblastosis virus.

G2/M progression of the macrophage cell cycle (6). In BMM cells arrested in G1 by cAMP, expression of both cyclin D1, the major D-type cyclin in macrophages, and Cdk4 is suppressed (17, 18). However, BAC1.2F5A variants which are capable of expressing cyclin D1 and Cdk4 in the presence of cAMP are still sensitive to cAMP-mediated G1 arrest (6), suggesting that cyclin D1 and Cdk4 are not the only targets of the cAMP pathway. In these particular cells, addition of cAMP induces the accumulation of a Cdk inhibitor, p27^{Kip1}, which binds to and inhibits cyclin D1/Cdk4 complexes (6). However, the mechanism by which cAMP regulates G2/M progression remains to be investigated.

The cyclin B/Cdc2 complexes play a central role in G2 and M phase progression and are subject to multiple positive and negative regulatory mechanisms (11, 19). As cyclin B proteins accumulate during G2 phase, they form a complex with Cdc2, which triggers both activating and inhibiting phosphorylation on a Cdc2 subunit by CAK, Weel, and Myt1 (11, 19). As cells approach the G2/M boundary, cyclin B/Cdc2 complexes, which are localized in the cytoplasm during G2, move into the nucleus and are dephosphorylated and activated by Cdc25 (11, 19). After metaphase/anaphase transition, cyclin B proteins are ubiquitinated by Anaphase Promoting Complex (APC) and degraded by 26S proteasome (20, 21). Activation of cyclin B/Cdc2 complexes are absolutely necessary for cells to enter M phase (11, 19). Cyclin B mRNA begins to accumulate in late S phase and reaches its maximum level around the G2/M boundary, which accounts for the accumulation of cyclin B proteins during S and G2 phases. However, the levels of cyclin B mRNA gradually decline in G1, when cyclin B proteins exist at undetectable levels. This is because once the degradation system for cyclin B proteins such as APC is activated in M phase, its activity is maintained during G1 phase (22). The machinery governing the DNA replication and repair checkpoints mainly regulates cyclin B/Cdc2 kinase activity by inhibitory phosphorylation of Cdc2 (11, 19), but some DNA damaging agents delay expression of cyclin B mRNA during G2 arrest (23), indicating that complex mechanisms underlie the regulation of cyclin B/Cdc2 complexes and G2/M progression in response to anti-proliferative signals.

In the present study, we examined the effects of cAMP on G2/M progression in a mouse macrophage cell line, BAC1.2F5A. Our findings indicate that cAMP treatment resulted in the delay of G2 progression, which was accompanied by the delay of the activation of cyclin B1/Cdc2 complexes and the accumulation of cyclin B1 proteins. Slow accumulation of cyclin B1 proteins is primarily due to instability of the protein, suggesting a role for the cAMP/PKA pathway in regulating the stability of the cell cycle regulator.

MATERIALS AND METHODS

Cell Culture, Synchronization, and Cell Cycle Analysis

Mouse BAC1.2F5A macrophage cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 10% L cell-conditioned medium (LCM) as a source of CSF-1. Macrophages were arrested in early G1 by CSF-1 starvation for 22 hr and stimulated with CSF-1 to reenter the cell cycle synchronously. Since addition of cAMP reagents in early to mid G1 blocks cells from progressing to S phase (6, 16), we performed a series of kinetic experiments and found that addition of 8Br-cAMP (1 mM) and IBMX (1 mM) 8 hours after CSF-1 stimulation allowed more than 80% of cells to enter S phase. To specifically investigate S/G2/M progression, we used these conditions for all synchronized experiments.

For flow cytometric analysis of DNA content, cells were suspended in a 1 ml solution of 0.1% sodium citrate and 0.1% Triton X-100 containing 50 μ g/ml of propidium iodide and treated with 1 μ g/ml of RNase for 30 min at room temperature. Fluorescence from the propidium iodide-DNA complex was measured with a FACScan flow cytometer (Becton Dickinson), and the percentages of cells in G1, S, and G2/M phases of the cell cycle were determined.

To specifically determine percentages of cells in mitosis (mitotic index), metaphase chromosome spreads were prepared as follows. Cells were washed with PBS, and suspended and incubated in 1 ml of 75 mM KCl for 15 min. Then, 0.2 ml of ice-cold Carnoy's fixative agent (Methanol:Acetic acid = 3:1 (vol/vol)) was added. Cells were collected by centrifugation and resuspended in ice-cold Carnoy's fixative agent. The cell suspension was dropped onto a wet glass slide and dried. Metaphases per total number of nuclei were counted under a light microscope.

Protein Analyses

Cells were lysed in SDS-sample buffer (40 mM Tris-HCl, pH 6.8, 0.1 M DTT, 1% SDS, 10% glycerol, and 0.05% Bromophenol Blue) and boiled for 4 min. The cell lysates clarified by centrifugation were separated on 10% SDS-polyacrylamide gels under reducing conditions, transferred to a PVDF membrane (Millipore), and immunoblotted with rabbit polyclonal antibody specifically recognizing rodent cyclin B1, but not B2 or B3 (this antibody is also capable of immunoprecipitating active cyclin B1/Cdc2 complexes; Santa Cruz Biotechnology), followed by detection with the ECL blotting system (Amersham) according to the manufacturer's instructions.

For immunoprecipitation, cells were lysed for 30 min on ice in EBC buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% NP-40, and 1 mM EDTA) containing 5 μ g/ml of aprotinin, 1 mM PMSF, 0.1 mM NaF, 0.1 mM NaVO₄ and 1 mM DTT. After clarification by centrifugation, lysates containing an equal amount of proteins were incubated with antibody to cyclin B1 for 2 hours. After 1 hour incubation with protein A-Sepharose beads, immunoprecipitates were collected by

centrifugation and washed three times with ice-cold EBC buffer. For an in vitro kinase assay, cyclin B1/Cdc2 complexes concentrated by immunoprecipitation were washed with kinase buffer (50 mM HEPES pH 7.5, 10 mM MgCl₂) twice and incubated for 15 min at 30°C in 25 μ l of kinase buffer supplemented with 1 mM DTT, phosphatase inhibitors (NaF and NaVO₄, see above), 5 μ g of histone H1, and 3.3 μ Ci (0.12 MBq) of [γ -³²P] ATP (NEN). Phosphorylated histone H1 was separated by SDS-polyacrylamide gel electrophoresis and quantified for ³²P incorporation by a Fuji BAS-2000 image analyzer.

For biosynthetic studies, cells were incubated in methionine-free medium supplemented with dialyzed FBS and dialyzed LCM for 30 min and then metabolically labeled for 1 hour with 60 μ Ci of [³⁵S]methionine (TRAN³⁵S-LABEL, 1175 Ci/mmol, ICN) in the same medium. After being extensively washed with PBS, cells were incubated for indicated times in medium containing FBS, LCM, and 25 μ g/ml of cold methionine. Cyclin B1 proteins were isolated and separated as described above and the radioactivities contained in cyclin B1 were quantified using a Fuji BAS-2000 image analyzer.

Quantitative RT-PCR Analysis

Total RNA was prepared from macrophage cells using Iso-gene (Nippon Gene), and cDNA was synthesized from 1 μ g of RNA template with oligo dT primers and Avian Myeloblastosis Virus reverse transcriptase using a Takara RNA LA PCR Kit (AMV) according to the manufacturer's instructions.

PCR amplification of cyclin B transcripts was performed with oligonucleotide primers specific for coding sequences of cyclin B1 (5'-GTGCAGATGGAGGCGCTAGC-3' and 5'-CAGGATCTGAGAAGCGCAGG-3') and cyclin B2 (5'-GAGGTGGAAGTTGCTGAGCC-3' and 5'-GTTATGCCTTTGTCACGGCC-3') with 28 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 2 min). PCR products were electrophoresed in 1% agarose gels, visualized by staining with EtBr, and quantitatively analyzed using a Densitograph (ATTO). Specific amplification of cyclin B1 and B2 sequences was confirmed by digestion with restriction enzymes specific to each of them (*SaI* and *SacI*, respectively) and by sequencing of amplified fragments. Experiments conducted using various amounts of input template ensured that the assay was quantitative under these conditions.

RESULTS

Effect of cAMP on G2/M Progression in Murine Macrophage Cells

The mouse macrophage cell line, BAC1.2F5A, is dependent upon CSF-1 for proliferation and survival, and randomly distributes throughout the cell cycle when proliferating in medium containing CSF-1 (6). Addition of cAMP analogs (dibutyryladenosine 3':5'-cyclic monophosphate or 8Br-cAMP) or cAMP-elevating agents (prostaglandin E₂, IBMX, or cholera toxin) into medium arrests cell cycle progression at two points. The majority of cells accumulate in G1 phase, whereas the

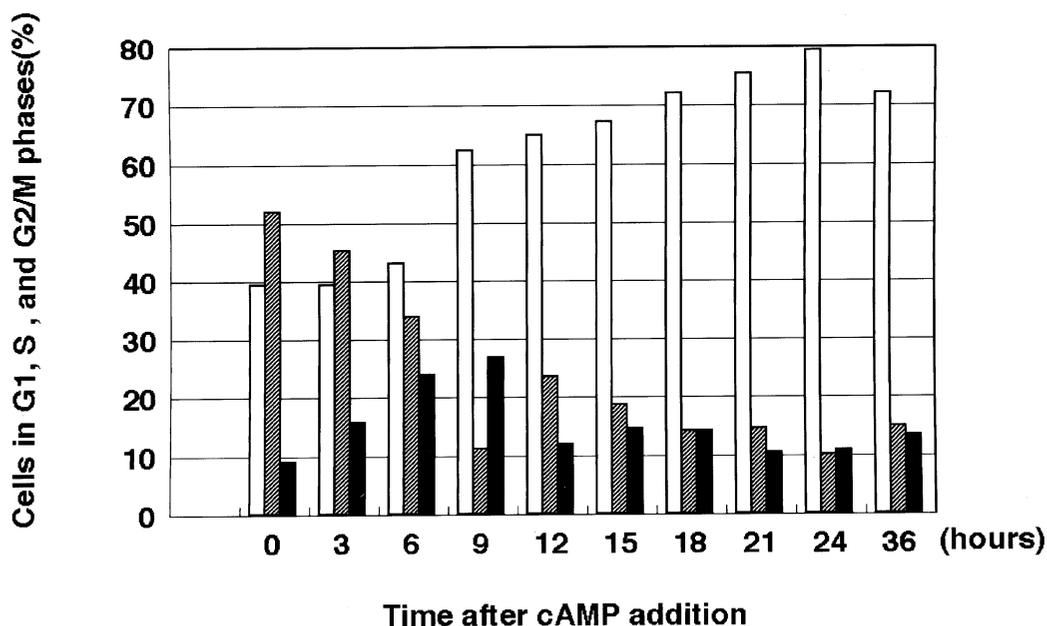


Fig. 1. Effect of cAMP on cell cycle distribution of asynchronously proliferating macrophage cells. 8Br-cAMP and IBMX were added to asynchronously growing Bac1.2F5A cells and the cell cycle distribution was determined by flow cytometric analysis of DNA content at the times indicated (□; G1 phase, ▨; S phase, and ■; G2/M phase).

remainder arrest in G2 (6). In contrast, other inhibitors of cell cycle progression such as rapamycin block proliferating cells only in G1 phase (6). Thus, because inhibition of G2 progression is correlated with nothing else (the solvent used to dissolve agents or any other particular structure of the chemicals) other than an activator of PKA, we concluded that activation of PKA, but not any kind of toxic effect, is responsible for G2 block. However, the amount of cells arrested in G2 differed from one experiment to another. To investigate the effect of cAMP more precisely, we added cAMP-elevating reagents (in this particular experiment shown in Fig. 1, we used 8Br-cAMP and IBMX, but other cAMP agents exhibited the same effects; data not shown) to asynchronously growing cells and determined cell cycle distribution by flow cytometric analysis of DNA content every 3 hours (Fig. 1). The population of cells in G1 phase with a 2n DNA content gradually increased after a short time and that of cells in S phase reciprocally decreased. In contrast, the number of cells in G2/M phase with a 4n DNA content elevated during the first 9 hours after stimulation and declined thereafter.

Macrophage cells arrested in early G1 by deprivation of CSF-1 for 22 hours were stimulated with the growth factor to reenter the cell cycle synchronously and 8Br-cAMP and IBMX were added 8 hours after stimulation, at which time cAMP was no longer able to arrest cells in G1 (data not shown, but see Fig. 2 and Experimental Procedures), allowing us to investigate the effect of cAMP primarily on G2/M. Cell cycle distribution of synchronized cells in the presence or absence of cAMP-reagents was determined every 3 hours (Fig. 2). With or without cAMP, cells entered S phase between 8 and 11 hours after CSF-1 release, and exited from S phase by 17 hours after release. The second cell cycle was initiated without cAMP, but G1 arrest occurred in its presence. The population in G2/M phase began to increase between 11 and 14 hours after release. Untreated cells exited from M phase between 17 and 20 hours after release, but the G2/M population of cAMP-treated cells continued to increase, maximally accumulating at 17 hours after release, and then declined. These results, consistent with those obtained from the asynchronized cultures, clearly indicate that the cAMP-mediated signal temporarily blocks progression through G2 and M phases without affecting G1/S transition, S phase progression, or S/G2 transition.

To determine the exact time point of cAMP action, we measured the mitotic index of synchronized cells in the absence and presence of cAMP (Fig. 3). Normally proliferating cells began to enter M phase 13 hours after CSF-1 release, reaching maximum numbers at around 17 hours, whereas the M phase population of cAMP-treated cells began to increase 17 hours after re-

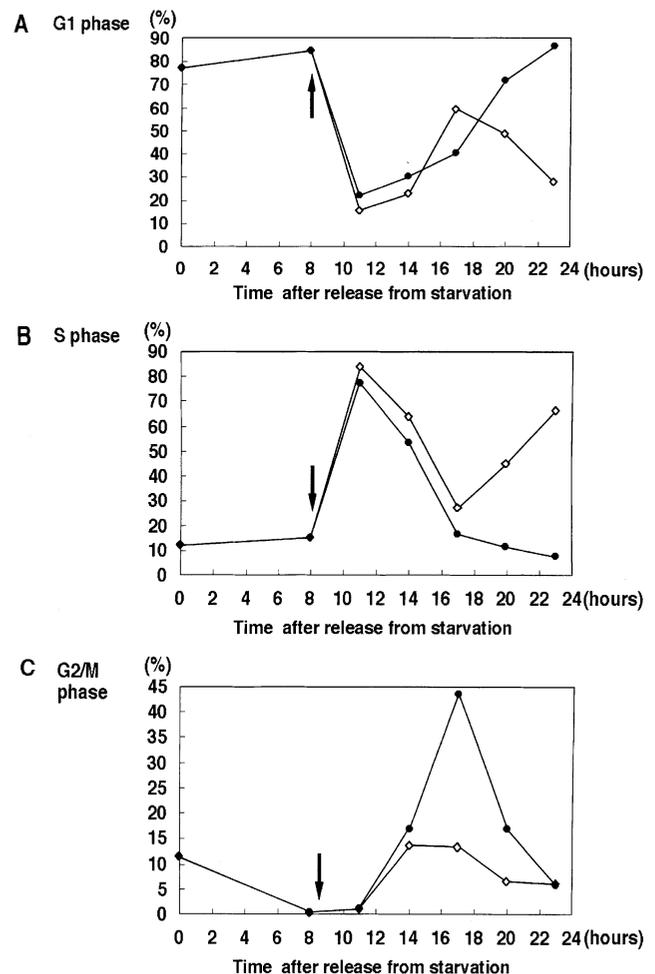


Fig. 2. Effect of cAMP on progression from S to G1 phase of the synchronized macrophage cell cycle. Bac1.2F5A cells arrested in early G1 were stimulated with CSF-1 to enter the cell cycle synchronously and the population of cells in G1 phase (A), S phase (B), or G2/M phase (C) in the presence (●) and absence (◊) of cAMP was determined by flow cytometric analysis at the times indicated. The arrow indicates the time of 8Br-cAMP and IBMX addition.

lease with a maximum accumulation around 21 hours after release. Note that at their peaks, the maximum mitotic indices were very similar (3–4%), which is in striking contrast to the results obtained from flow cytometric analysis that shows G2 and M phases as one population. Thus, activation of the cAMP-mediated pathway delayed G2 progression and/or G2/M transition by about 4 hours.

The delay of cyclin B1/Cdc2 kinase activation and accumulation of cyclin B1 proteins by cAMP

Activation and inactivation of cyclin B/Cdc2 complexes are absolutely required for transition from G2 to M and M to G1, respectively (19). Two subtypes of cyclin B (cyclins B1 and B2) have been reported (24),

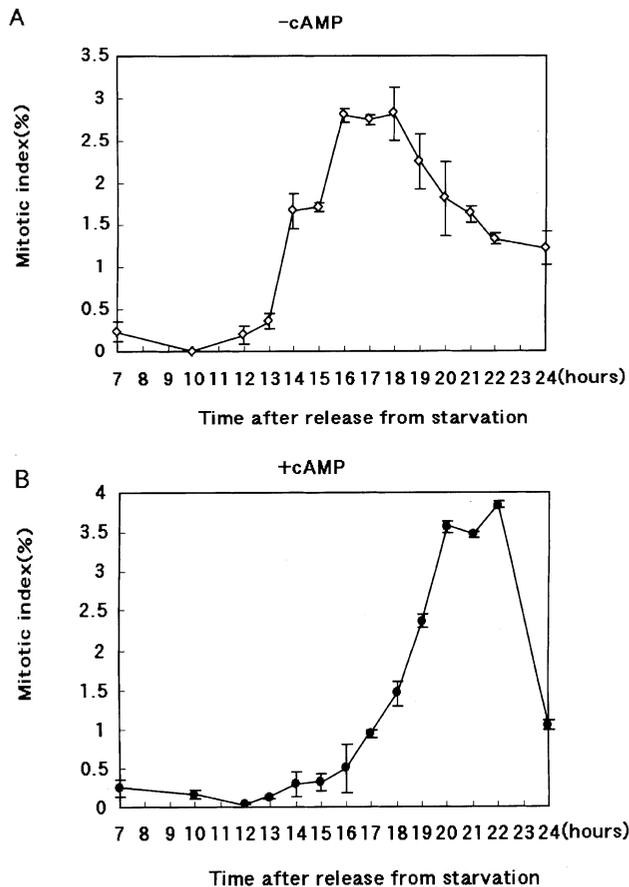


Fig. 3. Effect of cAMP on entry into M phase. Percentages of synchronized cells in metaphase (mitotic index) in the presence (panel B) and absence (panel A) of cAMP were determined by analyzing metaphase chromosome spreads prepared as described in Experimental Procedures.

among which cyclin B1 plays an essential role in regulation of G2/M transition. Therefore we focused our attention on the effect of cAMP on the timing of cyclin B1/Cdc2 kinase activation. Macrophage cells arrested in early G1 by CSF-1 deprivation synchronously reentered the cell cycle on stimulation with the growth factor, and 8Br-cAMP and IBMX were added 8 hours after stimulation to exclusively examine the effect of cAMP on G2/M. Cells in G2 and M phases were harvested, and cyclin B1/Cdc2 complexes were immunoprecipitated from each cell lysate with antibody specifically recognizing murine cyclin B1 and were assayed for their kinase activity *in vitro* using histone H1 as a substrate (Fig. 4). Cyclin B1/Cdc2 kinase was maximally activated 17 hours after release in normally proliferating cells, whereas cAMP-treatment delayed its activation by about 4 hours with a peak 21 hours after release. The relatively high level of cyclin B1/Cdc2 kinase activity observed before entering M phase was

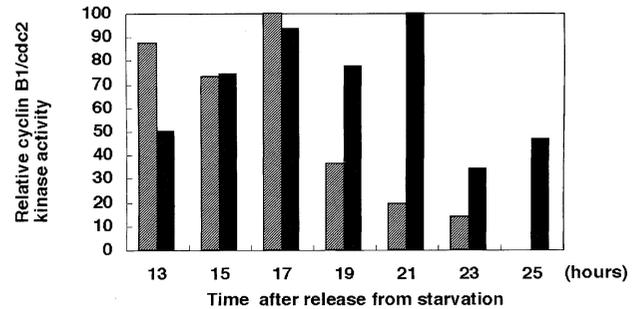


Fig. 4. Effect of cAMP on cyclin B1/Cdc2 kinase activation. Synchronized BAC1.2F5A cells incubated with (■) or without (◻) cAMP were harvested at the times indicated, and cyclin B1/Cdc2 complexes were immunoprecipitated from each cell extract and assayed for histone H1 kinase activity *in vitro*.

mostly due to the partial activation of the cyclin B1/Cdc2 complex which occurred during immunoprecipitation by an unknown mechanism. However, in agreement with the results obtained from the *in vitro* kinase assay, loss of the inhibitory phosphorylation of the Cdc2 catalytic subunit, which can be easily and accurately detected by examining the mobility shift of Cdc2 in SDS-polyacrylamide gel electrophoresis using the direct lysates, occurred between 15 and 17 hours after release in normally proliferating cells, but it took place between 19 and 21 hours after release in the presence of cAMP (data not shown). Thus, cAMP-treatment did delay the timing of cyclin B1/Cdc2 kinase activation by about 4 hours, but did not sustain the high level of cyclin B1/Cdc2 activity. The timing of maximum activation of cyclin B1/Cdc2 kinase in both cells coincides with the M phase peaks identified by mitotic index measurement (see Fig. 3). The maximum levels of cyclin B1/Cdc2 kinase activity were very similar in both cases.

We next analyzed induction of cyclin B1 protein expression in synchronized cells with and without cAMP-treatment (Fig. 5). Cyclin B1 proteins were first detected in late S phase (11 hours after release), accumulated gradually during G2 phase (between 11 and 15 hours after release), reached their maximum level in M phase (15 to 17 hours after release), and then rapidly declined thereafter. In the presence of 8Br-cAMP and IBMX, accumulation of cyclin B1 proteins was slowed and the peak of the maximum cyclin B1 protein expression was delayed about 4 hours. The maximum expression levels of cyclin B1 proteins in M phase were indistinguishable with or without cAMP-treatment. Furthermore, anti-cyclin B1 immunoprecipitates contained the amounts of Cdc2 subunit in proportion to cyclin B1 expression (data not shown), implying that the complex formation between the two subunits was not affected. These results indicate that cAMP-mediated signals de-

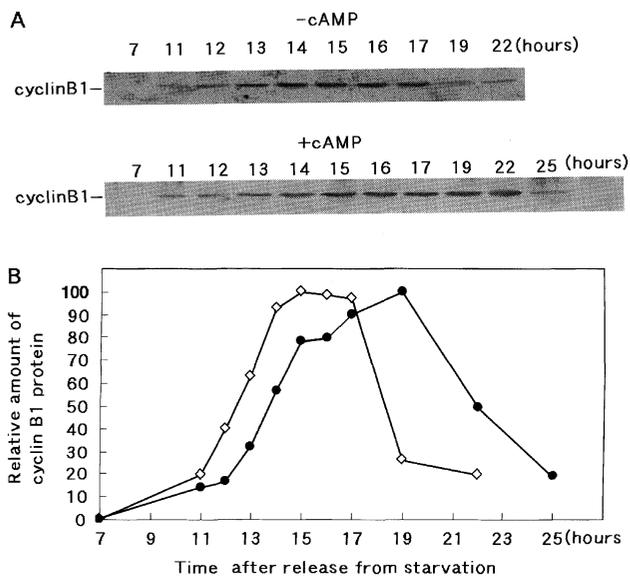


Fig. 5. cAMP delays accumulation of cyclin B1 proteins. (A) Synchronized BAC1.2F5A cells were cultured with (lower panel) or without (upper panel) cAMP, and analyzed for cyclin B1 protein expression by immunoblotting. (B) Quantitative analysis of the data shown in panel A (◇ untreated cells, ● cAMP treated cells).

lay the timing of cyclin B1/Cdc2 kinase activation by hindering cyclin B1 proteins from efficiently accumulating.

To directly investigate whether slow accumulation of cyclin B1 proteins is the primary cause of G2 delay, we attempted to overexpress cyclin B1 proteins in BAC1.2F5A cells. However, the transfection efficiency onto macrophage cells was about 1000 times lower than that on any other types of cells by the methods we used, which include calcium phosphate transfection, electroporation, liposome-mediated transfection, and infection with retrovirus vectors. Therefore, the extremely low transfection efficiency of macrophages prevented us from directly testing whether upregulation of cyclin B1 proteins can override the G2 delay induced by the cAMP/PKA pathway.

The accumulation of cyclin B1 mRNA was not significantly affected by cAMP during G2

To examine whether slow accumulation of cyclin B1 proteins in the presence of cAMP is due to inhibition of the cyclin B1 gene transcription or instability of cyclin B1 mRNA, we analyzed expression of cyclin B1 mRNA in synchronized cultures in the presence and absence of cAMP. To specifically detect cyclin B1 mRNA in total RNA which contains highly related cyclin B2 mRNA, we utilized quantitative reverse transcription and polymerase chain reaction (RT-PCR) using specific primers (Fig. 6A). A single band was obtained by

RT-PCR using a set of primers specific to cyclin B1 or B2 cDNA sequences (lanes 1 and 6). Nucleotide sequences of cyclin B1 and B2 cDNA revealed that the digestion sites of *SalI* and *SacI* are specifically contained, respectively, in the regions between primers. PCR products obtained using cyclin B1 primers were refractory to *SacI* digestion but completely digested by *SalI* (lanes 2 and 3), whereas cyclin B2 products were digested by *SacI* but not by *SalI* (lanes 4 and 5), confirming that each RT-PCR product was specific to cyclin B1 or B2 mRNA. No DNA fragments were amplified without RTase (data not shown), indicating that PCR products were derived from cDNA but not from contaminated genomic DNA. The amount of PCR products proportionally increased as more template cDNA was used (Fig. 6B), confirming that the PCR conditions we used are suitable for quantitative analysis.

The total RNA was isolated from synchronized cells at various times in the presence or absence of 8Br-cAMP and IBMX, and was analyzed for cyclin B1 mRNA expression by RT-PCR using specific primers (Fig. 6C). Very little cyclin B1 mRNA was detected 11 hours after release, and its amount increased as cells progressed through G2 and M phases. The kinetics and the amount of cyclin B1 mRNA expression are very similar until 17 hours after release in the presence and absence of cAMP, which suggests that cAMP-mediated signals do not inhibit cyclin B1 mRNA accumulation during G2 phase (between 11 and 15 hours after release), when cyclin B1 proteins accumulate with slower kinetics in the presence of cAMP (see Fig. 5). However, it is interesting to note that cyclin B1 mRNA expression continued to increase after entry into M phase and peaked at around M/G1 transition, and that addition of cAMP agents seemed to prevent efficient accumulation of cyclin B1 mRNA during this period, resulting in about 2 hours delay of the maximum expression.

Decrease of cyclin B1 protein stability during G2 by cAMP

Since cAMP-treatment did not significantly alter the kinetics of cyclin B1 mRNA induction during G2 phase (see above and Fig. 6C) or the rates of cyclin B1 translation (data not shown), we next analyzed the stability of cyclin B1 proteins during G2 with and without cAMP treatment (Fig. 7). To examine the half life of cyclin B1 proteins specifically in G2, synchronized early G1 cells were stimulated to progress to late S phase (11 hours after release) by incubating them in CSF-1, metabolically labeled with [³⁵S]methionine for 1 hour, and chased for the times indicated in Fig. 7 (4 hours at most) in medium containing excess cold methionine and CSF-1 in the presence or absence of 8Br-cAMP and IBMX. Following quantitative immunoprecipitation, the relative [³⁵S] activities contained in cyclin B1 proteins were

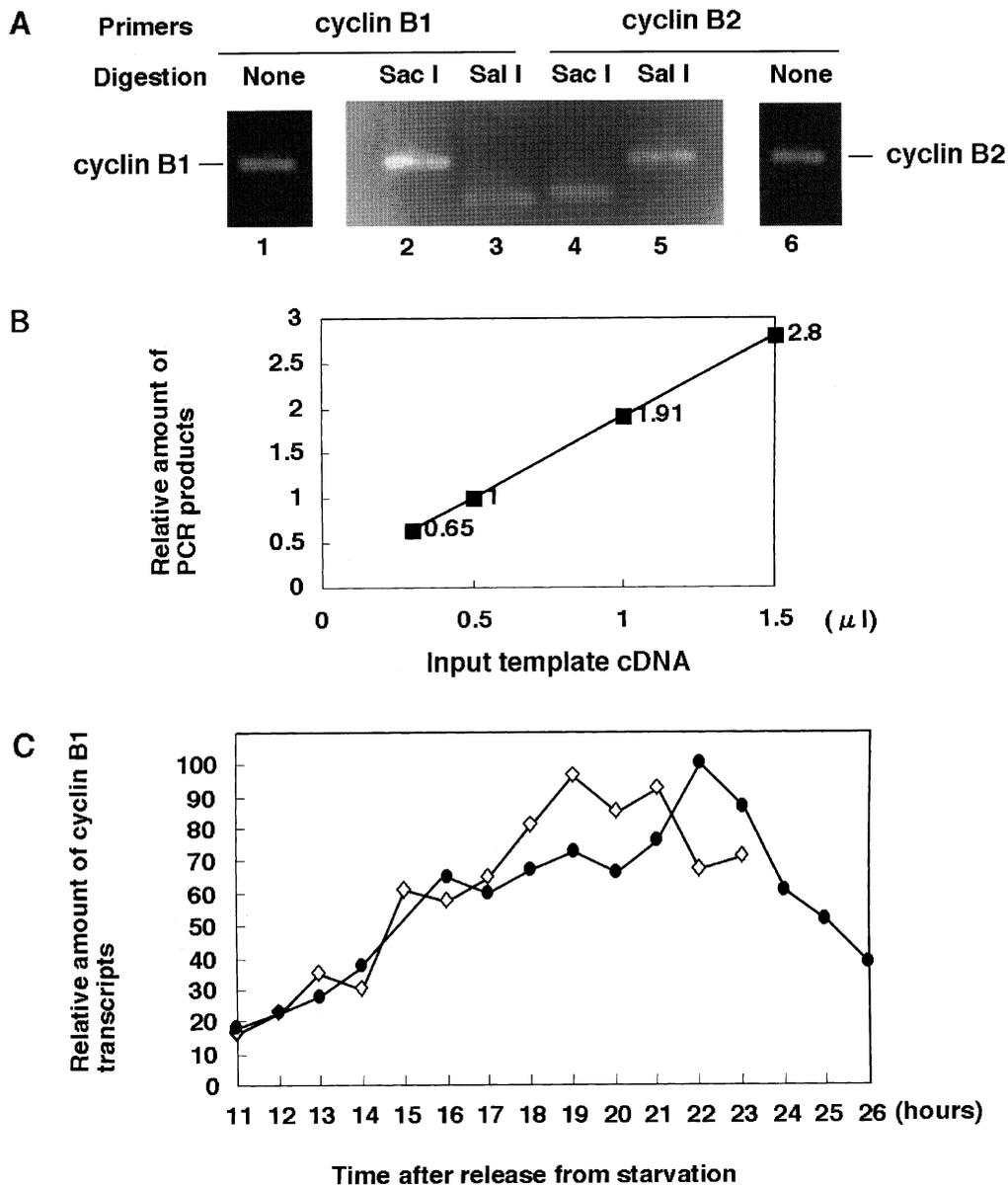


Fig. 6. Quantitative analysis of cyclin B1 mRNA expression by RT-PCR in synchronized macrophages. (A) Specific amplification of cyclin B1 and B2 transcripts. PCR products amplified with cyclin B1 primers (lanes 1–3) or cyclin B2 primers (lanes 4–6) were digested with restriction enzyme SacI (lanes 2 and 4) or SalI (lanes 3 and 5). (B) Quantitative amplification of cyclin B1 transcripts. The relative intensities of PCR products are proportional to the amount of input template cDNA. (C) Analysis of cyclin B1 mRNA expression in synchronized macrophages in the presence (●) and absence (◇) of cAMP. The data are mean values from 4 independent experiments.

measured. As cells exit from M phase, cyclin B1 proteins rapidly degrade (19), which prevents precise measurement of the half life of cyclin B1 protein. To avoid this, nocodazole was added to control cultures. Cyclin B1 proteins were quite stable in G2 phase. Note that during incubation with nocodazole, the number of round cells, which can easily float in medium, increased and these cells were not completely recovered. Therefore, in Fig. 7, the half life of cyclin B1 in

the control culture was underestimated. In contrast, cAMP treatment rendered cyclin B1 proteins quite unstable. During chase incubation in the presence of cAMP, round cells were rarely observed and flow cytometric analysis revealed that less than 10% of the cells reentered G1 phase during this period. Furthermore, the half life of cyclin B1 protein calculated in the presence of both nocodazole and cAMP was not significantly different from that on cAMP-treatment alone.

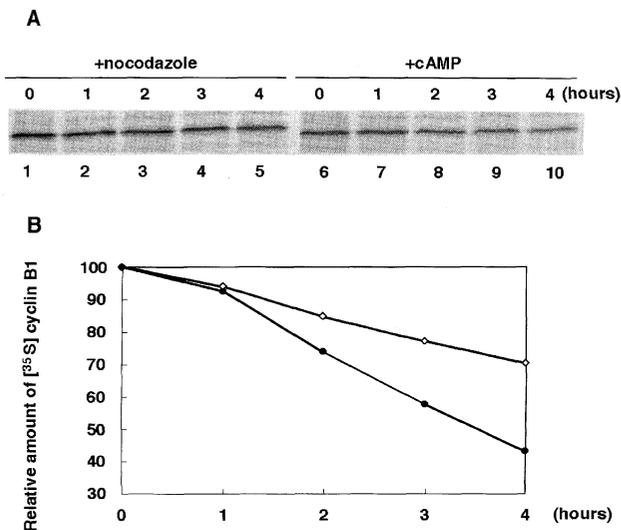


Fig. 7. cAMP decreases the stability of cyclin B1 proteins during G2. (A) Synchronized BAC1.2F5A cells were labeled with [³⁵S]methionine in late S phase, chased for the times indicated on the top in the presence of CSF-1 and excess methionine together with nocodazole (lanes 1–5) or cAMP (lanes 6–10), and analyzed for the [³⁵S] activities contained in cyclin B1 by immunoprecipitation. (B) Quantitative analysis of the data shown in panel A using a Fuji BAS-2000 image analyzer (◇ nocodazole treated cells, ● cAMP treated cells). The results are representative of 4 independent experiments.

Thus, activation of the cAMP pathway markedly reduces the stability of cyclin B1 proteins during G2 phase, which would account for the delay of cyclin B1 protein accumulation and most probably for the delay of cyclin B1/Cdc2 kinase activation and G2 progression.

DISCUSSION

cAMP functions as an activator of PKA in the signal transduction pathway initiated by a variety of hormones (12, 13). The effect of cAMP on cell proliferation varies from one cell type to another (12), but the signals mediated by the cAMP/PKA pathway are eventually transmitted to the machinery which governs cell cycle progression. Inhibition of cell cycle progression by cAMP has been well characterized using the macrophage cell system (6, 15, 16). In G1-arrested macrophages, the activity of cyclin D/Cdk4 kinase, which plays an essential role in G1 progression, is down-regulated either by preventing both subunits from being expressed (17, 18) or by inducing a Cdk inhibitor, p27^{Kip1} (6). Introduction of the plasmid expressing antisense p27^{Kip1} cDNA allowed macrophage cells to progress into S phase in the presence of cAMP (J.-Y. Kato, unpublished data), suggesting that p27^{Kip1} plays an important role in G1 arrest mediated by cAMP.

cAMP-mediated signals appear to affect G2 progression of the macrophage cell cycle as well (6). The number of cells in G2/M temporally increased after the addition of cAMP agents to asynchronous cultures and then declined, whereas the G1 population continuously expanded. Using synchronized cells, we found that cAMP signals exhibited little effect on S phase progression or M/G1 transition, but transiently arrested cells in G2, resulting in about a 4-hour delay of G2 progression, which coincided with slower accumulation of cyclin B1 proteins. Accumulation of cyclin B1 mRNA was not retarded during G2 phase, but the half life of cyclin B1 proteins was significantly shorter in the presence of cAMP. These results indicate that cAMP signals regulate G2 progression and cyclin B1/Cdc2 kinase activity by altering the protein stability of the cyclin B1 regulatory subunit, but not the promoter activity or the mRNA stability. Treatment of HeLa cells with DNA damaging agents delays expression of cyclin B1 mRNA, resulting in slow accumulation of cyclin B1 proteins during the G2 arrest induced by these agents (23), suggesting that inhibition of cyclin B1 protein accumulation is not specific to the cAMP/PKA pathway, but could be a part of the G2 checkpoint control mechanisms. However, our results do not exclude the possibility that negative phosphorylation of the Cdc2 subunit is also involved in cAMP-mediated G2 regulation.

In M phase, cyclin B1 proteins are rapidly degraded through ubiquitination by APC followed by proteolysis mediated by 26S proteasome (20, 21). In fission yeast, the cAMP/PKA pathway inhibits APC complex formation and activation (25). In the mammalian system, Polo-like kinase and PKA positively and negatively regulate the APC activity, respectively, by specifically phosphorylating the APC subunits (26). The level of PKA activity in NIH3T3 cells rapidly increases at the beginning of mitosis, reaches maximum in early mitosis, and drops at metaphase (26). These results appear to indicate that activation of PKA inhibits APC activity and prevents cyclin B degradation. However, the experiments using cycling *Xenopus* egg extracts indicate that activation of the cAMP/PKA pathway is required for induction of cyclin B degradation and exit from M phase (27). Furthermore, the effect of cAMP differs from one cell type to another (12) and addition of 8Br-cAMP and IBMX did not inhibit growth of NIH3T3 cells (J.-Y. Kato, unpublished observation). Thus, it is most likely that the cAMP/PKA pathway targets different molecules in different types of cells. In macrophage cells, which are sensitive to cAMP-mediated growth arrest, premature activation of PKA in late S to G2 phase may trigger initiation of some portions of the M phase specific program, such as APC activation, thereby preventing efficient progression from G2 to M phase. Alternatively, macrophages may express specific

PKA-target proteins through which cAMP decreases the stability of cyclin B1 proteins and inhibits progression of the cell cycle.

If the ubiquitination pathway is involved in accelerated cyclin B1 turnover during G2 in cells treated with cAMP, the modified cyclin B1 proteins with increased molecular mass should be accumulated. However, we were unable to detect the slower-migrating forms of cyclin B1 proteins in cells treated with cAMP agents either by Western blotting or by metabolic labeling followed by immunoprecipitation (unpublished observation). This may suggest that the unidentified degradation machinery unique to cAMP-sensitive cells is activated through PKA. However, we cannot rule out the possibility that the antibody to cyclin B1 we used in these experiments may not efficiently recognize ubiquitinated cyclin B1, or that only a limited amount of modified cyclin B1 proteins exist with a very short half life.

Taking into consideration that cAMP treatment increases expression of p27^{Kip1} during G1 by altering its stability (6 and J.-Y. Kato, unpublished observation), the cAMP/PKA pathway activated by either extracellular or intracellular signals plays an important role in the regulation of cell cycle progression in both G1 and G2 phases by controlling the activity of the cell cycle-specific degradation machinery.

Acknowledgments. This work was supported by Grants-in-Aid for Scientific Research and for Cancer Research from the Ministry of Education, Science, and Culture of Japan, and by the Asahi Glass Foundation.

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(Received for publication, August 5, 1998
and in revised form, October 9, 1998)