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The involvement of diadenosine 5′,5‴-P^1,P^4-tetraphosphate in cell cycle arrest and regulation of apoptosis

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Abstract

Diadenosine oligophosphates (Ap_nA) have been proposed to function as intracellular and extracellular signaling molecules in animal cells. Here, we have examined the cellular and molecular mechanisms underlying the induction of apoptosis by diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A). We have shown a dose-dependent apoptotic response in cells treated with Ap_4A. Flow cytometric analysis indicated an involvement of Ap_4A at an early stage of G1/S arrest. No difference in the amount of $p21^{waf1}$ was observed in HL60 cells treated with Ap_4A compared to control cells. The level of retinoblastoma protein (pRb) dropped dramatically when apoptosis was extensive. The cleavage of pRb was abrogated if Ap_4A-treated cells were incubated with general caspase inhibitor zVAD-fmk. Ap_4A also induced a profound decrease in the level of the Bcl-2 protein. The lack of effect of Ap_4A on CDK1 activity indicated that Ap_4A is not involved in "aberrant mitosis". We suggest that *in vivo* Ap_4A may play a significant role in tumor growth suppression by inducing apoptosis.

Keywords: Ap_4A; HL60; CEM-SS; Apoptosis; Cell cycle; Bcl-2

1. Introduction

Ap_4A and diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A) are synthesized in cells by aminoacyl-tRNA synthetases from aminoacyl adenylates and ATP or ADP, respectively, and are found in mammalian cells at basal concentrations ranging from 0.01 to 5 μM [1]. Extracellular signaling roles for these and related dinucleoside polyphosphates in the cardiovascular and nervous systems are now well established [2,3]. However, until recently the intracellular role(s) of these dinucleotides in higher eukaryotes remained obscure, with early suggestions that they were involved in the regulation of DNA replication and/or repair remaining unsubstantiated [4]. The stress-inducibility of these compounds is well documented and evidence is now emerging that supports a role for diadenosine polyphosphates as signals in a number of essential cellular functions [5,6]. In particular, several physiological and pathological effects have been found to be associated with an alteration in the intracellular Ap_3A/Ap_4A ratio. For example, differentiation and apoptosis of cultured cells have significant and opposite effects on this ratio: differentiation is associated with an increase in the ratio, and apoptosis with a decrease [7]. Furthermore, we have shown that Ap_4A induces apoptosis in reversibly permeabilized human HL60, U937, Jurkat, CEM and mouse MVRO cells whereas Ap_3A is a co-inducer of the differentiation of HL60 cells [8]. It now seems timely to consider the whole Ap_nA family as a new class of signaling molecule used by eukaryotic cells to regulate many housekeeping and specialized functions [5].

Apoptotic cell death is an important part of the natural defense of the organism against primary tumor cells, which in most cases depend on growth factors for their proliferation and survival [9–11]. In the course of progression to a fully malignant tumor, early primary tumors often undergo mutations that inactivate apoptotic pathways, resulting in increased tumor recurrence. This makes induction of apoptosis an important and often crucial step in the regression of many tumors.

In this study we focus on the cellular and molecular mechanism(s) underlying the induction of apoptosis by

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Abbreviations: Ap_3A, diadenosine 5′,5‴-P^1,P^3-triphosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Ap_nA, diadenosine oilgophosphates; pRb, retinoblastoma protein; CDK, cyclin dependent kinase.

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Ap_4A. Our experiments suggest that Ap_4A is involved at an early stage of G1/S-phase arrest by activation of pRb *via* its dephosphorylation and *via* a reduction in cyclin dependent kinase 2 (CDK2) kinase activity. We suggest that *in vivo* Ap_4A may play a significant role in tumor growth suppression by inducing apoptosis.

2. Materials and methods

2.1. Cell growth and permeabilization

The human promyelocytic cell line HL60, the HL60/Bcl-2 variant and the human T cell line CEM-SS were grown in suspension at 37° in an atmosphere of 5% CO_2 in RPMI 1640 supplemented with 10% fetal calf serum. Cells in logarithmic phase were harvested by centrifugation at 1000 *g* for 10 min at 4°, washed with PBS, suspended in permeabilizing buffer (10 mM Tris–HCl, pH 7.8, 30 mM 2-mercaptoethanol, 10 mM EDTA, 4 mM $MgCl_2$) [12] and incubated for 15 min at 4° with or without Ap_4A. Then, cells were centrifuged, washed twice with PBS and seeded in complete RPMI 1640 medium at 37° in an atmosphere of 5% CO_2. Cellular viability was determined by trypan blue exclusion.

2.2. Induction of apoptosis and analysis of cell morphology

Cells were subjected to cold shock in the presence of 1 or 10 μM Ap_4A for 15 min at 4° as described above, centrifuged, washed twice with cold, serum-free RPMI 1640 and cell smears were prepared and stained with 1 μg/mL Hoechst 33258 (Sigma) in PBS and embedded in glycerol. Morphological assessment was performed with a Leitz fluorescent microscope (60× objective). The type of cell death in Ap_4A-treated cells was determined on the basis of morphological features characteristic of apoptosis (cell shrinkage, nuclear condensation and extensive formation of membrane blebs and apoptotic bodies). DNA was extracted 10 hr after cell seeding using a DNA isolation kit (Roche). Electrophoresis was carried out in a 1% agarose gel containing 0.5 μg/mL ethidium bromide.

2.3. Flow cytometric analysis of DNA content

For quantitative estimation of the number of apoptotic cells, HL60 cells in the log phase of growth were cold-shocked in the presence of Ap_4A as described earlier, washed with PBS and seeded in complete RPMI 1640 medium. Cells (1×10^6) were incubated in 0.5 mL of medium containing 0.2% Triton X-100, 0.1 mg/mL RNAase A and 25 μg/mL propidium iodide at room temperature for 10 min. Analysis was performed with an EPICS-C Flow cytometer with an INNOVA-90-6 Argon laser working at 488 nm and 100 mW power. Cell fluorescence was registered through the 600 nm long-pass filter. Cell cycle distribution was analyzed with the CELL FIT software package.

2.4. Immunoprecipitation and immunoblotting

Cellular proteins were extracted from HL60 and CEM-SS cells in buffer containing 50 mM HEPES-NaOH, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5 mM DTT, 0.5% Nonidet P-40 and 10 μg/mL protease inhibitor cocktail (Sigma). Aliquots (300 μL, 2 mg/mL total cellular protein) of the lysates were incubated with primary antibodies—monoclonal anti-human Rb, polyclonal anti-human cyclin B, polyclonal anti-human CDK2, polyclonal anti-human CDK1 (all from Oncogene Research Products), polyclonal anti-human $p21^{waf1}$ or monoclonal anti-human Bcl-2 (Santa Cruz Biotechnology). The immunocomplexes were adsorbed on to protein A-Sepharose 4B (Pharmacia) and washed twice with SNNTE buffer (50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 5% sucrose, 0.5 M NaCl, 1% Nonidet P-40) and once with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate). Immunoprecipitates were dissolved in sample buffer and subjected to SDS–PAGE in 10 or 15% gels. The proteins were then transferred to a nitrocellulose membrane which was then incubated with anti-Rb, anti-cyclin B, anti-CDK2, anti-CDK1, anti-Bcl-2 or anti-$p21^{waf1}$. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgGs (Oncogene Research Products) were used as secondary antibodies as appropriate. The immunoreactive bands were visualized on X-ray film (Hyperfilm ECL) using a chemiluminescent substrate (Amersham). To be sure that each probe contained the same amount of protein, control experiments with housekeeping protein β-actin were performed.

2.5. Phosphorylation assay

Cell lysates (1 mg) were incubated with anti-CDK2 antibody for 1 hr at 4°. The immunocomplexes were adsorbed on to protein A-Sepharose 4B and washed extensively with SNNTE and RIPA buffers and then once with kinase buffer (50 mM HEPES-NaOH, pH 7.0, 10 mM $MgCl_2$ 1 mM DTT). Immunoprecipitates were incubated at 30° for 20 min with 1.5 μg histone H1 and 5 μCi [γ-^{32}P]ATP (2000 Ci/mmol) in 30 μL kinase buffer. Samples were analyzed by SDS–PAGE followed by autoradiography. Histone H3 phosphorylation by CDK1 was measured in the same way.

2.6. In vitro caspase assay

The activity of caspase-3 was determined in cell extracts using fluorometric substrate, i.e., DEVD-AMC. Cell lysates and the peptide substrate (100 μM) were combined in a standard reaction buffer (25 mM HEPES, pH 7.4, 1.0 mM

EGTA, 10 mM dithiotreitol, 0.1% (w/v) 3-[(3-chloramidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) and complete EDTA-free protease inhibitor cocktail and incubated for 2 hr at 37°. Cleavage of fluorogenic peptide substrate was monitored by AMC liberation using 360 nm excitation and 475 nm emission wavelengths [42]. Fluorescence units were converted to pmol of AMC using a standard curve generated with free AMC. Baseline activities were determined in samples from untreated HL60 cells.

3. Results

3.1. Ap_4A induces apoptosis in a variety of mammalian cells

The concentration of Ap_4A in human myeloid HL60, U937 and lymphoid CEM-SS cells is considerably lower (0.008–0.01 μM) than the concentration of Ap_3A. To change the level of intracellular Ap_4A experimentally, cells were permeabilized by cold shock in the presence of Ap_4A. Cold-shocked cells were morphologically intact and more than 95% of the treated cells remained viable after resealing as shown by trypan blue exclusion. Uptake of Ap_4A at different concentrations in cold shock buffer was studied. About 0.7–0.9% of Ap_4A penetrated into the cells under the condition of cold shock (data not shown). Cells permeabilized in the presence of Ap_4A displayed cytoarchitectural features typical of programmed cell death, such as membrane blebs and apoptotic bodies. The proportion of apoptotic HL60 cells increases in parallel with the elevation of Ap_4A concentration from $5 \pm 2\%$ at 0.1 μM Ap_4A to $12 \pm 2\%$ at 1 μM Ap_4A and reached $27 \pm 2\%$ of the total cell number after incubation with 10 μM Ap_4A compared to a level of 2–3% in control cells (Fig. 1). The same correlation was observed for the other promyelocytic cell line U937 cells (Fig. 1). Thus, cells with the highest Ap_4A concentration enter an apoptosis first. To confirm the specificity of Ap_4A for the observed effect we have also investigated the breakdown products of the dinucleotide as inducers of apoptosis. Eukaryotic Ap_4A hydrolase cleavages Ap_4A into ATP and AMP. The inability of 1–10 μM ATP or AMP to induce apoptosis in HL60 cells (data not shown) clearly indicated that Ap_4A alone but not the breakdown products is responsible for the observed effect. Human lymphoid T cell line, CEM-SS cells were more sensitive to the alteration of the intracellular Ap_4A concentration as compared with promyelocytic HL60 cells. The number of cells undergoing apoptosis increased from 8 ± 2 to $36 \pm 2\%$ measured at the same of Ap_4A concentrations. No significant differences in the extent of apoptosis was observed when cells were treated with 100 μM Ap_4A: the number of apoptotic cells increased about 4–6% above the values obtained with 10 μM Ap_4A in both cell lines. Ap_4A also induced apoptotic DNA fragmentation in human K-562, Jurkat T-cells and mouse adenocarcinoma VMRO cells (data not shown).

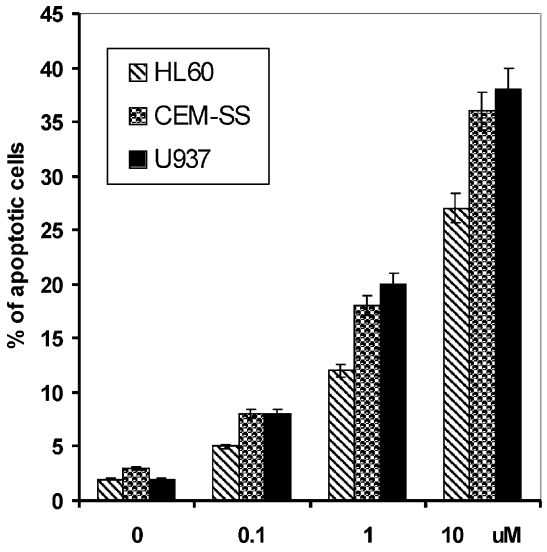

Fig. 1. The effect of different Ap_4A concentration on apoptosis in HL60, U937 and CEM-SS cells. Control experiments were performed without treatment with Ap_4A.There was correlation between the proportion of cells undergoing apoptosis and Ap_4A concentration.

3.2. Ap_4A-induced apoptosis is accompanied by modulation of pRb without a change in $p21^{waf1}$

The pRb and $p21^{waf1}$ proteins are known to be important regulators of cell proliferation and apoptosis. We have investigated whether changes in pRb and $p21^{waf1}$ accompany apoptosis induced by Ap_4A. The proteins were immunoprecipitated from extracts of Ap_4A-treated HL60 and CEM-SS cells and immunoblots of the precipitates were probed with appropriate antibodies. $p21^{waf1}$ has been identified as a cell cycle inhibitor that specifically binds to cyclin–CDK complexes, preventing the phosphorylation of pRb [13]. The low level of $p21^{waf1}$ detected in both cell lines was not altered by Ap_4A treatment (Fig. 2A).

The pRb tumor suppressor protein is regulated by the degree of phosphorylation, which is determined by the net activity of the cyclin/CDK kinases and their inhibitors [14]. pRb normally appears on Western blots as a series of bands representing different phosphorylated forms. Slower moving forms are hyperphosphorylated while inactive, faster moving forms are hypophosphorylated and are active in binding to transcription factor E2F and in blocking the cell cycle [15]. In HL60 cells a significant change in the phosphorylation state of Rb was observed upon Ap_4A treatment (Fig. 2B). In control (untreated) cells, the hyperphosphorylated form of pRb was predominant; however, pRb was converted to higher mobility hypophosphorylated active forms 10 hr after treatment with Ap_4A. By 18 hr, when apoptosis was extensive, the level of both forms of pRb dropped dramatically. A similar situation was found with CEM-SS cells, the level of the hypophosphorylated

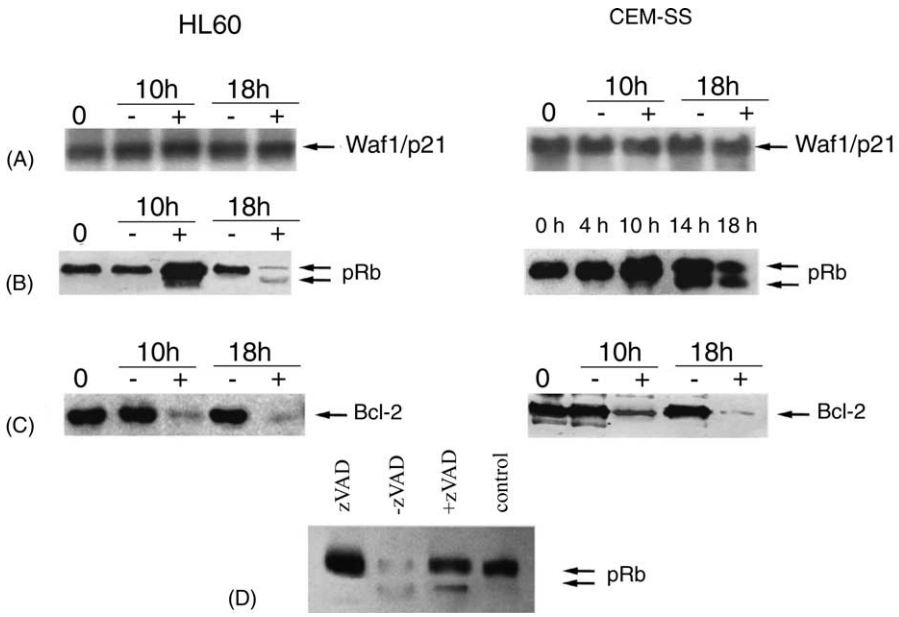

Fig. 2. The effect of Ap_4A on $p21^{waf1}$, pRb and Bcl-2 in HL60 and CEM cells. Cells were incubated in complete RPMI 1640 for the indicated periods of time after cold shock in the presence (+) or absence (−) of 10 μM Ap_4A. (A) $p21^{waf1}$, (B) pRb, and (C) Bcl-2 in cell extracts were estimated by immunoblotting using appropriate antibodies. In the case of pRb in CEM-SS cells, all incubations shown were in the presence of 10 μM Ap_4A, (D) incubation of HL60 cells with the general caspase inhibitor zVAD-fmk blocked the emergence of Ap_4A-induced pRb cleavage. A total of 20 μM of cell permeable inhibitor zVAD-fmk were added to cells cold shocked in the presence of 10 μM Ap_4A. Cells were harvested after 15 hr and analyzed by Western blot as decribed earlier. The addition of zVAD-fmk alone did not affect cell viability.

pRb increasing from 10 to 14 hr, and all forms diminishing after 18 hr (Fig. 2B). After incubation of Ap_4A-treated cells in the presence of general caspase inhibitor zVAD-fmk for 15 hr the cleavage of pRb was abrogated (Fig. 2D). To get the direct evidence that ''effector'' caspase activation is induced by Ap_4A we incubated extracts from cells treated with Ap_4A for up 20 hr with the fluoregenic substrate DEVD-AMC. The AMC release was markedly increased over time, with a 10-fold increase in activity within 20 hr of the addition of Ap_4A. A modest increase in DEVD-AMC cleavage under the conditions of cold shock (control, without Ap_4A) was also evident (Fig. 3) The results concerning Bcl-2 in Fig. 2 will be discussed later.

3.3. Ap_4A is involved at an early stage of G1/S phase arrest

HL60 cells cold-shocked in the presence of Ap_4A were incubated in complete RPMI 1640 medium for up to 18 hr. Flow cytometric analysis of the DNA content in these Ap_4A-treated cells as determined by propidium iodide staining showed the accumulation of a sub-G1 peak of hypodiploid cells (Ap) to the left of the G1 peak, which was presumed to represent apoptotic cells (Fig. 4A). The first apoptotic cells appeared 4 hr after treatment with Ap_4A. Prolonged (>10 hr) incubation with Ap_4A increased the number of apoptotic cells due to the loss of cells in S- and G2-phases. The broadening of the G1 peak (18 hr) and the appearance of a slope in the S-phase region of the histograms (10 and 18 hr) indicated that not only G1-phase cells were condemned to apoptosis but also S-phase cells. Fig. 3 is derived from the histograms presented in Fig. 4A. The percentage of different cell populations plotted on the graph was obtained by deconvolution of the total histogram data using the standard EPICS-C instrument software. Apoptotic cells comprised 28–30% of the Ap_4A-treated cells, compared with 6% of the untreated cells. The gradual decrease in S-phase cells from 28 to 8% during the course of incubation with Ap_4A was observed in parallel with an increase in G1-phase cells from 42 to 52% (Fig. 4B). These observations indicated that Ap_4A is somehow involved in

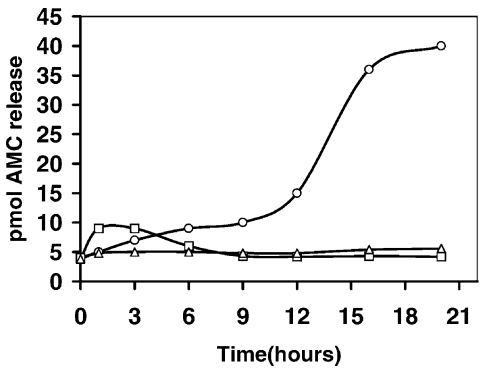

Fig. 3. Ap_4A triggers the activation of ''effector'' caspase in HL60 cells. Time-course of caspase activation in Ap_4A-treated (○) and non-treated (△) HL60 cells. Cells were lysed at the indicated time points and the *in vitro* cleavage of the fluorogenic peptide substrate DEVD-AMC (100 μM) was measured. A modest increase in DEVD-AMC cleavage is evident (□) under the conditions of cold shock without Ap_4A. Data shown are the average of two independent experiments performed in duplicate.

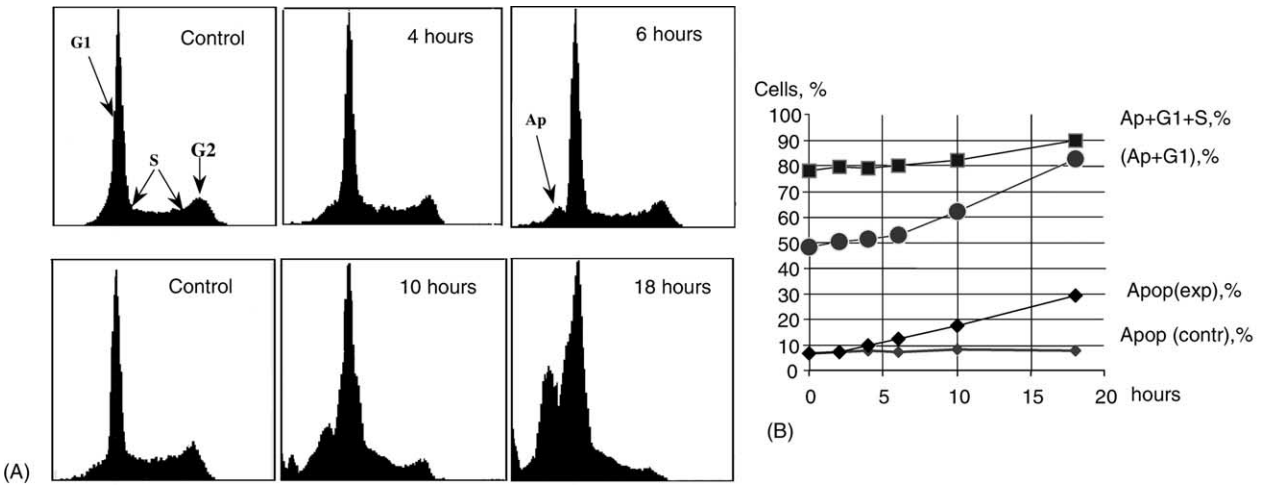

Fig. 4. Flow cytometric analysis of Ap_4A-treated HL60 cells. (A) Cytofluorimetric histograms of cells 0 hr (control), 4, 6, 10 and 18 hr after treatment with 10 μM Ap_4A. Apoptotic cells (Ap) appear as a sharp peak to the left of the G1 peak; (B) cell subpopulation kinetics showing the percentage of cells in different peaks (Ap, G1 and S) after treatment with 10 μM Ap_4A and the percentage of apoptotic cells (Apop) at different times after treatment with (exp) or without (contr) 10 μM Ap_4A. The results shown are typical of the appearance of cells in three identical experiments.

G1/S-phase arrest. However, this arrest was manifested only at the early stage: the G2-peak did not disappear and 8% of the cells remained in S-phase even at full apoptosis.

3.4. Ap_4A reduces the activity of CDK 2

Cyclin A or E-dependent kinase 2 (CDK2) activity has been shown to be required for DNA synthesis and is responsible for the phosphorylation of pRb and execution of S-phase [16]. The expression of CDK2 was also probed by immunoblotting. A 2.3- and 2.1-fold decrease in the level of immunoreactive CDK2 in response to Ap_4A was found in both HL60 and CEM-SS cells at full apoptosis (Fig. 5A and B). Activation of CDK2 occurs as a result of the dephosphorylation of tyrosine 15 and threonine 14, while at the same time an independent increase in phosphorylation of threonine 160 occurs. When the CDK2 protein was immunoprecipitated from HL60 cell lysates and subjected to a kinase activity assay using histone H1 as substrate, the degree of histone H1 phosphorylation produced by CDK2 from Ap_4A-treated cells was significantly lower than that produced by CDK2 from control cells (Fig. 4). Densitometric analysis and measurement by liquid scintillation counting showed a reproducible 2-fold decrease in histone H1 phosphorylation after treatment with Ap_4A. Thus, the onset of Ap_4A-induced apoptosis could also involve the activation of pRb *via* a reduction in CDK2-mediated phosphorylation.

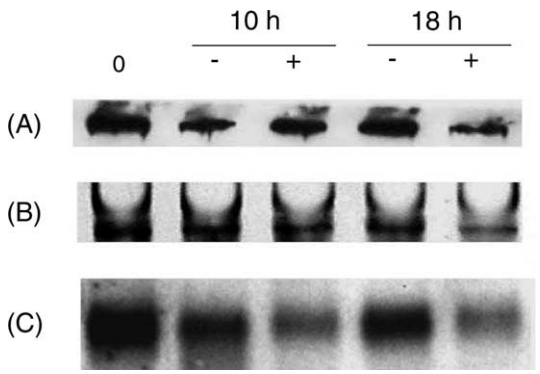

Fig. 5. CDK2 content and activity after cold shock in the presence of Ap_4A. Cells were incubated in complete RPMI 1640 medium for the indicated periods of time after cold shock in the presence (+) or absence (−) of 10 μM Ap_4A. CDK2 content in HL60 (A) and CEM-SS (B) cells estimated by immunoblotting; (C) phosphorylation of histone H1 by CDK2 immunoprecipitated from HL60 cells determined by autoradiography.

3.5. Ap_4A is not involved in "aberrant mitosis"

The formation of the CDK1/cyclin B complex leads to the phosphorylation of lamins, histones H1 and H3 and centrosomal and other proteins that need to be displaced from chromatin to allow chromosome condensation. Such events take place during mitosis and apoptosis. The uncoupling of the timing of CDK1 activation and the completion of DNA replication results in a so-called "aberrant mitosis". By virtue of its close structural similarity to ATP, Ap_4A may bind to kinases as an ATP analog. Densitometric analysis showed no differences in the level of CDK1 protein in Ap_4A-treated (18 hr) compared to control cells (Fig. 6A). Using histone H3 as substrate and immunoprecipitated CDK1 as a source of kinase activity, no alterations in CDK1/cyclin B complex activity were observed even at full apoptosis (Fig. 6B) despite the fact that immunoblotting for cyclin B showed an increased level of this protein in response to Ap_4A treatment in both HL60 (Fig. 6C) and CEM-SS cells (data not shown).

3.6. Bcl-2 is abolished in Ap_4A-treated cells

To determine whether a change in the level of the anti-apoptotic Bcl-2 protein was associated with the apoptotic

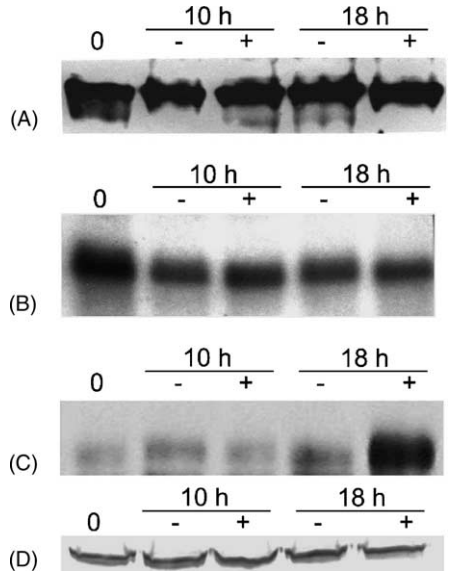

Fig. 6. Influence of Ap_4A on CDK1 content and activity and on cyclin B content in HL60 cells. After cold-shock in the presence (+) or absence (−) of 10 μM Ap_4A, cells were incubated in complete RPMI 1640 medium for the indicated periods of time. (A) The level of CDK1 estimated by immunoblotting; (B) phosphorylation of histone H3 by CDK1 immunoprecipitated from HL60 cells. The kinase reaction was performed as described in Section 2. Samples were analyzed by SDS–PAGE followed by autoradiography. (C) Cyclin B content in HL60 cells estimated by immunoblotting. (D) A control blot, β-actin content in HL60 cells.

response to Ap_4A, the expression of Bcl-2 was probed by immunoblotting. A dramatic decrease in Bcl-2 content was found in both HL60 and CEM-SS cells 10 hr after treatment with Ap_4A (Fig. 2C). By 18 hr, when apoptosis was extensive, the level of Bcl-2 was low or even undetectable.

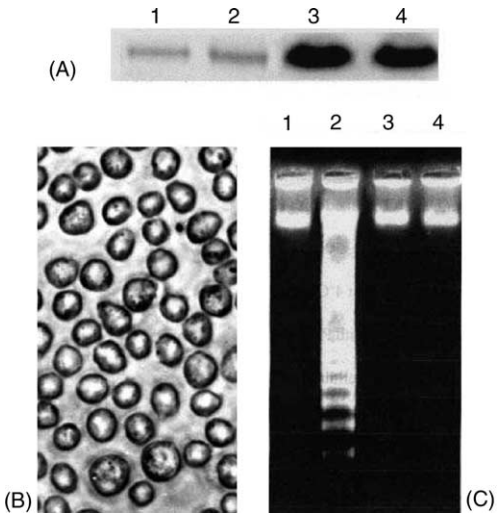

Fig. 7. Lack of induction of apoptosis by Ap_4A. (A) Bcl-2 content estimated by immunoblotting in HL60 (lanes 1 and 2) and HL60/Bcl-2 cells (lanes 3 and 4) with (lanes 2 and 4) or without (lanes 1 and 3) cold shock. (B) Phase-contrast microscopy of HL60/Bcl-2 cells treated with 50 μM Ap_4A. (C) Apoptotic DNA degradation in Ap_4A-treated cells. DNA was extracted from HL60 (lanes 1 and 2) and HL60/Bcl-2 (lanes 3 and 4) cells 18 hr after treatment with 10 μM Ap_4A (lane 2), 50 μM Ap_4A (lane 4) or without Ap_4A (lanes 1 and 3) and electrophoresed in a 1% agarose gel.

The loss of Bcl-2 was surprising as normally it has a long half-life. To get independent evidence for the involvement of Bcl-2 in Ap_4A-induced apoptosis, we examined a derivative of the HL60 cell line that overexpresses Bcl-2. When subjected to cold shock, these HL60/Bcl-2 cells retained their ability to express a high level of Bcl-2 (Fig. 7A). When permeabilized in the presence of Ap_4A, HL60/Bcl-2 cells showed no evidence of plasma membrane ruffling, chromatin collapsing into crescents along the nuclear envelope or the appearance of apoptotic bodies, in contrast to control HL60cells (Fig. 7B). Moreover, DNA isolated from HL60/Bcl-2 cells cold-shocked in the presence of different concentrations of Ap_4A did not display the characteristic apoptotic ladder. The cells yielded intact high molecular weight DNA, again in contrast to control cells (Figs. 1 and 7C).

4. Discussion

We have previously observed that apoptosis is associated with a dramatic inversion of the Ap_3A/Ap_4A ratio [7,8]. This inversion was due to elevation of Ap_4A concentration in apoptotic cells. Here, we have shown that an increase in the intracellular concentration of Ap_4A achieved by reversible permeabilization of human myeloid and lymphoid cells by cold shock in the presence of this dinucleotide induced a significant proportion of the cells to undergo apoptosis. There was a correlation between the intracellular concentration of Ap_4A and the fraction of cells undergoing apoptosis: cells with the highest Ap_4A concentration go through apoptosis first. The effect was also specific for Ap_4A, since (i) ATP or AMP, the breakdown products of Ap_4A, were unable to induce apoptosis in HL60 cells; and (ii) hydrolysis-resistant analogues of Ap_4A were inactive as apoptotic inducers [8]. A further specific aspect of the action of Ap_4A was the sensitivity of only certain cell types.

One possible mechanism for the induction of cell death by Ap_4A would be an involvement in cell cycle arrest. This has been observed in cells exposed to a variety of apoptosis-inducing agents, such as topoisomerase inhibitors, bacterial toxins, viruses and γ-irradiation [17–19]. It is known that the initial two thirds of G1 phase is the time window during which the mammalian cell makes most of its decisions regarding growth vs. or quiescence [3]. pRb allows the cell cycle clock to open the gate and permits the cell to proceed into late G1. Through the early hours of G1, pRb is found in a hypophosphorylated form, while during the latter hours of G1 it is hyperphosphorylated. Several lines of evidence indicate that pRb loses its multiple phosphate groups only upon emergence from mitosis. The phosphorylation state of pRb is regulated by (i) cyclin D/CDK4 or CDK6 kinases in early G1; (ii) cyclin E/CDK2 kinase in mid-to-late G2; and (iii) $p21^{waf1}$, a protein inhibitor of CDK4 and CDK6, the expression of which is under the control of the p53 transcription factor [20].

We have studied the influence of all three possible factors on pRb phosphorylation. By immunoblotting, we have shown a significant change in the phosphorylation state of pRb after treatment with Ap_4A, with a transition towards the hypophosphorylated active form that was temporally correlated with the time of Ap_4A treatment (Fig. 2). The data obtained raised two major questions: does Ap_4A inhibit the phosphorylation of pRb *via* binding to kinases, possibly by virtue of its close similarity to ATP, or does it somehow activate pRb dephosphorylation? Given the sequence homology between prokaryotic Ap_4A hydrolases and mammalian serine-threonine protein phosphatases [21,22], a direct effect of Ap_4A on pRb dephosphorylation cannot be excluded. Another feature of Ap_4A-induced apoptosis is the low content of pRb at full apoptosis (Fig. 2). Hyperphosphorylated pRb is predominant in growing cells. During growth suppression, the amount of this form decreases with an increase in the amount of the hypophosphorylated form, while at full G1/S-phase arrest, cells were found to contain only hypophosphorylated pRb [23]. Based on our data, we assume that Ap_4A is able to retard the transition from G1- to S-phase but it is unable to induce a complete arrest. The above interpretation is consistent with the flow cytometric data.

Flow cytometric analysis points to a possibility of an early G1/S-arrest (Fig. 4). A reduction in the number of S-phase cells and an increase in the G1 peak are features characteristic of the early stage of G1/S-phase arrest although the arrest was incomplete because some cells were either still cycling or paused at the G2 phase (Fig. 4).

There is growing evidence that successive phosphorylation of pRb by both cyclin D in complex with CDK4 or CDK6 and cyclin E/CDK2 is needed to completely inactivate pRb and allow progression through cell cycle. CDK2 can also phosphorylate E2F in complex with cyclin A, and this phosphorylation inhibits E2F binding to DNA [24]. This is a potential mechanism for ‘‘turning off’’ transcriptional activation by E2F in S-phase. It was of interest to determine whether CDK2 phosphorylation might be regulated by Ap_4A. About 2-fold reduction in the expression of CDK2 was found after treatment of cells with Ap_4A in both HL60 and CEM cells, at 18 hr and when CDK2 kinase was immunoprecipitated from Ap_4A-treated HL60 cells and its activity measured *in vitro*, the phosphorylation of a histone H1 substrate was significantly decreased (Fig. 5). Thus, the onset of Ap_4A-induced apoptosis could involve the CDK2 action.

We have also checked the expression of $p21^{waf1}$ in response to Ap_4A. No differences in the amounts of $p21^{waf1}$ were observed in HL60 or CEM cell lysates after incubation with Ap_4A (Fig. 2), suggesting a p53-independent effect of Ap_4A. This observation is consistent with the induction of apoptosis in HL60 cells, which are known to be p53 null cells [25].

In *Escherichia coli*, the elevation of intracellular Ap_4A induces cell division and produces many small cells, each with a single nucleoid [26]. If the same were true in mammalian cells, then Ap_4A may somehow be related to the uncoupling of the timing of mitotic activation and completion of DNA replication. However, no significant change in CDK1 content was observed in Ap_4A-treated HL60 cells compared with control HL60 cells (Fig. 6). Phosphorylation of histone H3, which is associated with mitotic chromosome condensation [27], did not change either in spite of the fact that immunoblotting showed an increased level of cyclin B in response to Ap_4A treatment (Fig. 6). Ap_4A does not produce ‘‘aberrant mitosis’’. Thus, our working hypothesis that Ap_4A by virtue of its close similarity to ATP may bind to CDK1 as an ATP analogue and reduce the histone phosphorylation has not been supported experimentally: Ap_4A did not act as ATP analogue in histone phosphorylation reactions.

Two major features of apoptosis are caspase activation and organelle dysfunction. The anti-apoptotic Bcl-2 protein inhibits the caspase pathway by preventing cytochrome *c* release from mitochondria. Overexpression of Bcl-2 has been shown to be a crucial event in suppressing cell death induced by the v-Cbl oncogene, TGF-β1 and IL-3 [28]. Ap_4A-induced apoptosis is accompanied by a significant reduction in the Bcl-2 content of HL60 and CEM-SS cells. The loss of Bcl-2 was surprising: normally, it has a long half life. Moreover, recently the non-caspase cleavage of Bcl-2 in B lymphoma cell line RL-7 treated with the Zn^{2+} chelator was observed [29]. Contrary to Ap_3A, Ap_4A is a strong Zn^{2+} chelator [30]. To get an independent evidence of Bcl-2 involvement in Ap_4A-induced apoptosis we used Bcl-2-expressing HL60 cells line. Ap_4A did not induce apoptosis in HL60/Bcl-2 cells. Since Bcl-2 was shown to be a substrate of caspase-3 in myeloid leukemic cells [31] we suggest that Ap_4A is, somehow, involved in the cascade of events leading to ‘‘effector’’ caspase activation. This is supported by the observation that pRb level dropped dramatically in Ap_4A-treated cells (Fig. 2). Direct cleavage of pRb by caspase-3 was shown recently [32]. To confirm further, that Ap_4A is involved in caspase activation, we incubated Ap_4A-treated HL60 cells in the presence of the general caspase inhibitor zVAD-fmk (Fig. 2D). The cleavage of pRb was abrogated under these conditions, indicating that pRb cleavage in HL60 cells in response to Ap_4A is, indeed, caspase-mediated. In the present study we could detect the activation of ‘‘effector’’ caspase activity in Ap_4A-treated cells, as evidenced by the *in vitro* cleavage of the fluorogenic substrate DEVD-AMC (Fig. 3). Future studies will address the question of whether Ap_4A is a low molecular weight activator of caspases.

Association of Ap_4A with the induction of apoptosis is consistent with a number of earlier observations. The action of apoptosis-inducing agents causing the accumulation of DNA strand breaks is accompanied by an increase in the intracellular level of Ap_4A [4,33]. The action of other agents known to increase the percentage of apoptotic cells,

such as arsenite, cadmium and heat shock, is also accompanied by an elevation in the level of Ap_4A [4,34]. *In vivo*, elevation of Ap_4A could be achieved by direct upregulation of the aminoacyl-tRNA synthetases [1,4], downregulation of the specific degradative Ap_4A hydrolases or both, in response to appropriate signals. Alternatively, a limitation in the supply of tRNAs as acceptors of aminoacyl moieties from aminoacyl adenylates or excessive amounts of ATP available to split aminoacyl adenylates might lead to a specific increase in Ap_4A, thus providing a link between apoptosis and the protein-synthesizing apparatus.

The majority of current anti-cancer therapies eliminate tumor cells *via* apoptosis [35]. Defects in the intra- or extra-cellular pathways are an important cause of resistance to cytotoxic agents and can lead to cancer. A variety of conditions and agents have been identified during the last decade as inducers of programmed cell death [9]. To this list Ap_4A can now be added. Here, we have shown that an elevated level of Ap_4A within the cell appears to function as a factor leading to cell loss *via* apoptosis. We suggest that agents increasing the synthesis or, alternatively, decreasing the hydrolysis of Ap_4A may interfere with the malignant proliferation of human hematopoietic cells.

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