Deficiency in the Catalytic Subunit of DNA-Dependent Protein Kinase Causes Down-Regulation of ATM

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Abstract
Previous reports have suggested a connection between reduced levels of the catalytic subunit of DNA-dependent protein kinases (DNA-PKcs), a component of the nonhomologous DNA double-strand breaks end-joining system, and a reduction in ATM. We studied this possible connection in other DNA-PKcs–deficient cell types, and following knockdown of DNA-PKcs with small interfering RNA, Chinese hamster ovary V3 cells, lacking DNA-PKcs, had reduced levels of ATM and hSMG-1, but both were restored after transfection with PRKDC. ATM levels were also reduced in murine scid cells. Reduction of ATM in a human glioma cell line lacking DNA-PKcs was accompanied by defective signaling through downstream substrates, post-irradiation. A large reduction of DNA-PKcs was achieved in normal human fibroblasts after transfection with two DNA-PKcs small interfering RNA. Within hours after transfection, a decline in PRKDC mRNA was seen, followed by a more gradual decline in DNA-PKcs protein beginning 1 day after transfection. No change in ATM mRNA was observed for 2 days post-transfection. Only after the DNA-PKcs reduction occurred was a reduction in ATM mRNA observed, beginning 2 days post-transfection. The amount of ATM began to decline, starting about 3 days post-treatment, then it declined to levels comparable to DNA-PKcs. Both proteins returned to normal levels at later times. These data illustrate a potentially important cross-regulation between the nonhomologous end-joining system for rejoining of DNA double-strand breaks and the ATM-dependent damage response network of pathways, both of which operate to maintain the integrity of the genome. (Cancer Res 2005; 65(5): 1670-7)

Introduction
DNA double-strand breaks, which occur under normal circumstances to initiate essential processes such as V(D)J and meiotic recombination, are efficiently rejoined (1). Breaks also occur spontaneously, or as a result of exposure to ionizing radiation and certain chemical agents (2, 3). Failure to process these correctly can be lethal or mutagenic. In most higher eukaryotic cells, error-prone nonhomologous end-joining (NHEJ) is one of the principal rejoining processes (4). This is facilitated by DNA-dependent protein kinases, comprised of a catalytic subunit (DNA-PKcs), and the end-binding Ku70/Ku86 subunits (5). These proteins are also associated with telomeres; the normal double-stranded ends of chromosomes (6). Homology-directed repair (HDR), a slower error-free process that operates primarily during S and G2 phase, can also repair double-strand breaks in DNA (7–9). It is an especially favored double-strand break repair system for lower eukaryotes and certain highly recombinogenic vertebrates (8). There is also ample evidence that one or more other end-joining systems may operate. Knocking out key components of both the NHEJ and HDR systems described above does not, by any means, completely eliminate rejoining of radiation-induced DNA double-strand breaks (10). Little is known concerning the extent to which these pathways overlap.

ATM, the gene defective in ataxia-telangiectasia (A-T), plays a central role in signaling double-strand breaks to cell cycle checkpoints but is also implicated in break repair (11–15). Early cytogenetic data revealed both constitutive chromosomal instability and elevated levels of radiation-induced chromosome breakage in A-T cells (14). Several reports have provided evidence for the persistence of residual breaks in DNA (13–15) as well as excess mis-rejoined interphase chromosome fragments long after post-irradiation in cultured noncycling G0 A-T cells (12). In the latter case, because G0 cells have little or no Rad51 (16, 17), and the same levels of residual and mis-rejoined breaks occur in the first post-irradiation mitosis after subculture as were present in the G0 cells (18), HDR or cell cycle checkpoint defects could not account for the chromosomal hypersensitivity. Recently, sensitive assays quantitatively reflecting levels of DNA breaks produced, as well as their rejoining, has led to the demonstration of defect in noncycling G0 A-T cells (13). Distinctly different rejoining kinetics and residual break levels occur in A-T relative to NHEJ-defective cells and of course both differ with respect to normal cells (13). These results are consistent with the cytogenetic data, and further point to the possibility that ATM plays a role in double-strand break rejoining unrelated to its known connection to HDR, and perhaps involving alternative end-joining systems or unknown interactions with the known NHEJ system. Although ATM phosphorylates a host of substrates, very few of those studied to date are linked to the various manifestations of hyper-radiosensitivity associated with A-T. Recent data shows that ATM phosphorylates the structural maintenance of chromosomes protein 1 (SMC1) at two sites and mutations at these sites sensitize cells to radiation (19). SMC1 is part of the RC-1 complex, which promotes recombination; therefore, this radiosensitization is likely an HDR-related effect associated with an alteration of the response of S and G2 cells in the asynchronous HeLa cells studied (19).
Normal levels of DNA-PKcs have been reported in A-T cells where loss of ATM function occurs (20). However, a human glioma cell line, MO59J, which is characterized by hypersensitivity to radiation and lack of DNA-PKcs protein, has extremely low levels of ATM protein (21). Reverse transcription-PCR failed to detect a difference in ATM mRNA in MO59J and a related cell line with normal DNA-PKcs. Complementation of MO59J cells with a fragment of chromosome 8 containing the PRKDC gene restored levels of DNA-PKcs, reduced the sensitivity to radiation, and partially restored ATM levels (22). Subsequent studies, however, revealed that MO59J cells possessed two different aberrant ATM transcripts, predicted to cause premature truncations of the ATM protein (23). There are, in fact, many differences in the genetically unstable MO59J tumor cell lines relative to its sister cell line, MO59K, derived from the same human tumor, and further differences relative to normal cells. Although no PRKDC mutants other than the MO59J line are available for humans, there are several Prkd mutant rodent cell lines (24–32). Rodent cells normally express DNA-PKcs at levels that are only about 2% to 4% of that for human cells, although there are large differences in expression levels among cells from different tissues (31). The ideal approach for examining a possible cause and effect relationship between a DNA-PKcs deficiency and reduced levels of ATM would require the comparison of ATM levels in normal cells and in PRKDC mutant cells with an otherwise identical genetic background, but no such comparisons have been made.

This issue was addressed in the present study using radiosensitive V3 Chinese hamster ovary (CHO) cells deficient in DNA-PKcs and V3 cells transfected with full-length PRKDC cDNA (32, 33). We further determined the expression of ATM in mouse cells showing different levels of deficiency of DNA-PKcs, and measured radiation-induced ATM signaling in human MO59J cells, already shown to be deficient in ATM. We report here the universality of the observation that DNA-PKcs deficiency is accompanied by a decrease in ATM protein and this is reflected in deficient ATM signaling post-irradiation. Under those conditions, another member of the phosphoinositide 3-kinase related family, hSMG-1 (34), which resembles ATM both in terms of substrate specificity and sensitivity to wortmannin, was also reduced in amount. Finally, using low passage normal human fibroblasts, we established a causal relationship between deficiency in DNA-PKcs and reduced ATM following targeted specific degradation of PRKDC mRNA using small interfering RNA (siRNA). We show that reduction of DNA-PKcs by siRNA precedes a drop in ATM protein levels, and that this is also reflected in a decrease in PRKDC and ATM mRNAs. These data point to a potentially important cross-regulation between the NHEJ system for rejoining of DNA double-strand breaks and the ATM-dependent damage response network of pathways.

Materials and Methods

Cells. The cultured low passage normal human fibroblasts, GM08399, used for the siRNA knockdown of PRKDC gene products, were obtained from the National Institute of General Medical Sciences Coriell Cell Repositories. These were grown as described previously (35). For studies involving expression of ATM, we used L3 and C3 ABR lymphoblastoid cells for negative and positive controls, respectively (36). The MO59J and MO59K human tumor cell lines were kindly supplied by Dr. Allalunis-Turner (Cross Cancer Institute, Alberta, Canada) and grown as previously described (37).

CHO V3 cells are a radiosensitive mutant in which DNA-PKcs protein is absent (32). V3+ cells have wild-type radiosensitivity restored by stably transfecting cells with a plasmid containing and expressing wild-type human DNA-PKcs. The V3 cells used in this study as controls were V3 cells with the same vector but did not contain the DNA-PKcs gene (38).

siRNAs. The siRNAs used were described previously (35). One (sequence A) targeted a sequence 5′-GAUGGACUCUACUCUGUU-3′ in PRKDC mRNA 293 bases downstream from the start codon, and the other (sequence B) targeted the sequence 5′-CUUAGGUGGCGCAUGGAG-3′ in the kinase domain. The control siRNA sequence, 5′-GAAGGACUCUACUGUU-3′ was similar to sequence A above, but with a U-to-A substitution at position 3 from the 5′-end and an AC-to-CA substitution in positions 12 and 13 from the 5′-end. All the siRNAs were synthesized with 3′dTdT overhangs on both strands.

Transfection. Transfections with siRNA, facilitated with oligofectamine, were carried out one day after inoculating 10⁶ GM08399 human fibroblasts into a six-well polystyrene culture dish. Two days later, a second transfection was carried out. The protocol was very similar to that described previously (35), but the concentration of siRNA was 16 nmol/L.

Immunoblotting. Levels of the proteins of interest in cells relative to those in control cells were measured by immunoblot analysis as previously described (35, 36). For GM08399 human fibroblast cells, DNA-PKcs was detected by mouse antibody Ab4 (Neomarkers, Fremont, CA) and ATM was detected with rabbit antibody NB 100-104 (Novus, Littleton, CO). For detection of ATM in CHO cells, the ATM monoclonal antibody 2C1 (Genetex, San Antonio, TX) was employed, DNA-PKcs was detected with a monoclonal antibody (Oncogene Science, Boston, MA) and hSMG-1 was detected with a polyclonal antibody (ATX-1) raised in sheep. A polyclonal antibody raised in rabbits was used to detect phosphorylation in human cells and ser18 phosphorylation in murine cells. Secondary detection of these was done with horseradish peroxidase-labeled goat anti-mouse, goat anti-rabbit, or goat anti-sheep antibodies. Ku80, which had previously been shown to be unaffected in changes in DNA-PKcs, or β-actin, were used for the loading controls for the siRNA knockdown experiments. The Ku80 antibody was mouse anti-Ku80 Ab-2 (Neo-markers/Lab Vision) and the β-actin antibody was mouse anti-β-actin (NB 600-501; Novus). The horseradish peroxidase-labeled secondary antibodies were goat anti-mouse (Amersham Biosciences, Piscataway, N.J.). To accurately quantify the time course of changes in DNA-PKcs or ATM after siRNA knockdown of DNA-PKcs, enhanced chemiluminescence reagent was used for X-ray film detection and densitometry or Enhanced Chemiluminescence Plus reagent for the image analysis detection system (Storm Scanner System, Amersham Biosciences).

Immune Complex Protein Kinase Assay. ATM kinase assays were done as described (39). Briefly, cells were lysed and centrifuged at 13,000 × g for 10 minutes. Precleared cell lysate (1 mg) was used for immunoprecipitation with ATM antibody. The immune complex was absorbed onto protein G-Sepharose and washed. The immunoprecipitates were resuspended in kinase buffer and ATM kinase assays were carried out for the substrate phosphorylation of Chk2. The reaction products were separated by SDS-PAGE.

Immunocytochemistry. For immunocytochemistry, cells were grown and transfected on two-chamber slides (Nunc, Rochester, NY) as described previously (35). After fixation and permeabilization, the cells were incubated in 3% bovine serum albumin in PBS containing mouse anti-DNA-PKcs Ab-4 and rabbit anti-ATM polyclonal antibody (Novus). The primary antibodies were then detected with rhodamine-labeled goat anti-mouse for DNA-PKcs and FITC-labeled goat anti-rabbit for ATM. Slides were counterstained with 4',6-diamidino-2-phenylindole in antifade, mounted with a cover slip, and were viewed under an Olympus Provis fluorescent microscope.
Real-time Reverse Transcription-PCR. RNA was extracted from cells and aliquots of total RNA were reversetranscribed to first-strand cDNA. The forward and reverse primers used to detect PRKDC cDNA were 5'-ACAGGACATCTGACAACTTTATTTGTG-3' and 5'-AAAGGCACTAACTCACTGAGACT-3'. The TaqMan probe, 5'-CCATGGAGCTGGCCCGTGTATG-3', was labeled with the fluorescent dye FAM on the 5' end and quencher BHQ1 on the 3' end. For ATM cDNA, the primers were 5'-AGGCTGTCTCCAT-TACTGATAGATC-3' and 5'-TCCGTAAGGCATGTCAACACAATA-3'. The TaqMan probe, 5'-CAGCTTCTACCCAAACAGCGCATGG-3', was labeled with fluorescent dye and quencher as above. The forward and reverse primers used for an internal reference control to detect α-tubulin cDNA were 5'-GCAAATCCTGTGACCA-3' and 5'-CCTAAATCCTAAGGACGACGACT-3'. The TaqMan probe, 5'-CACCGGTTCTACGGGCTCTTGTTG-3', was labeled with fluorescent dye FAM on the 5' end and quencher TAMRA on the 3' end. Five replicate sample tubes were included for each determination. The PCR reaction was carried out at 95°C for 2 minutes and at 95°C for 10 minutes to activate the AmpliTaq Gold Polymerase, followed by 50 cycles at 95°C for 15 seconds, and at 60°C for 1 minute using an iCycler iQ real-time PCR detection system. The relative DNA-PKcs and ATM mRNA levels were calculated by normalizing mRNA level of the x-tubulin and PCR efficiencies for each individual sample. Data were processed as described by Muller et al. (40). To measure the level of ATM mRNA in the V3 cells and the rate of mRNA decay, actinomycin D was added to a final concentration of 2 μg/mL. ATM mRNA was then measured using real-time PCR and the level normalized to β-actin.

ATM Promoter Activity. The activity of the ATM promoter in the V3 and M059 cells was measured using the ATM promoter luciferase reporter construct as described by Queven et al. (41). Briefly, subconfluent cells were transfected with 1 μg of the reporter construct. To standardize transfection efficiencies, 0.2 μg of Renilla-luciferase plasmid pRL-cytomegalovirus (picomega) was also cotransfected. After 48 hours, the cells were lysed and the luciferase activity determined.

Results

Cells Deficient in DNA-PKcs Have Reduced ATM Protein. Previous results have shown that the absence of DNA-PKcs in a human glioma cell line M059J is accompanied by reduced levels of ATM (21). The present study aimed to determine whether this was a specific feature of this particular cancer cell line, or whether it represented a more general and causal relationship between DNA-PKcs and ATM. Immunoblotting of extracts from a hamster cell line that is radiosensitive (V3) confirmed the presence of low levels of DNA-PKcs relative to V3+ cells corrected with PRKDC cDNA (Fig. 1A). The level of ATM was also lower in the V3 cell line compared with V3+ cells. These results again show a concordance between the level of ATM and the status of DNA-PKcs. We compared the levels of ATM protein in mouse cells exhibiting different degrees of DNA-PKcs deficiency. Very low levels of ATM were observed in scid mice (negative for DNA-PKcs) and these did not change after radiation exposure (Fig. 1B). ATM protein was readily detectable in both C57BL/6 and BALB/c mice. (Fig. 1B). In vivo activation of ATM, determined by ser18 phosphorylation of p53, failed to detect activity for the scid protein, in keeping with reduced ATM, but there was no detectable difference in the activity following 10 Gy irradiation seen in BALB/c and C57BL/6. These data were confirmed by immunoprecipitating ATM from scid, BALB/c, and C57BL/6 splenocytes and measuring kinase activity with p531-44 as a substrate. Again, this revealed no activity in scid but this time C57BL/6 had greater activity than BALB/c 1 hour after 10 Gy irradiation (Fig. 1B, bottom). It is of interest that another member of the PI-3 kinase family, hSMG-1 (34), is present at negligible levels in V3 cells, but is restored to normal levels in PRKDC-corrected V3+ cells (Fig. 1A). This was also the case for cells from scid relative to C57BL/6 mice and to a somewhat lesser extent in the human glioma cells (Fig. 1A).

We also determined the effect of reduced levels of ATM in M059J cells on radiation-induced signaling. Results of these experiments are summarized in Fig. 2, where levels of the various proteins and their phosphorylated products in M059J and M059K cells are shown initially and at various times after 10 Gy irradiation. As described previously (21, 42), DNA-PKcs was not detected in these cells and ATM was reduced compared with the M059K cell line, derived from the same tumor, which is proficient in DNA-PKcs. The reduction in ATM was accompanied by a marked reduction in ATM activation, post-irradiation, as determined by autophosphorylation on ser184. In keeping with these results, radiation-induced, ATM-dependent phosphorylation of SMC1 was delayed in M059J cells. There also seemed to be some reduction in radiation-induced phosphorylation of p53 on ser15, but this was attenuated by the lower levels of p53 protein in M059J relative to M059K cells.

To further test for a causal relationship between DNA-PKcs deficiency and ATM levels, we used RNA interference to knock down DNA-PKcs so that ATM levels could be compared in cells of identical genetic backgrounds. Here we used low passage cultured normal human fibroblasts and used the siRNA approach involving transient transfections with double-stranded oligonucleotides to better measure the time course of changes.

To verify that specific siRNA targeting of DNA-PKcs mRNA was achieved, we adhered to suggested control criteria for studies involving the use of RNA interference (43). Figure 3A shows a...
Western blot of samples for measurement of both DNA-PKcs and ATM proteins 5 days after the start of transfections of low passage normal human fibroblast cultures with two PRKDC siRNA sequences (lane 3), a control siRNA sequence (lane 4), and a control sample from untransfected cells (lane 5). The PRKDC siRNA transfection specifically reduced the levels of both DNA-PKcs and ATM in the sample taken 5 days after transfection. Lanes 1 and 2 are controls from two different untransfected lymphoblastoid cell lines, one from a patient with A-T (L3 ATM-deficient, lane 1), and the other from a normal individual (C3 ABR, lane 2). β-Actin served as a loading control, and because we have previously shown that Ku80 is unaffected by DNA-PKcs knockdown, the latter also served the same purpose. Both of the PRKDC-targeted siRNA sequences used here were effective in knocking down DNA-PKcs, although both used together were slightly more effective, and were used for the remainder of experiments relating to effects on ATM. Regarding the criteria for specificity of RNA interference knockdown referred to above (43), (a) the siRNAs we used were effective in the nanomolar concentration range, (b) the levels of other proteins measured were not affected, (c) no evidence for induction of an IFN response was seen, though such a response (PKR phosphorylation) could be induced in these cells by high concentrations of copolymers of polynucleosides and polycytidylic acids, (d) slightly altered siRNA control sequences were totally ineffective in altering levels of any of the proteins measured, and (e) human Genbank searches for the oligonucleotide siRNA sequences did not return any matches except for the PRKDC target, and the closest match to the ATM mRNA involved a sequence with a 13-bp mismatch. Furthermore, the time course experiment shown in Fig. 4 is inconsistent with an off-target siRNA or mRNA effect on ATM.

To further show concordance between reduced DNA-PKcs and ATM, we used immunocytochemistry to measure ATM and DNA-PKcs simultaneously in individual cells 5 days after transfection. With PRKDC siRNA, knockdown of both DNA-PKcs and ATM was observed in the majority of cells (Fig. 3B, top). Two cells, however, seem to be unaffected for both DNA-PKcs and ATM. In our experience, it is typical that some cells in a culture do not become transfected by the procedure, so it is not surprising that Western blots can never show 100% knockdown. The fact that a few cells were not successfully transfected was advantageous here, because a 1:1 correspondence between DNA-PKcs depletion and a reduced level of ATM was then apparent.

The kinetics of the reduction of DNA-PKcs and ATM were also investigated. The results in Fig. 4A show that the level of DNA-PKcs was not greatly changed at day 1 after transfection but had dropped to 40% of that for untreated cells by day 2 and fell to ~15% to 20% of that for untreated cells from days 2 to 5. There was no change in ATM protein up to 3 days post-transfection, but by day 4, a small drop was evident (70-80% of untreated) and ATM protein was reduced to nearly the same relative extent as DNA-PKcs by days 5 to 6 (Fig. 4A). We also examined the kinetics of degradation of PRKDC mRNA (the target of the siRNA) as well as the ATM mRNA (Fig. 4B). PRKDC mRNA was markedly reduced at the earliest sampling time, 4 hours after transfection, and to ~35% of that for untreated cells within the first day. After a second transfection at day 2, a further decrease in PRKDC mRNA occurred, and reached a plateau level ~20% relative to controls between days 3 and 4. These data are consistent with the kinetics of reduction of DNA-PKcs protein which were delayed compared with mRNA loss. No change in ATM mRNA was seen up to 2 days post-transfection, and only after DNA-PKcs protein had decreased did ATM mRNA decrease to 25% of untreated between days 3 and 5.
on the ATM to samples collected from two independent experiments. Although the PRKDC measurements of levels, also as a function of time after the start of the PRKDC B, with exposures adjusted to lie in the linear range of exposure versus density.

In the independent experiment where quantification was by film densitometry to pretransfection levels. Error bars for the estimates shown in (A), protein level ( ), had already dropped. After about 7 days, the levels returned to pretransfection levels. It is noteworthy that the percentage increase noted above are quantities relative to the whole cultures. Only over the following 2 days does it begin to decrease. After 6 days, the levels of mRNAs and proteins begin to return to 40% of untreated, whereas the level of ATM protein is unchanged. Only over the following 2 days does it begin to decrease. After 6 days, the levels of mRNAs and proteins begin to return to 40% of untreated, whereas the level of ATM protein is unchanged. Only over the following 2 days does it begin to decrease. After 6 days, the levels of mRNAs and proteins begin to return to pretransfection levels. It is noteworthy that the percentage increases noted above are quantities relative to the whole cultures. These are likely to be minimum relative reductions because the immunocytochemistry results (e.g., Fig. 3) show that perhaps 10% to 20% cells in the cultures remain untransfected after the procedure and only the remaining 80% to 90% of the cell populations are subject to any reduction in DNA-PKcs or ATM following siRNA treatment.

It is also of interest that in the V3 CHO and scid cells there was ~50% less ATM mRNA compared with the corresponding controls but this did not match the extent of reduction in ATM protein (Fig. 5A). The results obtained with siRNA for DNA-PKcs, where reduced levels of DNA-PKcs protein lead to a dramatic decrease in the level of ATM mRNA, suggest that DNA-PKcs may have a role in controlling the transcription of ATM mRNA or in maintaining its stability. To address this, we blocked de novo transcription with actinomycin D and showed that the rate of loss of ATM mRNA was similar in both V3 and V3+ cells, indicating that DNA-PKcs was not controlling the stability of mRNA (Fig. 5B). Because DNA-PK has been shown to phosphorylate and alter the function of different transcription factors (44), it was possible that it controlled ATM expression at this level. The results in Fig. 5C using an ATM promoter-luciferase construct reveal that reporter activity is similar for both V3 and V3+ cells. This was also the case for M059J and K cells, showing that any such effect was not evident when a minimal ATM promoter was employed.

Discussion

The recognition, repair, and signaling of double-strand breaks in DNA represents a series of coordinated processes essential for maintaining the integrity of the genome (45). These breaks are largely repaired by NHEJ or HDR, which are thought to be distinct processes although they have been shown to have overlapping roles in maintaining genome integrity (8). Coupling of these two pathways can preserve the integrity of the genome for repair of a single double-strand break (10). We have shown here that the amount/activity of DNA-PKcs, an essential component of the NHEJ pathway influences the level of ATM, a HDR and checkpoint control factor. Loss or diminution of DNA-PKcs is associated with reduced levels of ATM. This relationship was first reported in the glioma cell line M059J (21). The possible conclusion that DNA-PKcs levels affect ATM was complicated by the fact that this was a tumor cell line that would be expected to carry a variety of genetic changes. In fact, it was later shown that there were truncating mutations in both ATM alleles in these cells (23). However, in a variety of A-T cell lines carrying two ATM truncating mutations (46), there was no evidence that these mutations caused its mRNA destabilization. Furthermore, the relationship between DNA-PKcs and ATM in M059J cells was not a general feature of other glioma cell lines (21). Both cell lines also contain mutant p53 (47). This was also evident from the results described here where basal levels of p53 were elevated to different extents and did not change appreciably after radiation. On the other hand, it was possible to detect a time-dependent increase in ser15 phosphorylated p53 in both cell lines and, whereas the response was weaker in M059J cells, when normalized to p53, it was not significantly different. It is evident that reduced levels of ATM in M059J results in a much reduced capacity for ATM activation and also leads to a delay in radiation-induced phosphorylation of SMC1. The reduced but appreciable p53 response in M059J cells suggests that residual ATM activity remains. This is in keeping with a normal ATM-dependent G2-M arrest in these cells (48). However, it also suggests that there may be some residual activity of p53 in these cells. A series of other reports show that the p53 response to DNA damage is functional in mouse embryonic fibroblasts lacking DNA-PK activity (49–51). Thus, there seems to be reduced but sufficient ATM levels in DNA-PK deficient cells to maintain cell cycle control.

In the light of these observations with M059J cells, it was important to test the universality of the DNA-PKcs/ATM relationship in other cell systems. This relationship was shown to occur in

![Diagram](https://example.com/diagram.png)
Figure 5. A, relative ATM mRNA levels in V3 cells and in mouse tissue. mRNA was extracted from the V3 radiosensitive (V3) cells and scid spleens and compared with the mRNA levels in the radioresistant (V3+) cells and in C57BL/6 mouse tissue. B, ATM message stability in V3 cells. De novo transcription was blocked by the addition of actinomycin D (1 μg/mL) to the cultures of both V3+ and V3 cells. Following the addition of actinomycin D, the relative abundance of ATM message present in the two cell lines at 0, 12, 24, and 36 hours was determined. C, ATM promoter activity in V3, V3+, and M059J and M059K cells. Cell extracts were prepared 48 hours after transfection with the ATM promoter-luciferase construct (41) and the luciferase activity determined for each lysate. Results shown are a summary of three independent experiments.

the V3 radiosensitive CHO cell line, and once the amount of DNA-PKcs was restored to normal levels by transfection with DNA-PKcs, so too were the levels of ATM protein. Different degrees of deficiency of DNA-PKcs in murine cells from different strains were also associated with reduced Atm. We also showed that the reduced ATM in M059J cells was characterized by reduced ATM kinase activity in response to radiation damage to DNA. Taken together, these results suggest that either the amount of DNA-PKcs and/or its activity influence expression of ATM. Knockdown of DNA-PKcs by specific siRNA provided the definitive experiment to show that its loss was followed by a delayed decrease in the amount of ATM. In addition, the response kinetics revealed that abrogation of PRKDC mRNA preceded the decrease in DNA-PKcs protein and this in turn was followed by reduction in the amount of ATM mRNA, eventually leading to loss of ATM protein. Reduced ATM mRNA (down by 40%) was also observed in V3 CHO cells compared with those corrected with PRKDC cDNA, and in cells from scid (down by 50%) versus C57BL/6 murine cells. Promoter studies using the minimal ATM promoter region showed no reduction in luciferase activity in the absence of PRKDC protein, suggesting that other regulatory elements may be encoded within the ATM message downstream of this region. It is therefore difficult to reconcile the larger differences in ATM protein levels in these cells based on the minimal promoter activity and on ATM mRNA levels alone, suggesting that DNA-PKcs might be controlling ATM expression at more than one level.

Analyses of gene expression in cells lacking DNA-PK have identified genes that are either up-regulated or down-regulated in response to the loss of this protein (52–54). Using the glioma cell lines, M059J/M059K, microarray analysis revealed differential expression of 14 genes. However, only one of these, replication factor C (38 kDa subunit mRNA), was down-regulated in the cell line lacking DNA-PKcs (52). More recently, Ai et al. (54) have shown that the transcripts of melanoma antigen subfamily A genes are strongly down-regulated in M059J cells compared with M059K cells and these are restored to normal levels in PRKDC-complemented cells. They also showed that the MAGE-A1 promoter is methylated in M059J cells and demethylated in the M059K and PRKDC-complemented cells. However, another set of genes were up-regulated in M059J cells and differential expression did not correlate with promoter methylation. Given the rapidity of the effect of loss of DNA-PKcs on ATM mRNA and protein in the siRNA experiment, this down-regulation does not seem to be explained by methylation changes to the promoter. Analysis of gene transcription in murine cells lacking DNA-PK activity identified only one gene, laminin 4, whose expression was reduced in DNA-PK null cells (53). There was evidence of up-regulation of this gene after transient transfection of Prkdc into DNA-PK-null mouse embryo fibroblasts.

The down-regulation of ATM mRNA prior to ATM protein in response to PRKDC siRNA suggests that DNA-PKcs may be positively regulating ATM expression at the transcriptional level. Evidence for the lack of a positively acting regulatory factor has been reported in DNA-PKcs-deficient CHO cells (55). Extracts from DNA-PKcs-deficient cell lines had a 2- to 7-fold decrease in the level of ATM mRNA prior to ATM protein expression compared with matched controls and transcription could be restored by addition of small amounts of extract from DNA-PK-proficient cells. This factor was not DNA-PKcs or Ku because purified forms of these proteins failed to restore transcription. Possible candidates include, (a) TATA-binding protein and transcription factor TFIIH because phosphorylation of these proteins by DNA-PK stimulated the formation of P-TATA-binding protein-TFIIH-TFII-F-Pol II complex and basal transcription (44), and (b) regulation by RNA pol II, because it has been shown that transcription activators function more efficiently in the presence of DNA-PKcs (56). DNA-PK also phosphorylates a host of other factors implicated in transcription, some of which lead to up-regulation of genes, others causing down-regulation (57–59). Another alternative is that it is not the kinase activity, but another interacting domain of DNA-PKcs that is involved. We have carried out preliminary experiments with radiosensitive CHO cell lines, irs-20 and xrs-5. The former has only about 5% to 10% of the normal levels of a mutant DNA-PKcs, but the protein has a greatly reduced kinase activity (28–30). This mutant cell line has only slightly reduced levels of ATM, although Ku70/Ku80-deficient CHO cells do have an appreciably reduced ATM (data not shown). The fact that irs-20 cells are also radiosensitive serves to clarify and emphasize the point that radiation hypersensitivity of DNA-PKcs mutant cells cannot be explained by parallel decreases in ATM.

Why is it that in A-T cells normal levels and activity of DNA-PK are maintained, yet in DNA-PKcs-deficient cells ATM is
down-regulated? This may well relate to the central role of DNA-PKcs in repairing DNA double-strand breaks, whereas ATM is primarily a cell cycle checkpoint activator with a less critical role in double-strand break repair. Thus, it is possible that in a cell compromised for NHEJ of DNA double-strand breaks, ATM is down-regulated to avoid its futile constitutive activation, thus favoring conditions for apoptotic death in proliferating cells. Whatever the explanation, it seems clear that DNA-PKcs can control the expression of ATM in human cells and DNA-PKcs deficiencies could have consequences in processes connected to the network of damage response pathways controlled by ATM that have not been recognized previously.

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Deficiency in the Catalytic Subunit of DNA-Dependent Protein Kinase Causes Down-Regulation of ATM


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