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ATM Is Involved in Cell-Cycle Control Through the Regulation of Retinoblastoma Protein Phosphorylation

Javier G. Pizarro,[1] Jaume Folch,[2] Aurelio Vazquez de la Torre,[1] Felix Junyent,[1,2] Ester Verdaguer,[1] Joaquin Jordan,[3] Merce Pallas,[1†] and Antoni Camins[1*,†]

[1] *Unitat de Farmacologia i Farmacognosia, Institut de Biomedicina (IBUB), Centro deInvestigacion Biomedica en Red de Enfermedades Neurodegenerativas (CIBERNED), Facultat de Farmacia, Universitat de Barcelona, Nucli Universitari de Pedralbes, Barcelona E-08028, Spain*

[2] *Unitat de Bioquimica, Facultat de Medicina i Ciencies de la Salut, Centro de Investigacion Biomedica en Red de Enfermedades Neurodegenerativas (CIBERNED), Universitat Rovira i Virgili, C./St. Llorenc 21, Reus E-43201 (Tarragona), Spain*

[3] *Facultad de Medicina, Departamento de Ciencias Medicas, Universidad de Castilla-La, Mancha, Avda. Almansa, 14, Albacete E-02006, Spain*

ABSTRACT

Ataxia telangiectasia mutated protein (ATM) is a member of the phosphatidylinositol-3 kinase (PI3K) family, which has a role in the cellular response to DNA double-strand breaks (DSBs). In the present study, we evaluated the role of ATM in cell-cycle control in dopaminergic rat neuroblastoma B65 cells. For this purpose, ATM activity was either inhibited pharmacologically with the specific inhibitor KU-55933, or the ATM gene was partially silenced by transfection with small interfering RNA (siRNA). Our data indicate that although ATM inhibition did not affect the cell cycle, both treatments specifically decreased the levels of cyclin A and retinoblastoma protein (pRb), phosphorylated at Ser780. Furthermore, ATM inhibition decreased the active form of p53, which is phosphorylated at Ser15, and also decreased Bax and p21 expression. Using H_2O_2 as a positive control of DSBs, caused a rapid pRb phosphorylation, this was prevented by KU-55933 and siRNA treatment. Collectively, our data demonstrate how a new molecular network on ATM regulates the cell cycle through the control of pRb phosphorylation. These findings support a new target of ATM. J. Cell. Biochem. 110: 210–218, 2010.

KEY WORDS: ATM; APOPTOSIS; KU-55933; siRNA; CELL CYCLE; RETINOBLASTOMA PROTEIN

DNA damage induces a variety of responses that restore specific DNA lesions [Ding et al., 2006]. The mechanism of DNA repair and the kind of molecules involved depend on the type of DNA damage, as well as other factors including the response of signal transduction pathways and the efficiency of DNA repair [Rotman and Shiloh, 1997; Zhang et al., 2007]. Ataxia telangiectasia mutated protein (ATM) is activated in response to double-strand break (DSB) damage. ATM belongs to the family of phosphatidylinositol-3 kinases (PI3K), which is activated by autophosphorylation at Ser1981 (Ser1981) [Shiloh, 2003; Khanna et al., 2001]. Likewise, mutation of ATM occurs in human autosomal recessive disorder ataxia telangiectasia, which is characterized by progressive neurodegeneration, cancer, immune system defects, and hypersensitivity to ionizing radiation [Lavin and Shiloh, 1996; Lee and McKinnon, 2000]. Other PI3K enzymes in the mammalian DNA damage response that is key regulators of cell-cycle checkpoint pathways are ATR (ATM- and Rad3-related) and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) [Savitsky et al., 1995; Kurz et al., 2004].

In eukaryotic cells, the DNA damage response is involved in numerous physiological alteration processes by means of a series of steps. As mentioned above, an early event following DSBs is nuclear

Javier G. Pizarro and Jaume Folch contributed equally to this work.

[†]Senior co-author.

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*Correspondence to: Antoni Camins, Unitat de Farmacologia i Farmacognosia i Institut de Biomedicina, Facultat de Farmacia, Universitat de Barcelona, Nucli Universitari de Pedralbes, E-08028 Barcelona, Spain.
E-mail: camins@ub.edu

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ATM autophosphorylation at Ser1981, which is the primary DSB transducer [Rotman and Shiloh, 1997; Yoo et al., 2007]. In a second step, active ATM phosphorylates and activates different substrates involved in the DNA damage response, including the tumor suppressor protein p53. Moreover, in vitro studies have shown that ATM is a component of the cell-cycle checkpoint machinery that causes growth arrest after ionizing radiation-induced DNA damage [Keramaris et al., 2003; Wyttenbach and Tolkovsky, 2006]. Likewise, ATM is an upstream regulator of the apoptosis pathway, since unrepaired DNA damage can trigger apoptosis that is typically mediated by the ATMp53 pathway in post-mitotic neurons [Herzog et al., 1998; Khanna et al., 2001].

Pharmacological ATM inhibitors have been developed that have potential applications in cancer treatment. Recent studies identified a novel, specific small molecule inhibitor of ATM: 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one, termed KU-55933 [Hickson et al., 2004]. Studies performed in human tumor cell lines, such as U2OS, LoVo, SW620, HeLa B, the A-T fibroblast cell line AT4 and the normal human fibroblast 1BR, showed that KU-55933 resulted in significant sensitization to the cytotoxic effects of ionizing radiation and DNA DSBs-inducing chemotherapeutic agents like tenoposide, doxorubicin, and camptothecin [Hickson et al., 2004]. In this report, we study the role of ATM in the control of cell-cycle progression using two strategies: we exposed neuroblastoma B65 cells to the pharmacological inhibitor of ATM, KU-55933; and we used a small interfering RNA (siRNA) sequence targeting mRNA ATM. In the present study, we suggest that the enzyme ATM controls cell-cycle machinery in B65 neuroblastoma cells through the modulation of retinoblastoma protein (pRb) phosphorylation. To our knowledge, this is the first study in which a role of ATM in pRb phosphorylation is suggested.

MATERIALS AND METHODS

MATERIALS

The pharmacological agents used in this study included KU-55933 from Sigma Chemical Co. (St. Louis, MO). Cell culture media and fetal calf serum (FCS) were from GIBCO (Life Technologies, Paisley, UK). Flow cytometry experiments were carried out using an Epics XL flow cytometer. Optical alignment was based on an optimized signal from 10 nm fluorescent beads (Immunocheck, Epics Division). Stained cells were visualized under UV illumination, using the 20× objective of a Nikon Eclipse fluomicroscope. Western blot analysis was performed with polyvinylidene fluoride (PVDF) sheets from ImmobilonTM-P (Millipore Corp., Bedford, MA) and a transblot apparatus (BioRad). Monoclonal antibodies against the following were used: cyclin D1 (Cell Signalling Technology, Denvers, MA), Cyclin A (Abcam plc, Cambridge, UK), phospho-ATM (ser1981), phospho-Rb (Ser780), p53, phospho-53 (Ser15), Cdk4, Cdk2 and Cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase-conjugated IgG secondary antibody was also used (Amersham Corp., Arlington Heights, IL).

Immunoreactive protein was visualized using a chemiluminescence-based detection kit following the manufacturer's protocol (ECL kit; Amersham Corp.). The SilencerR Pre-designed siRNA sequence and siPORTTM NeoFXTM used for reverse transfection were purchased from Ambion (Applied Biosystems, Foster City, CA). pEGFP-C1 vector was purchased from Beckton Dickinson Biosciences Clontech. Other chemical reagents were of analytical quality and purchased from Sharlab (Barcelona, Spain).

CELL CULTURE AND TREATMENT

Dopaminergic neuroblastoma B65 cell line was purchased from the European Collection of Cell Cultures (ECACC, Aylesbury, UK). Cells were plated at 200 cells/mm^2 and cultured in DMEM media containing 10% FCS and 2 mM glutamine for 24 h prior to addition of KU-55933. In toxicity experiments, cells were exposed to the toxicity of H_2O_2 500 μM from 15 min to 6 h, either alone or in the presence of KU-55933.

Adherent cells from wells to be treated with small RNA were trypsinized 1 h beforetransfection and diluted in normal growth medium to 1×10^5 cells/ml. When 35 mm plates were used, the transfection complexes were first added to the empty plates. Then, a total of 2.3 ml of complete medium containing 2.3×10^5 cells was mixed with the complexes, and the plates were gently rocked back and forth to evenly distribute the cells.

ANALYSIS OF DNA FRAGMENTATION AND CELL-CYCLE PROGRESSION BY FLOW CYTOMETRY

We studied changes in the cell cycle and apoptosis after 24 h of either pharmacological or gene silencing treatments. DNA fragmentation was measured by flow cytometric analysis of propidium iodide-stained cellular DNA as previously reported [Yeste-Velasco et al., 2009]. In brief, DNA fragmentation was analyzed by flow cytometric detection of hypodiploid DNA. Cells were detached by trypsinization, combined with medium containing floating cells, and centrifuged at 100*g* for 5 min. The pellets were fixed in ice-cold 70% (v/v) ethanol in PBS overnight at 4°C by gradual addition while vortex mixing. The cells were subsequently stained with 10 μg/ml of propidium iodide and treated with 1 mg/ml RNase for 30 min at 37°C before analysis using a Beckman Coulter Epics XL flow cytometer (argon laser, excitation wavelength 488 nm). A minimum of 10,000 events were acquired in list mode while gating the forward and side scatters to exclude propidium iodide-positive cell debris and analyzed in FL-3 for the appearance of the sub-G1 peak.

PHARMACOLOGICAL INHIBITION AND GENE SILENCING OF ATM IN B65 CELLS

To investigate the effects of KU-55933 on B65 cells, several concentrations of the compound, ranging from 1 to 20 μM, were added to the culture media for 12–24 h. In the gene silencing experiments, we used a small interference sequence to target the boundary between exons 6 and 7 of the ATM mRNA (sense: GGCUAUUCAGUAUGCCAGAtt; antisense: UCUGGCAUAC UGAA-UAGCCtt). To transfect cells with siRNA, we followed the general recommendations of the purchaser. In brief, the lipid-based agent siPORTTM NeoFXTM was mixed with serum-free medium at a ratio of 5/100 μl for each 35 mm well, and the mix was incubated for 10 min at room temperature. Small RNA was then diluted in serum-free medium to a final concentration of 10 nM. For each 35 mm well, 12.5 μl of 2 μM siRNA was diluted with 100 μl of serum-free media. Both the diluted lipid transfection agent and the diluted siRNA were

then mixed together by pipetting up and down and the solution was incubated for 10 min at room temperature. Finally, the newly formed transfection complexes were dispensed into the empty wells of the culture plate and then mixed with the cell suspension. As a transfection control, both siRNA targeting ATM and pEGFPC1 were used. Fusions to the C terminus of EGFP retain the fluorescent properties of the native protein, which allows the localization of the fusion protein in vivo. The target gene should be cloned into pEGFP-C1 so that it is in frame with the EGFP coding sequences, with no intervening in-frame stop codons. The recombinant EGFP vector can be transfected into mammalian cells using any standard transfection method. If required, stable transformants can be selected using G418. pEGFP-C1 can also be used simply to express EGFP in a cell line of interest (e.g., as a transfection marker). Plasmids were cotransfected at a molar ratio of 3/1, respectively, and the percentage of green fluorescent cells was determined.

WESTERN BLOT ANALYSIS

Aliquots of cell homogenate, containing 25 μg of protein per sample, were analyzed by Western blot. In brief, samples were placed in sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-b-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95–100°C for 5 min. Samples were separated by electrophoresis on 10% acrylamide gels. Thereafter, proteins were transferred to PVDF sheets using a transblot apparatus. Membranes were blocked for 1 h with 5% non-fat milk dissolved in TBS-T buffer (50 mM Tris; 1.5% NaCl, 0.05% Tween-20, pH 7.5). They were then incubated with primary monoclonal antibodies against cyclin D1, cyclin E, and cyclin A, cdk4, cdk2, E2F-1, Bax, p21, phospho-ATM (ser1981), phospho-Rb (Ser780), p53, phospho-53 (Ser15) (1:500), and actin-beta (1:20,000). After 12 h of incubation, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG secondary antibody (1:3,000). Immunoreactive protein was visualized using a chemiluminescence-based detection kit. Protein load was monitored using phenol red staining of the blot membrane or immunodetection of actin-beta.

IMMUNOPRECIPITATION

To examine interactions of pRb protein, a coimmunoprecipitation was performed using Protein A-Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology). The starting amount of protein was 50 mg of clear cell lysates and the incubation time with anti-phospho-Rb antibody (1:100, Santa Cruz Biotechnology) was 6 h in agitation at 4°C. Then, 50 ml of protein A9 Agarose beads (50% suspension) was added into the mixture and incubated overnight in agitation at 4°C. The mixture was centrifuged at 1,000*g* for 5 min. The supernatant were discarded and pellet was washed twice with buffer containing 5 mg/ml aprotinine, 0.5 mM DTT, 0.2 mM PMSF and collected after centrifugation at 1,000*g* for 4 min. The pellets were re-suspended with 60 ml of 2× sample loading buffer. The final mixture was denatured by boiling for 5 min. After cooling, the mixture was centrifuged at 1,000*g* for 5 min and the supernatant was transferred to another tube. Twenty milliliters of the supernatant was loaded to conduct SDS–PAGE and Western blotting.

STATISTICAL ANALYSIS

Data are given as the mean ± SEM of at least three experiments for the protein determination and at least two independent experiments for the gene expression analysis. In the protein experiments, data were analyzed by ANOVA followed by post hoc Tukey–Kramer multiple comparison tests. *P*-values <0.05 were considered significant.

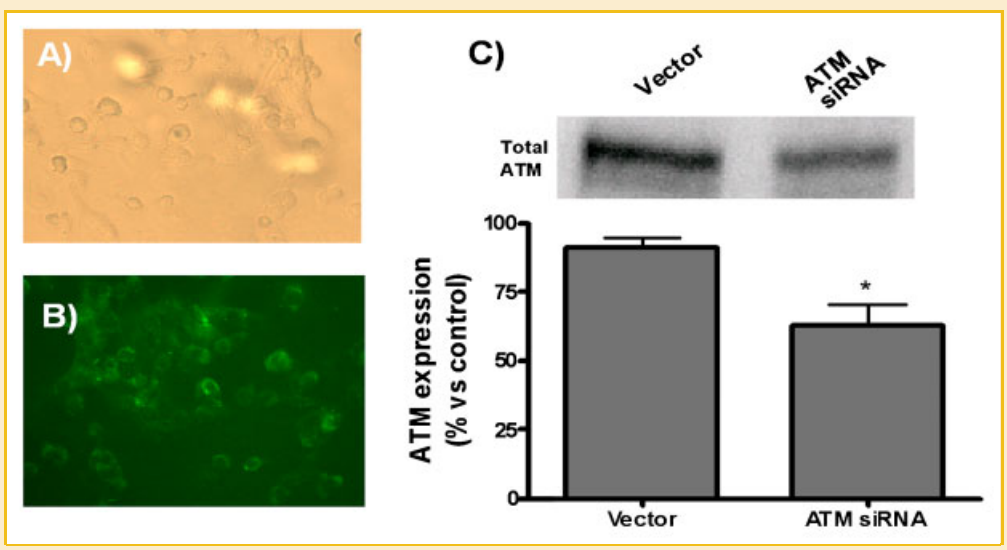

Fig. 1. Transfection efficiency was tested by cotransfecting siRNA for ATM and pEGFP-C1 plasmid at a molar ratio of 3/1, respectively. The percentage of greenfluorescent cells was determined (A), in comparison with the phase-contrast images (B). Results of the experiments showed that the efficiency of transfection was very high, with values of up to 90%. The siRNA sequence targeting ATM caused a significant decrease in total ATM content of about 40%, as shown in Western blot experiments (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS

EFFICIENCY OF siRNA TRANSFECTION AND GENE SILENCING

B65 cells were transfected with ATM siRNA and pEGFP-C1 plasmid for 24 h. Immunofluorescence images showed that there were up to 90% transfected cells. Therefore, the transfection efficiency was high (Fig. 1A,B). To confirm this result, an analysis of total ATM was undertaken by Western blot. Transfected cells showed a decrease in the expression of total ATM of about 40% at 24 h. This indicates that ATM was inhibited in ATM siRNA transfected cells (Fig. 1C).

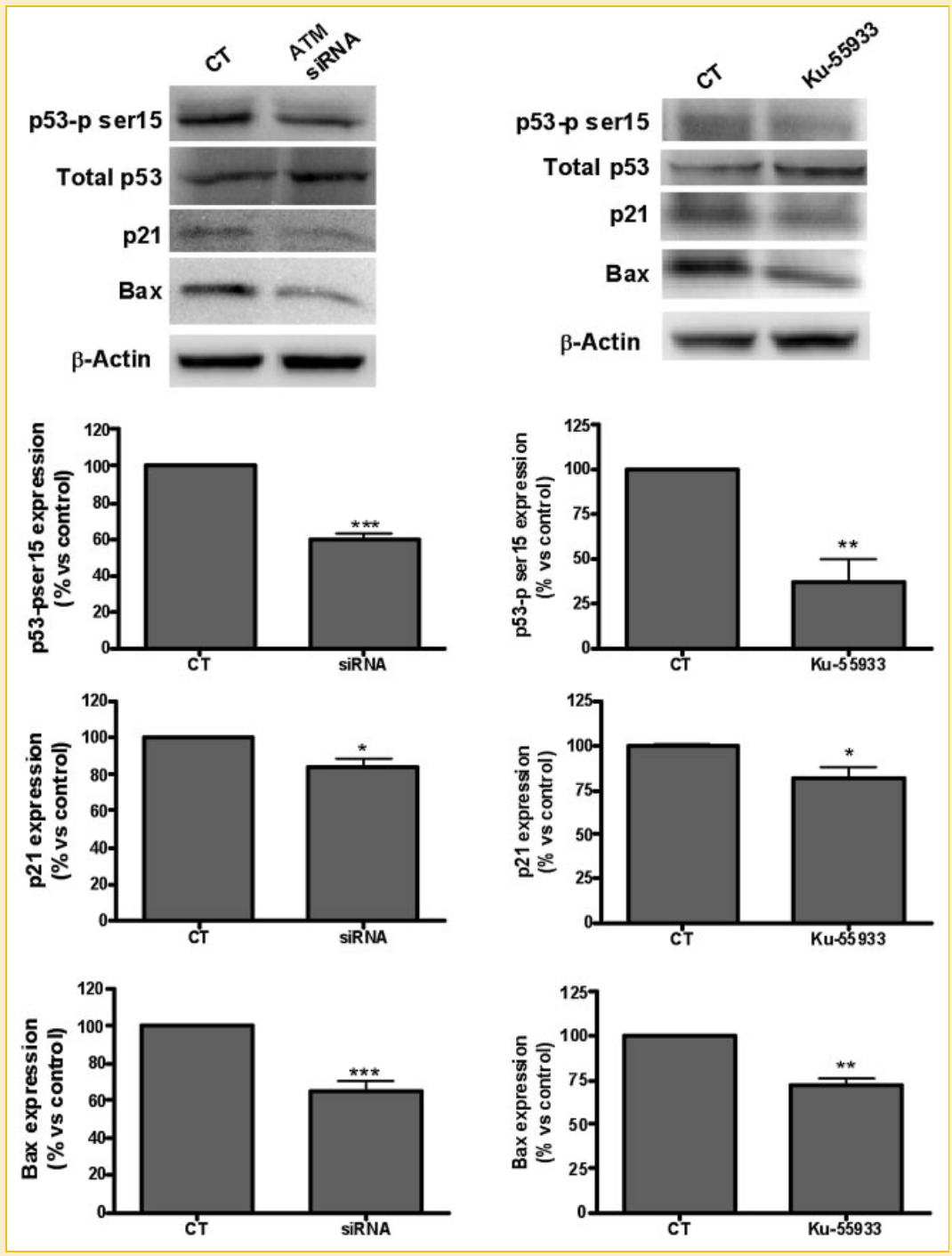

Fig. 2. Western blot of total p53 and p53 phosphorylated at ser15, p21, and Bax from B65 cells exposed for 24 h to siRNA targeting ATM and KU-55933 10 μM. The decrease in the content of the phosphorylated form of p53 was concomitant with an increase in the total p53, as shown in the bar graph, in which the ratio p53-ser 15/total p53 is represented. A significant decrease in the content of p21 and Bax was also observed at 24 h. The intensity of the bands was determined by densitometric analysis of at least three independent experiments. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control; $^{***}P < 0.001$ versus control.

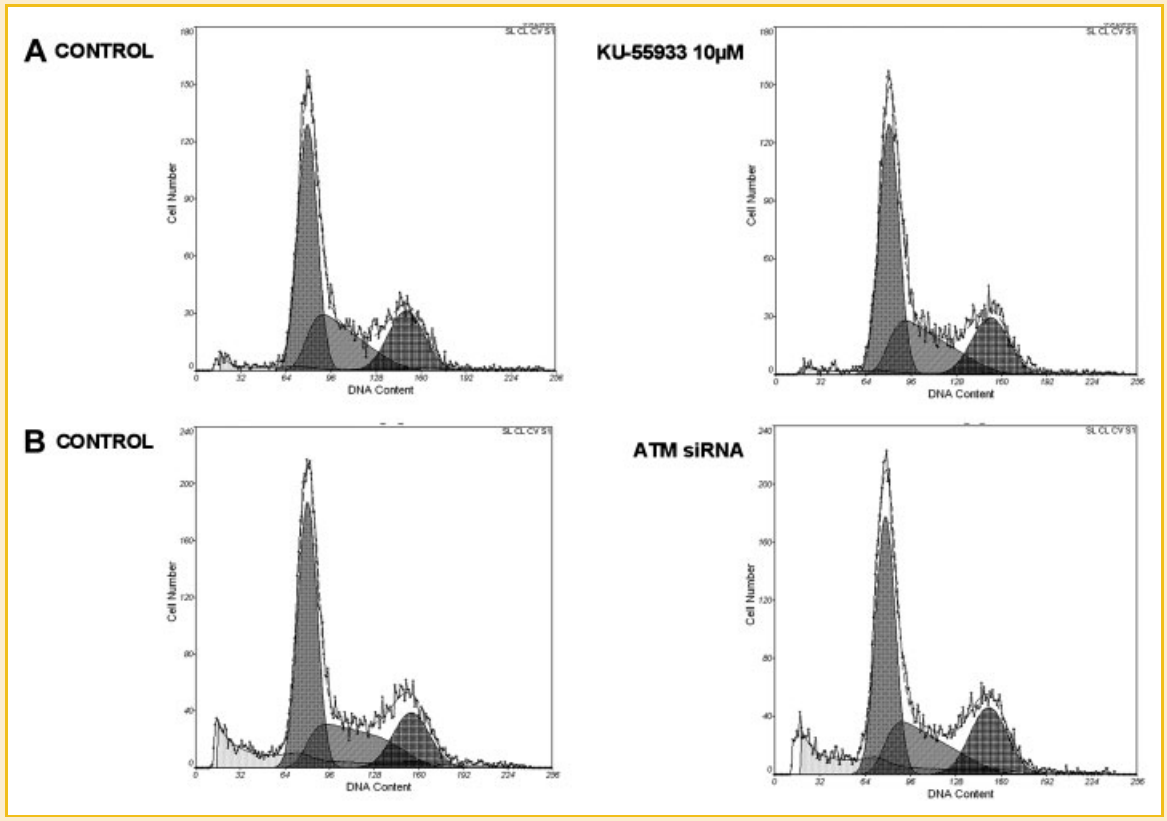

Fig. 3. Our data showed that neither KU-55933 nor siRNA caused a significant increase in the fraction of apoptotic cells, as shown by flow cytometry analysis. A: Cells exposed to KU-55933; (B) Cells exposed to siRNA.

EFFECTS OF KU-55933 AND GENE SILENCING ON THE ATM/P53 PATHWAY

Following the inhibition of ATM through pharmacological and knockdown strategies, we studied the expression levels of p53, which is a direct substrate of ATM, and p21, which is a downstream target of p53 that is involved in cell-cycle arrest, in order to evaluate the effects of ATM on the G1-S checkpoint. We found a significant decrease in p53 phosphorylated at Ser15 and in p21 expression after 24 h of ATM inhibition, either when we treated B65 cells with KU-55933 or when they were cotransfected with ATM siRNA (Fig. 2). It is well known that p53 activates the expression of genes related to death, among them Bax. Therefore, we also studied the expression of the proapoptotic protein Bax after ATM inhibition and found that its expression was also down-regulated (Fig. 2).

EFFECTS OF KU-55933 AND GENE SILENCING ON B65 CELL-CYCLE CONTROL

Previous investigations showed that the pharmacological ATM inhibitor KU-55933 alone did not affect cell-cycle proteins. However, it increased apoptosis when it was administered with antitumor drugs. Therefore, our data showed that neither KU-55933 nor siRNA alone caused a significant increase in the apoptotic fraction of cells, as shown by the flow cytometry analysis (Fig. 3; Table I). To assess whether inhibition of ATM affected the proliferation of B65 neuroblastoma cells, we examined the effects of KU-55933 10 WM and siRNA targeting ATM by flow cytometry after 24 h of treatment. As shown in Table II, the inhibition of ATM did not significantly change the fraction of cells in each phase of the cell cycle. However, Western blot analysis showed a significant decrease in the expression of pRb phosphorylated at Ser780 when B65 neuroblastoma cells were treated with either siRNA or KU-55933 (Fig. 4). pRb restricts cell-cycle progression, plays a key role in the control of proliferation and regulates the activity of a family of transcription factors known as E2F. Moreover, both pharmacological or ATM siRNA inhibitions significantly decreased the expression levels of cyclin A (an S phase marker) after 24 h. This supports our hypothesis that pRb is a target of ATM in proliferating cells. Cells treated with KU-55933 also showed a decrease in cyclin D1, which is the protein involved in the G0/G1 cell-cycle phase (Fig. 5). This result was unexpected.

TABLE I. Apoptosis Was Evaluated by DNA Fragmentation by Flow Cytometry at 24 h of Exposure to siRNA Targeting ATM and KU-55033

Treatment	Apoptosis (%)
Control	6.35 ± 1.37
KU-55933 10 μM	7.00 ± 1.09
Control	2.97 ± 0.23
siRNA ATM	2.36 ± 0.45

TABLE II. Cell-Cycle Analysis of B65 Cells at 24 h of Exposure to KU-55933 10 μM and After Transfection With siRNA for ATM

Cell-cycle phase/treatment	G1	S	G2/M
Control	50.11 ± 2.94	30.27 ± 1.33	19.61 ± 2.80
KU-55933 10 μM	46.29 ± 0.85	31.51 ± 2.49	22.19 ± 1.72
Control	50.83 ± 0.71	30.12 ± 0.51	19.05 ± 0.73
siRNA ATM	50.87 ± 0.65	30.94 ± 0.39	18.12 ± 0.82

EFFECTS OF H_2O_2 ON Rb PHOSPHORYLATION STATUS

As our data indicated that pRb could be a substrate of ATM, the next experiments were designed to evaluate the role of DSBs on pRb phosphorylation. In previous studies we have demonstrated that H_2O_2-induced DSBs with an activation of ATM pathway [Pizarro et al., 2009]. Compared with the low basal signal, H_2O_2 induced a significant increase in the expression of pRb phosphorylated at Ser780, which was maintained until 6 h of treatment, as shown in Western blot experiments (Fig. 6). ATM inhibitor KU-55933 was able to reverse the increase in the content of the phosphorylated form of both p53 and pRb, and also cells treated with siRNA showed a significant decrease in phosphorylated form of pRb which supports our hypothesis that ATM is involved in the regulation of pRb phosphorylation (Fig. 6).

ATM IS INVOLVED IN pRb AT Ser780 PHOSPHORYLATION

To further investigate the role of ATM on the regulation of pRb phosphorylation at ser780, we determined if pRb was phosphorylated directly or indirectly by ATM. For this purpose, we studied the pRb amino acid sequence surrounding the target phosphorylation site ser780 and were compared with consensus sequence motif of ATM phosphorylation. As shown in Figure 7A, the pRb amino acid in position +1 is Pro (P) whereas phospho-serine is followed by Gln (Q) in the consensus sequence motif of ATM phosphorylation. The sequence S/TQ is a minimal essential requirement for ATM kinase [Kim et al., 2005]. This observation confirmed that phospho-pRb ser780 was not phosphorylated directly by ATM.

Immunoprecipitation experiments were performed to determine if ATM and pRb ser780 coimmunoprecipitated together. Thus, we immunoprecipitated pRbser780 from cell lysates and as shown in Figure 7B, ATM does not coimmunoprecipitated with phospho-pRb ser780. This result show that ATM and pRb are not interacting.

DISCUSSION

The most important finding of the present study is the evidence of a new intracellular molecular network of pRb phosphorylation by ATM. Our hypothesis is supported by the fact that pharmacological and knockdown inhibition of ATM significantly and specifically inhibited the expression of cyclin A and of pRb phosphorylated at Ser780.

The process of pRb phosphorylation and cell cycle re-entry is mediated by the canonical, well-known pathway that is regulated by Cdk4/cyclinD and Cdk2/cyclinE [Baus et al., 2003; Kastan and Bartek, 2004]. However, new data support the involvement of additional proteins in the control of this process, such as calpains, GSK3b, and p38 [Hamdane and Buee, 2003; Camins et al., 2006; Yeste-Velasco et al., 2009]. In addition, recent studies have suggested that pRb could be directly phosphorylated by Cdk5 and

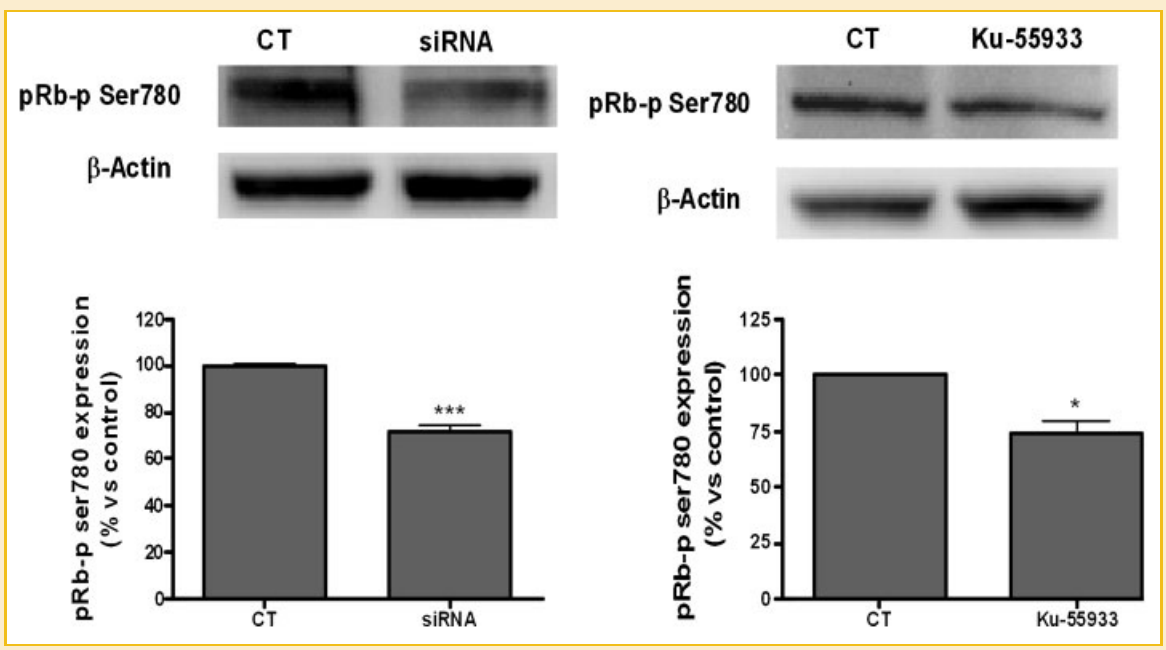

Fig. 4. Western blot of pRb phosphorylated at ser780 from cells exposed for 24 h to siRNA targeting ATM and to KU-55933 10 μM. Both treatments caused a significant decrease in the content of the phosphorylated form of the pRb protein at 24 h. The intensity of the bands was determined by densitometric analysis of at least three independent experiments. $^{*}P < 0.05$ versus control; $^{***}P < 0.001$ versus control.

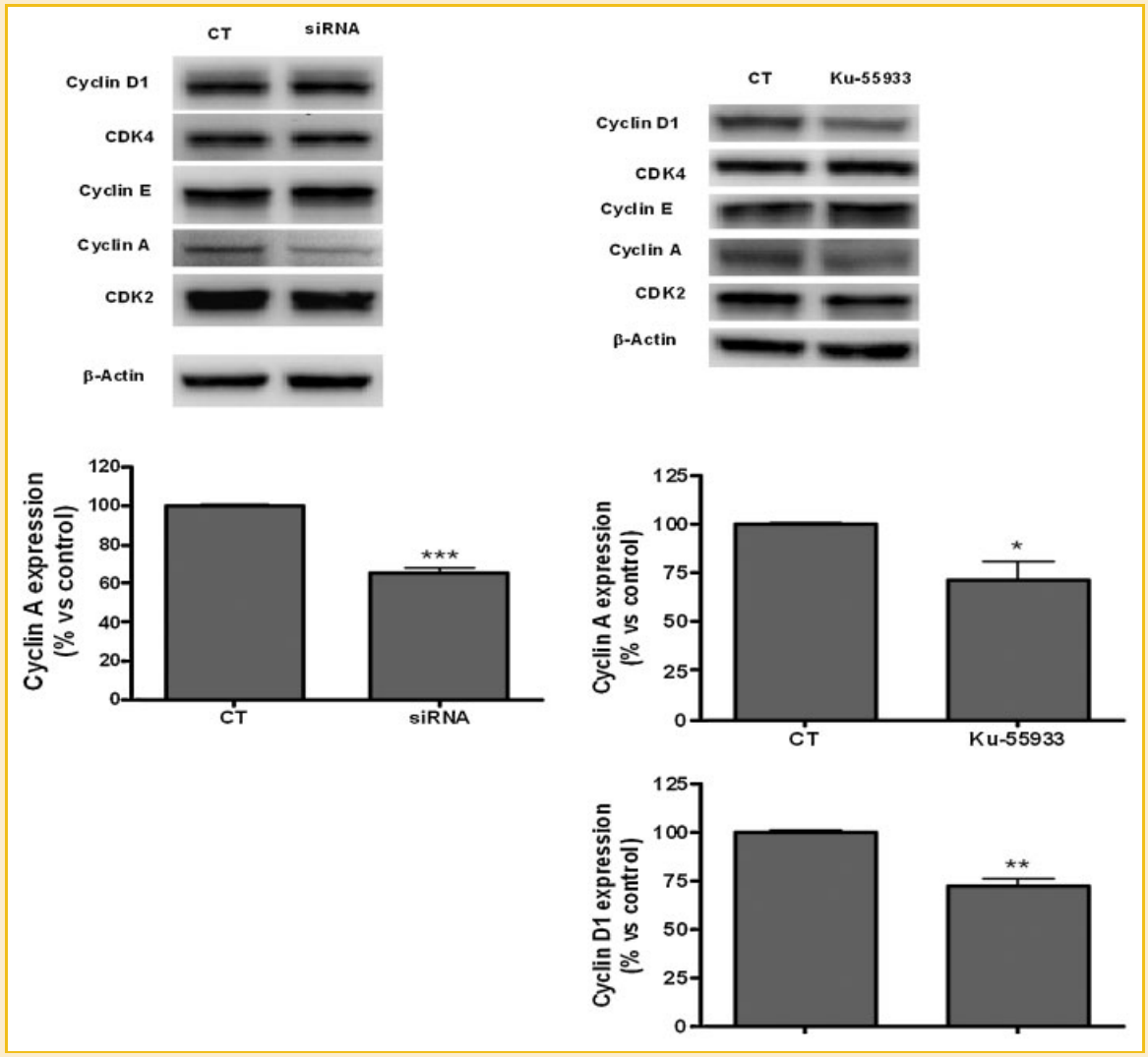

Fig. 5. Western blot of cyclin D1, cdk4, cyclin A, cyclin E, and cdk2 from B65 cells exposed for 24 h to siRNA targeting ATM and to KU-55933 10 μM. A significant decrease in the content of cyclin A was observed in both treatments, and in cyclin D1 in cells treated with KU-55933. We only show bar graphs that indicate significant expression changes. The intensity of the bands was determined by densitometry analysis of at least three independent experiments. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control; $^{***}P < 0.001$ versus control.

GSK3β [Hamdane and Buee, 2003]. In the present manuscript, we propose that a new enzyme/mechanism is involved in pRb regulation. Our hypothesis is based on the inhibitory effect of either the pharmacological ATM inhibitor KU-55933 or siRNA on pRb phosphorylation.

Furthermore, when ATM was targeted with both KU-55933 and siRNA ATM, a significant reduction in the phosphorylated form of p53 at Ser15 was found. This observation agrees with the well-documented involvement of ATM in the control of p53 activity [CRescenzi et al., 2008; Culmsee and Mattson, 2005; Nair 2006]. Upon phosphorylation by ATM, p53 becomes stabilized and triggers the activation of pro-apoptotic genes, including Bax [Gilman et al., 2003; Keramaris et al., 2003; Aleyasin et al., 2004; Otsuka et al., 2004]. In fact, the results of our experiments indicate that Bax activity is regulated by ATM, since inhibition of ATM caused a significant decrease in Bax content. When ATM/ATR signaling was blocked with KU-55933, it induced senescent breast, lung, and colon carcinoma cells to undergo cell death [Hickson et al., 2004]. The potential mechanism of action of this effect is via p21 (CIP1), which is known to act downstream of ATM. Our data shown that p21 levels declined after ATM inhibition. This finding could be related to the evidence that B65 cell-cycle progression was not affected by exposure to KU-55933 or siRNA targeting ATM. p21 (Cip1) has been shown to exert a key role in driving cells to the G2 exit, by inhibiting both cyclin B1/Cdk1 and cyclin A/Cdk1/2 complexes, which are involved in G2/M control progression, and by blocking the phosphorylation of pRb family proteins [Crescenzi et al., 2008]. In our experimental conditions, the decrease in the content of p21 suggests that this molecule is not involved in pRb phosphorylation. Although ATM inhibition has been proposed as a potential antitumor treatment, it has not yet been demonstrated that KU-55933 can induce apoptosis in neuroblastoma cells. Results from our

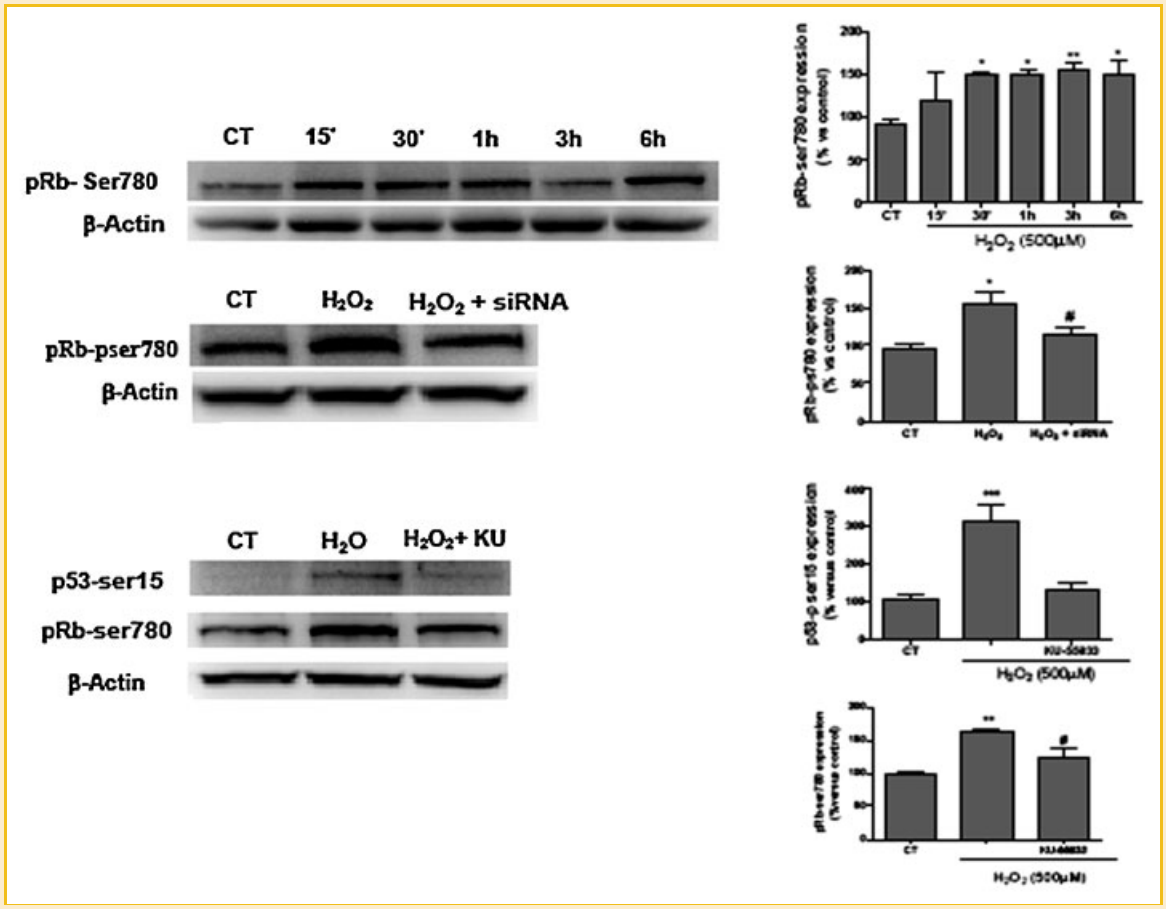

Fig. 6. Representative Western blot of time course of pRb phosphorylated at ser780 of B65 cells exposed to H_2O_2 500 WM for 15 and 30 min, 1, 3, and 6 h. Western blot of p53-p ser 15 and pRb ser780 for cells exposed to H_2O_2 500 μM for 3 h. The figure shows that the increased content of the phosphorylated form of both p53 and pRb was reversed by exposure to the ATM inhibitor KU-55933 and siRNA targeting ATM in the case of pRb. The intensity of the bands was determined by densitometry analysis 21 of at least three independent experiments. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ versus H_2O_2; $^{\#\#}P < 0.01$ versus H_2O_2.

experiments show that neither KU-55933 nor siRNA targeting ATM caused significant increases in the number of apoptotic cells at the concentrations used in the experiments. However, these results are in the line with previous studies in which, instead of killing tumor cells, KU-55933 sensitizes them to damage with a tumor drug, which then leads to apoptosis [Hickson et al., 2004].

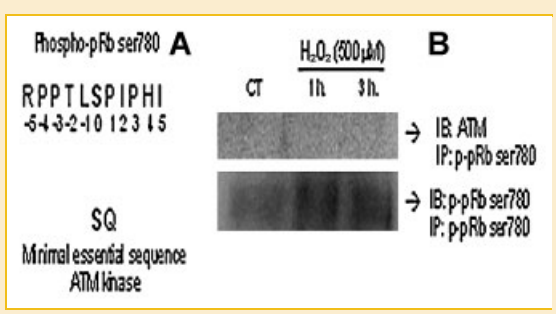

Fig. 7. A: pRb amino acid sequence surrounding the target phosphorylation site ser780 compared with minimal essential sequence requirement (SQ) for ATM kinase. The pRb amino acid in position +1 is Pro (P) whereas Gln is required to follow phospho-serine for ATM kinase recognition. B: Coimmunoprecipitation experiments show that ATM and phospho-pRb ser780 are not interacting although treating the cells with H_2O_2 500 μM.

The exposure of B65 cells to KU-55933 and siRNA did not significantly change the content of the proteins involved in the cell cycle. However, the results obtained by Western blot showed that either pharmacological inhibition or ATM siRNA decreased the expression of cyclin A. KU-55933 also induced a reduction in cyclin D1 expression. This data are probably due to the fact that while siRNA specifically targets and inhibits ATM, the addition of KU-55933 to ATM could affect others members of the family of phophatidylinositol-3 kinases that are involved in the control of the cell cycle, and specifically cyclin D [Liang and Slingerland, 2003; Kim et al., 2005]. Likewise, the key finding of the present research is the evidence that ATM exerts a role in the control of cell-cycle machinery through regulation of pRb phosphorylation. In previous studies we have demonstrated that H_2O_2 induced the activation of ATM pathway [Pizarro et al., 2009]. To confirm these findings, experiments on B65 cells with H_2O_2 were undertaken to evaluate whether DSBs of DNA acts on pRb. Thus, results from our experiments showed that damage to DNA increased the phosphorylated form of pRb, which could be reversed by KU-55933 and siRNA

targeting ATM. However, it still remains to be investigated whether phosphorylation of pRb by ATM is a common mechanism in other cell models, since ATM is widely expressed in all cell types. We suggest that the ATM/pRb pathway may be activated following DNA damage to various tissue types and regulation of ATM may have a role in the cellular response to a broad spectrum of signals.

We observed that pRb is not phosphorylated at ser780 directly by ATM, and we also determined that both proteins are not interacting. These results suggest that another kinase that functions downstream from ATM can phosphorylate pRb at ser780. Recently, Inoue et al. [2007] reported that pRb can be phosphorylated at ser612 by Chk1/2 after DNA damage. Thus, the implication of checkpoint effector kinases (Chk1/2), which are substrates of ATM, on pRb phosphorylation should be investigated. Likewise, consistent with this, ATM substrates are predominantly responsible of foci formation whereas cell-cycle checkpoint depends on Chk substrates [Inoue et al., 2007].

Along with these data, the present results lead us to conclude that ATM participates in cell-cycle checkpoint control by modulating the phosphorylation of pRb.

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