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Epidermal Growth Factor Sensitizes Cells to Ionizing Radiation by Down-regulating Protein Mutated in Ataxia-Telangiectasia*

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Epidermal growth factor (EGF) has been reported to either sensitize or protect cells against ionizing radiation. We report here that EGF increases radiosensitivity in both human fibroblasts and lymphoblasts and down-regulates both ATM (mutated in ataxia-telangiectasia (A-T)) and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). No further radiosensitization was observed in A-T cells after pretreatment with EGF. The down-regulation of ATM occurs at the transcriptional level. Concomitant with the down-regulation of ATM, the DNA binding activity of the transcription factor Sp1 decreased. A causal relationship was established between these observations by demonstrating that up-regulation of Sp1 DNA binding activity by granulocyte/macrophage colony-stimulating factor rapidly reversed the EGF-induced decrease in ATM protein and restored radiosensitivity to normal levels. Failure to radiosensitize EGF-treated cells to the same extent as observed for A-T cells can be explained by induction of ATM protein and kinase activity with time post-irradiation. Although ionizing radiation damage to DNA rapidly activates ATM kinase and cell cycle checkpoints, we have provided evidence for the first time that alteration in the amount of ATM protein occurs in response to both EGF and radiation exposure. Taken together these data support complex control of ATM function that has important repercussions for targeting ATM to improve radiotherapeutic benefit.

Functional loss of the ATM[1] protein, mutated in the human genetic disorder ataxia-telangiectasia (A-T) (1), leads to a pleiotropic phenotype including neurological abnormalities, immunodeficiency, and a predisposition to develop a number of malignancies, primarily leukemias and lymphomas (2). The characteristic most widely studied in A-T is hypersensitivity to ionizing radiation (3–5) that appears to be due to an inability of ATM to recognize and facilitate the repair of a subcategory of double strand breaks or a form of damage that is converted into a double strand break in DNA (6, 7). It is also likely that recognition of a specific form of DNA damage represents the trigger for ATM to activate a number of cell cycle checkpoints (8, 9). A-T cells are defective in the phosphorylation of p53 on serine 15 and serine 20 and dephosphorylation at serine 376 after exposure of cells to ionizing radiation (10–12). Evidence for a direct involvement of ATM with p53 was provided by the demonstration that these two molecules interact directly (13) and ATM phosphorylates p53 on serine 15 in response to DNA damage (14, 15). Pre-existing ATM protein is rapidly activated by ionizing radiation and radiomimetic agents by an undescribed mechanism (13–15). Exposure of cells to ionizing radiation fails to change either the subcellular distribution or the total amount of cellular ATM protein (16–18). Furthermore, ATM protein levels are relatively constant throughout the cell cycle in human fibroblasts (17). These data point to a post-translational mechanism of activation for ATM. However, there is evidence for alteration in the amount of ATM under certain conditions. Although ATM is not detectable by immunohistochemical staining in quiescent myoepithelial cells lining normal breast ducts, significant expression is observed in the proliferative myoepithelium of sclerosing adenosis (19). In addition the amount of ATM protein changes dramatically in quiescent lymphocytes (PBMCs) in response to mitogenic agents (20). In the latter study a 6–10-fold increase in ATM in PBMCs was observed in response to PHA, reaching a maximum by 3–4 days. As the amount of ATM protein increased, so too did its protein kinase activity. Clearly this is a slow response compared with the rapid activation of ATM post-irradiation. Alteration in the amount of the ATM protein could occur by transcriptional control at the promoter. The housekeeping gene *NPAT*/*E14*/*CAND3* lies ~0.55 kb from the 5′ end of the ATM gene (21–23). These genes share a bidirectional promoter that contains CCAAT boxes and 4 consensus sites for the Sp1 transcription factor (21, 22). Further delineation of the ATM promoter is required to assist in understanding the transcriptional regulation of ATM.

ATM is located predominantly in the nucleus of proliferating cells, which is in keeping with its role in DNA damage recognition and cell cycle control, but ATM has also been detected in cytoplasmic vesicles (16, 17, 24). Since ATM has been impli-

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[1] The abbreviations used are: ATM, protein mutated in ataxia-telangiectasia; EGF, epidermal growth factor, A-T, ataxia-telangiectasia; DNA-PKcs, catalytic substrate of DNA-dependent protein kinase; GM-CSF, granulocyte/macrophage colony-stimulating factor; IR, ionizing radiation; PBMCs, peripheral blood mononuclear cells; Gy, gray; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; kb, kilobase pair; bp, base pair; NPAT, nuclear protein at the A-T locus; CMV, cytomegalovirus.

cated in more general intracellular signaling and since it responds to mitogenic agents, we studied its possible regulation by epidermal growth factor (EGF) that alters cellular response to radiation. EGF enhances the radiosensitivity of some cell types (25–28) and increases the radioresistance of other cells (29). We show here that EGF enhanced the radiosensitivity of both fibroblasts and lymphoblasts, and this was associated with a decrease in ATM. Reduction in ATM protein was accompanied by a decrease in the amount of Sp1 DNA binding activity. We also demonstrate that ATM protein is rapidly restored to constitutive levels by both granulocyte/macrophage colony-stimulating factor (GM-CSF) and ionizing radiation, and this appears to be achieved by increasing Sp1 DNA binding activity.

EXPERIMENTAL PROCEDURES

Materials—Normal human skin fibroblasts HSF7 and NFF were from healthy donors expressing ATM protein; A-T fibroblasts GM03395 were obtained from Coriell Cell Repositories. Lymphoblastoid cells were established by Epstein-Barr virus transformation from healthy individuals, C3ABR, C28ABR, C35ABR, C2ABR, and C31ABR. All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium supplemented with 10% fetal calf serum. Irradiation of cells was performed at room temperature using a ^{137}Cs source delivering gamma rays at a dose rate of 2.8 Gy/min. EGF (R & D Systems) was added at a concentration of 50 ng/ml to the culture medium of log-phase cultures or serum-reduced fibroblasts for various times as indicated. The antibodies used are as follows: p53 monoclonal antibody (polyclonal antibody 1801) and polyclonal Sp1 antibody (Sp1pEp2) were from Santa Cruz Biotechnology; monoclonal α-actin (A4700) was from Sigma; polyclonal anti-Ku and DNA-PKcs antibodies were obtained from Susan Lees-Miller, University of Calgary, Canada; anti-phosphorylated p53 serine 15 was obtained from New England Biolabs; and monoclonal ATM (CT1) and polyclonal ATM raised in sheep (ATM5BA) antibodies were generated in this laboratory.

Cell Survival—Logarithmically growing lymphoblastoid cells were incubated with or without EGF (50 ng/ml) for 16 h prior to exposure to ionizing radiation (2.5 Gy/min, ^{137}Cs). Cell viability was determined at daily intervals between 1 and 4 days by adding 0.1 ml of 0.4% trypan blue to a 0.5-ml cell suspension as described previously, and viable cells were counted (30).

Immunoblotting—EGF treatment was carried out on log-phase cells for different times as indicated. After washing in PBS, 5×10^6 cells were resuspended in 40 μl of nuclear extraction buffer (25 mM Hepes, pH 8, 0.25 M sucrose, 1 mM EGTA, 5 mM $MgSO_4$, 50 mM NaF, 1 mM DTT, 1 mM PMSF) and disrupted by at least 4 freeze-thaw cycles. Insoluble material was removed by centrifugation at 13,000 $\times$ *g* for 20 min. Protein concentration was determined using a Bio-Rad DC protein assay kit according to the manufacturer's recommendations, and 50–100 μg was used per sample. Protein samples were separated on 5 or 10% denaturing gels and blotted on nitrocellulose membranes. After blocking in 4% milk powder, 0.1% Tween 20, PBS for at least 1 h, the blot was incubated for 1 h at room temperature or overnight at 4 °C, with the relevant primary antibody. The blot was washed three times in 0.1% Tween 20/PBS and incubated with secondary peroxidase-conjugated antibody for 1 h. Following several washing steps, the blot was developed using a chemiluminescence kit (DuPont).

mRNA Determination—Changes in ATM mRNA in lymphoblastoid cells were determined by quantitative PCR as described (31). In short, known amounts of a 402-bp DNA fragment corresponding to an ATM mutation lacking exon 38 (5319 G to A) was used as a competitor during amplification of the corresponding region in ATM cDNA from normal cells, which gives a 544-bp fragment (nucleotides 5122–5665). A series of 2-fold dilutions were employed to find the equivalence point and thus a measure of the amount of ATM mRNA. Expression of mRNA was also determined by Northern blotting. Briefly, total RNA was isolated from log-phase cells with an RNA isolation kit (Qiaquick, Qiagen) according to the manufacturer's instructions, and 20 μg of RNA/lane was separated on a 1% agarose gel. Blotting of RNA on nylon membrane (Hybond-N, Amersham Pharmacia Biotech) was performed overnight by capillary force followed by hybridization with an ATM cDNA probe.

Gel Retardation Assay (Electrophoretic Mobility Shift Assay)—The DNA binding activity of transcription factor Sp1 was determined by modification of a method described previously (32). Briefly, 10^7 cells were washed in ice-cold PBS, resuspended in 400 μl of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.2 mM PMSF), and incubated for 15 min on ice. After addition of 25 μl of 10% Nonidet P-40, the cells were subsequently vortexed for 10 s and the nuclei pelleted by centrifugation (1 min, 13,000 $\times$ *g* at 4 °C). The nuclear pellet was resuspended in 40 μl of buffer C (20 mM Hepes, pH 7.9, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, 0.2 mM PMSF) and rotated for 30–60 min at 4 °C to elute nuclear proteins. After additional centrifugation for 15 min at 4 °C the supernatant containing the nuclear proteins was aliquoted, frozen in liquid nitrogen, and stored at −70 °C until used in binding reactions. Approximately 35 fmol of ^{32}P-radiolabeled double-strand Sp1 consensus sequence (5′-ATTCGATCGGGGCGGGGCGAGC-3′; Promega) (labeled using T4 polynucleotide kinase) was incubated with 10 μg of nuclear extract in the presence of 1 μg of herring sperm DNA and 20 μg of BSA in binding buffer (10 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 1% Nonidet P-40, 5 mM DTT, 0.2 mM PMSF) in a 20-μl reaction. Binding was for 25 min at room temperature before DNA-protein complexes were separated on a native 5% acrylamide gel. Exposure time to x-ray film was usually 30–45 min.

Luciferase Assays—The ATM promoter was amplified from human genomic DNA using primers (forward, 5-TCCCCCGGGGGAGATCAA-AACCACAGCAGG, and reverse, 5-CCCAAGCTTGGGCGTTCTCTCG-CCTCCTCCCGTG). The 615-bp amplification product was cloned into the pGL3-basic luciferase reporter vector (Promega) using a *Sma*I/*Hin*dIII digest. This construct (pGL3-ATM) was cotransfected with the pRL-CMV vector (Promega) at a ratio of 2:1 into lymphoblastoid cells by electroporation (280 V, 960 microfarads, 1 pulse). Thirty six hours after electroporation the cells were incubated with 50 ng/ml EGF for a further 16 h before cell extracts were prepared. Luciferase activity was measured using the Dual Luciferase Assay (Promega, E1910). Briefly, 100 μl of firefly luciferase substrate (LARII) and 20 μl of cell extract were mixed, and the reaction was immediately measured for 10 s. Then 100 μl of *Renilla* luciferase substrate including an inhibitor for firefly luciferase (Stop & Glow) was added, and light emission was detected for another 10-s interval. The ratio of both measurements (pGL/pRL) results in the relative luciferase activity, avoiding variabilities in transfection. The absolute values of luminescence measured (relative light unit) was generally in the range of $5{\cdot}10^4$–$5{\cdot}10^6$. The untreated control was set to 1.

ATM Kinase Assay—ATM kinase activity after EGF treatment was determined using the method described by Canman *et al.* (15). For whole cell lysate isolation, cells were lysed on ice in TGN buffer (50 mM Tris, pH 7.5, 50 mM β-glycerophosphate, 150 mM NaCl, 10% glycerol, 1% Tween 20, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml pepstatin, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM DTT). After centrifugation at 13,000 $\times$ *g* for 1 min, 2 mg of extract was precleared with mouse immunoglobulin G and protein A/G-Sepharose beads. ATM was immunoprecipitated with anti-ATM antibody (ATM5BA) and kinase activity determined as described.

RESULTS

EGF Sensitizes Cells to Radiation—Engagement of the EGF receptor by EGF and other ligands initiates signal transduction pathways giving rise to mitogenesis and can also alter the radiosensitivity status of cells (25–29). We determined whether EGF might increase the sensitivity of human fibroblasts and lymphoblasts by incubating cells in EGF for 16 h prior to exposure to radiation (1–4 Gy). The results in Fig. 1*A* demonstrate that EGF-treated lymphoblastoid cells are more sensitive to radiation, and this is observed over the radiation dose range 1–4 Gy (Fig. 1*B*). The effect of EGF treatment was significant for both dose and time course experiments for C3ABR; $p = 0.0023$ (α), $p = 0.0053$ (β) for dose and $p = 0.0065$ (α) and $p = 0.07$ (β) for time course. On the other hand treatment of A-T cells with EGF failed to increase the radiosensitivity of these cells (Fig. 1, *A* and *B*). A similar sensitization was observed in EGF-treated control fibroblasts, but again no further increase in sensitivity was revealed in EGF-treated A-T fibroblasts (Fig. 1*C*). We observed that EGF receptor was activated under these conditions (results not shown).

EGF Treatment Reduces ATM Protein Expression—Since radiosensitivity and abnormalities in mitogenesis and other signaling pathways are characteristic of the A-T phenotype, we initially determined whether the radiosensitizing effect of EGF might be due to alteration in the amount of ATM protein in normal control cells. Immunoblotting with anti-ATM antibod-

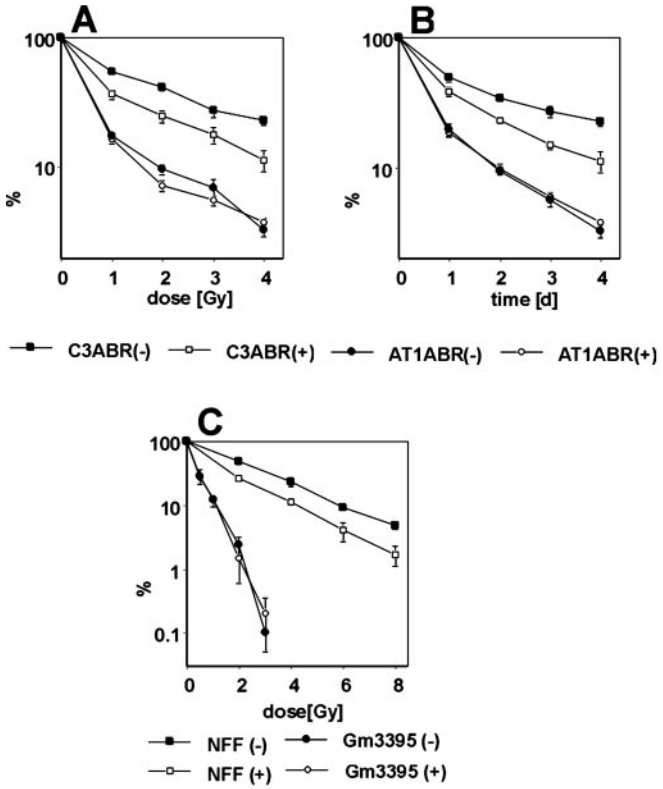

FIG. 1. **EGF sensitizes human cells to ionizing radiation.** *A,* effect of increasing radiation dose on survival of control (*C3ABR*) and A-T (*AT1ABR*) lymphoblastoid cells untreated or pretreated with EGF (50 ng/ml) for 16 h prior to irradiation. Viability was determined by trypan blue exclusion at 72 h post-irradiation. (−) and (+) refer to EGF addition. *B,* effect of γ-radiation (4 Gy) on survival of C3ABR and AT1ABR cells with time after irradiation. Cells were untreated and pretreated with EGF as in *A* above. *C,* effect of EGF (50 ng/ml) for 16 h on radiation killing in control (*NFF*) and A-T (GM 3395) fibroblast cells. In this case survival was determined by colony formation. In each case three independent experiments were carried out, and values were fitted by nonlinear repression and calculated curve parameters (α, β) subjected to Student's *t* test.

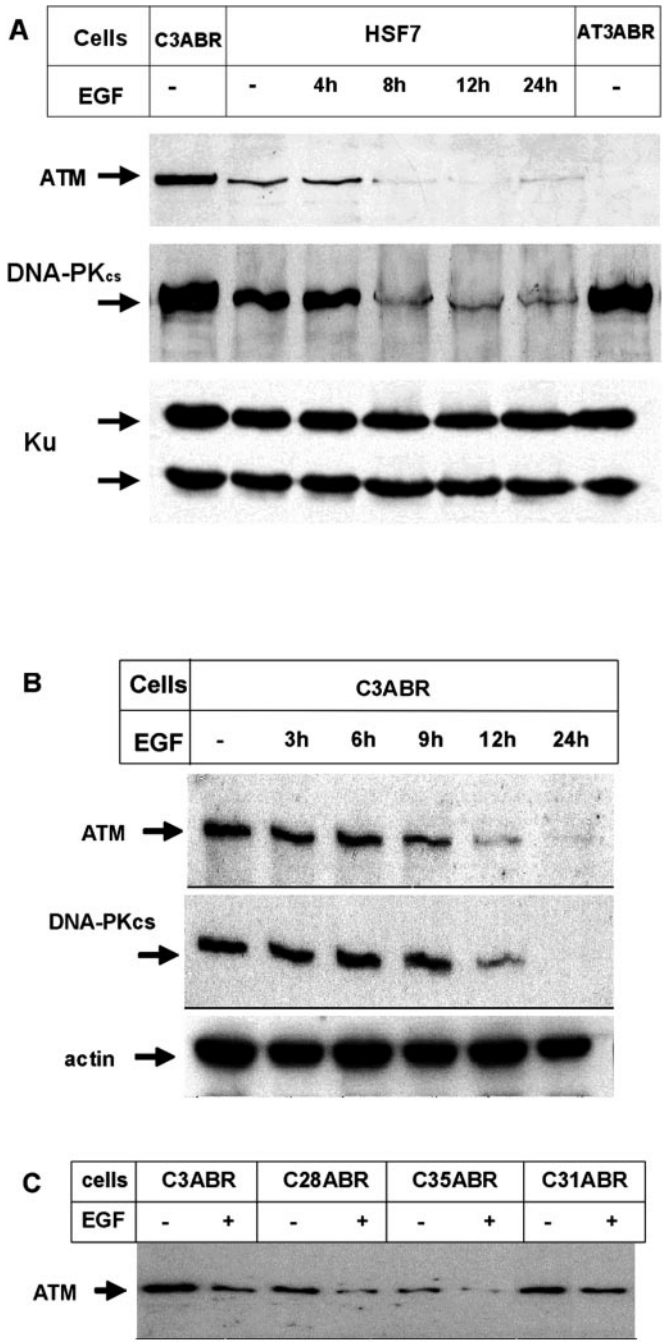

FIG. 2. **Effect of EGF incubation on the amount of ATM protein in control cells.** *A,* ATM protein levels in fibroblasts (HSF7) treated with EGF (50 ng/ml). 200 μg of HSF7 protein was loaded in each lane and immunoblotted with anti-ATM antibody (ATM-4BA) (16). Positive (C3ABR lymphoblastoid cell extracts, 100 μg) and negative (AT3ABR lymphoblastoid cell extracts, 100 μg) controls were also loaded. DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase, was determined using the antibody DPKI (kindly provided by Susan Lees-Miller, University of Calgary). The same cell lines were employed as controls for DNA-PKcs. Immunoblotting was also carried out for Ku70/86 using a mouse polyclonal antibody that detects both forms, and this acted effectively as a loading control. *B,* effect of EGF (50 ng/ml) incubation on ATM protein in (C3ABR) lymphoblastoid cells. Immunoblotting was carried out with anti-ATM antibody (CT-1) using 40 μg of extracts in each lane. DNA-PKcs was determined using immunoblotting with DPKI, and actin was used as a loading control. *C,* EGF reduces ATM protein in different lymphoblastoid cell lines. The cells were incubated with EGF (50 ng/ml) for 20 h and ATM levels determined by immunoblotting in C3ABR, C28ABR, C35ABR, and C31ABR. Protein loading in each lane was determined by Ponceau S staining (not shown).

ies showed that the amount of ATM protein was reduced significantly with time after incubation with EGF in normal fibroblasts (Fig. 2*A*). No ATM was detected in negative control AT3ABR cells, as expected (16). The same pattern of reduction was observed for the catalytic subunit of DNA-dependent protein kinase, DNA-PKcs, after EGF treatment, but Ku, the DNA damage recognition component of DNA-dependent protein kinase, remained largely unchanged (Fig. 2*A*). It is evident that the amount of DNA-PKcs is normal in the A-T cell line, AT3ABR, when compared with C3ABR. EGF treatment of C3ABR lymphoblastoid cells revealed a similar pattern of loss of expression of ATM, down to 30% by 9 h post-treatment (Fig. 2*B*). A similar pattern of decrease was also observed for DNA-PKcs (Fig. 1*B*). To test the universality of these observations we employed several control lymphoblastoid cell lines. In all four control lines investigated, ATM protein decreased significantly after EGF treatment (Fig. 2*C*). It is evident from the positive control (100 μg of extract from the lymphoblastoid cell line, C3ABR) that the amount of ATM protein in HSF7 fibroblasts (at equal loading) is considerably lower than that in lymphoblastoid cells (Fig. 2*A*).

Effect of EGF on mRNA Expression—Since EGF caused a marked decrease in the amount of ATM protein, it was possible that this was due to transcriptional down-regulation. To monitor changes to ATM mRNA in lymphoblastoid cells, we employed quantitative reverse transcriptase-PCR as described

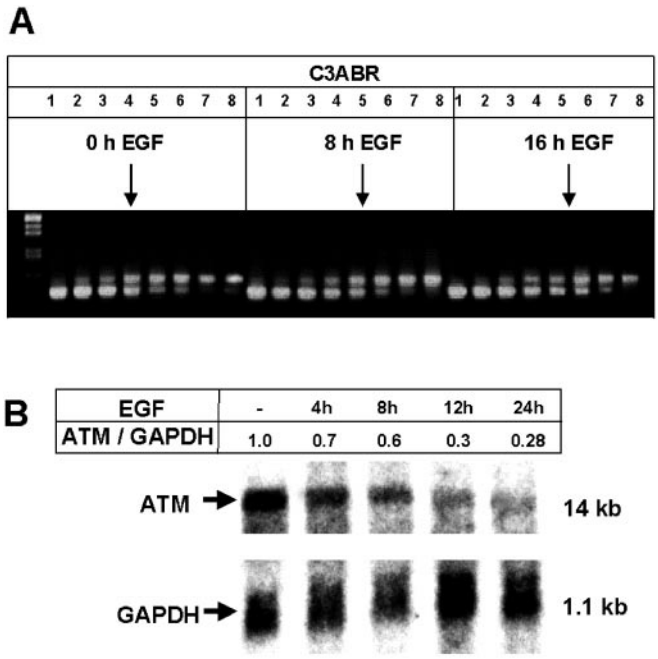

FIG. 3. **Determination of ATM mRNA transcript in lymphoblastoid cells after incubation with EGF.** *A,* quantitative reverse transcriptase-PCR was carried out to determine ATM mRNA in C3ABR lymphoblastoid cells as described previously (31). In these experiments a DNA fragment (402 bp) corresponding to an A-T mutant (G5319A) lacking exon 38 was employed as a competitor for ATM cDNA amplification from C3ABR cells (nucleotides 5122–5665, 544 bp) in a series of 2-fold dilutions. The amount of competitor DNA added to each sample was as follows: *lane 1*, 0.078 attomol/μl; *lane 2*, 0.156; *lane 3*, 0.313; *lane 4,* 0.625; *lane 5*, 1.25, *lane 6*, 2.5; *lane 7*, 5.0; and *lane 8*, 10 attomol/μl. Size markers appear on the *left-hand side* of the figure. Cells were incubated for 8 and 16 h with EGF (50 ng/ml) as indicated. These experiments were repeated three times. *B,* Northern blot analysis of ATM mRNA form normal human skin fibroblasts (HSF7) after treatment with EGF (50 ng/ml) for various times up to 24 h. Each lane contained 20 μg of total RNA, and glyceraldehyde-3-phosphate dehydrogenase was employed as a loading control. The ATM/glyceraldehyde-3-phosphate dehydrogenase ratio was determined by densitometry and represents the relative amounts of ATM mRNA present with time after EGF treatment.

previously (31). A DNA fragment (402 bp) corresponding to an ATM mutation (G5319A), which lacks exon 38, was used as a competitor for ATM cDNA amplification (nucleotides 5122–5665, 544 bp in size) in a series of 2-fold dilutions from C3ABR cells. After 8 h of treatment with EGF, an equivalent point was reached at 0.625 attomol/μl of competitor compared with 1.25 attomol/μl for untreated cells representing an ~2-fold reduction in ATM mRNA in response to EGF (Fig. 3*A*). After 16 h of treatment with EGF the equivalent point was reached at ~0.4 attomol representing a 3-fold reduction in ATM mRNA. Northern blot analysis revealed that ATM mRNA was reduced to 60% after incubation of normal fibroblasts (HSF7) with EGF for 8 h and reached a plateau at ~30% of the untreated value at 12–24 h post-treatment, which parallels the changes shown by reverse transcriptase-PCR in lymphoblastoid cells (Fig. 3*B*).

Reduced Sp1 Binding Activity after EGF Treatment—A similar genomic organization at the ATM locus exists for the human and mouse genes where ATM and nuclear protein at the A-T locus (NPAT) are arranged ~0.5 kb apart in a head-to-head configuration (Fig. 5*A*) (21, 22). These two genes are transcribed from a central bidirectional promoter that contains several binding sites for the transcriptional factor, Sp1 (33). Since ATM mRNA and protein were reduced after EGF treatment, we predicted that this might be due to interference with or reduced Sp1 DNA binding activity. Use of gel-shift analysis with an oligonucleotide-binding consensus sequence for Sp1 revealed the presence of a single, well defined, retarded band (Fig. 4*A*). The amount of Sp1 binding in extracts from C3ABR cells decreased with time after EGF treatment (Fig. 4*A*). A decrease of ~50% by 12 h post-treatment, leveling off at later times, paralleled the decrease in the amount of ATM mRNA and protein seen previously. To establish the specificity of this binding, we added anti-Sp1 antibody to the incubation mixture and observed a supershift of the retarded band (Fig. 4*A, lower panel*). A control antibody against p53 did not alter migration of the band. In addition excess cold Sp1 binding consensus oligonucleotide successfully competed for binding, whereas cold AP-1 binding oligonucleotide failed to do so (results not shown). Under these conditions the amount of Sp1 protein did not change (Fig. 4*B*). When Sp1 binding was determined in four additional cell lines a similar decrease was observed in all cases (Fig. 4*C*).

Effect of EGF on ATM Promoter—To confirm the effect of EGF on the transcriptional regulation of ATM, we cloned the common promoter region between ATM and NPAT into a luciferase reporter construct (Fig. 5) and carried out transient transfections in control lymphoblastoid cells. Exposure of transfected C3ABR cells to EGF for 16 h led to a significant down-regulation of luciferase activity in cells transfected with the ATM promoter construct in agreement with loss of Sp1 binding activity (Fig. 5).

Causal Relationship between Sp1 and ATM Down-regulation—The parallel decrease of ATM and Sp1 binding activity together with the down-regulation of ATM promoter activity after EGF treatment suggested that the decrease in Sp1 binding led to reduced ATM. To test this we employed GM-CSF that has been shown to increase markedly the DNA binding activity of Sp1 (34). As observed above, treatment of cells with EGF for 16 h down-regulated the amount of Sp1 binding activity (Fig. 6*A, 3rd lane*) and subsequent addition of GM-CSF to these cells caused a rapid (within 1 h) restoration of Sp1 binding activity (Fig. 6*A, 5th lane*). Incubation of untreated cells with GM-CSF also showed evidence of increased Sp1 binding activity (Fig. 6*A, 4th lane*). EGF caused a down-regulation of ATM protein (Fig. 6*B, 2nd lane*), and compatible with the increase in Sp1 binding activity there was also a rapid return of ATM to normal levels when cells were subsequently treated with GM-CSF (Fig. 6*B, 4th lane*). Under these conditions Sp1 protein levels did not change (Fig. 6*B*). To test whether the reduction in Sp1 binding activity and ATM down-regulation were responsible for the EGF-induced radiosensitivity, cells were exposed to radiation after pretreatment with EGF followed by GM-CSF incubation for 1 h. A normal pattern of radiosensitivity was observed compatible with restored levels of ATM protein under these conditions (Fig. 6*C*).

Radiation Induces ATM Protein—We investigated whether radiation might also attenuate the response, since it has been shown that Sp1 activity is up-regulated by radiation (32, 35), which in turn should increase the amount of ATM protein. When C3ABR cells were exposed to radiation (5 Gy) there was no change in the amount of Sp1 binding activity, but when these cells were pretreated with EGF initially for 16 h, Sp1 binding activity decreased and subsequent exposure to radiation restored the amount of Sp1 binding activity in 3 h (Fig. 7*A*). Under these conditions incubation of cells with EGF followed by radiation exposure led to an increase in the amount of ATM protein to untreated levels by 3 h post-irradiation (Fig. 7*B*). ATM kinase activity increased by 30 min post-irradiation, and the extent of this increase was somewhat lower in cells pretreated with EGF (Fig. 7*B*). By 3 h post-irradiation ATM kinase induction was the same in cells either untreated or

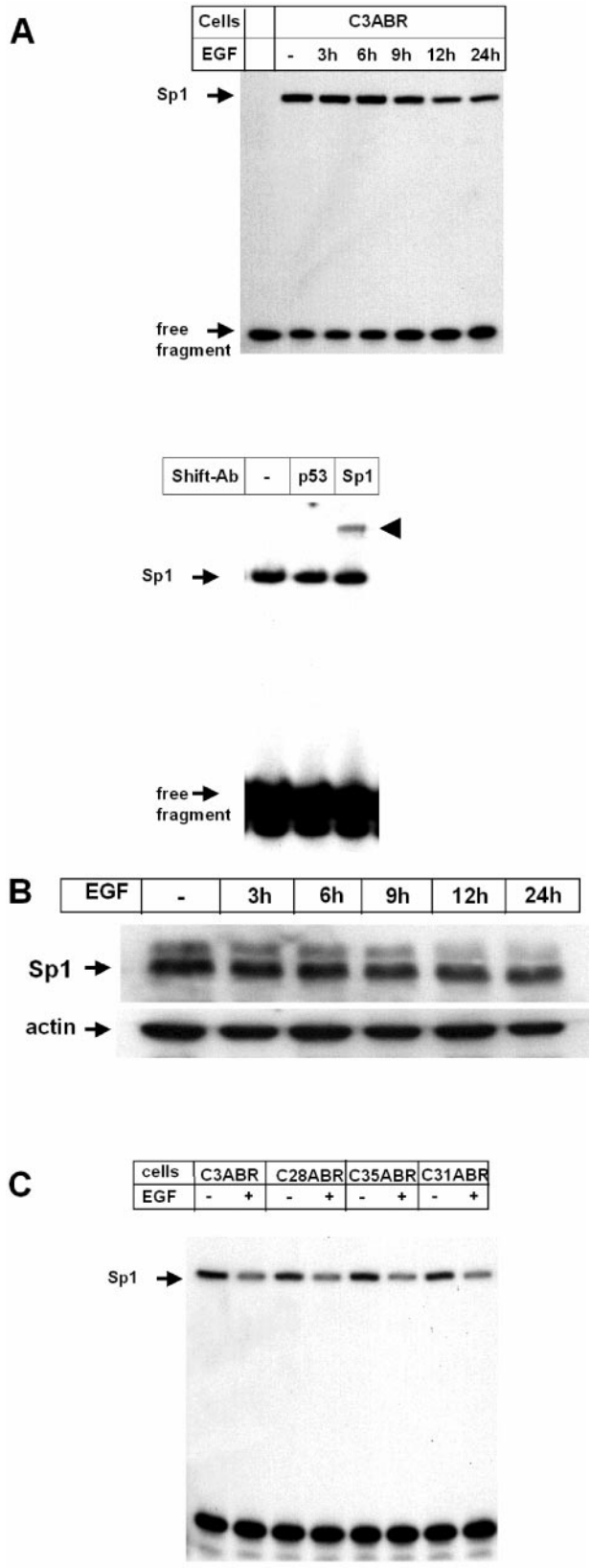

FIG. 4. **Effect of EGF on nuclear protein binding to an Sp1-binding consensus sequence in lymphoblastoid cells.** *A,* nuclear extracts (20 μg), prepared at different times after EGF addition from C3ABR, were incubated with ^{32}P-labeled double-stranded oligonucleotide (22-mer) containing a single Sp1-binding site. Free and retarded fragments were separated on native 5% acrylamide gels. *1st lane* is free fragment only. An Sp1 supershift assay was employed to establish the specificity of binding. Incubation of nuclear extract and labeled fragment was carried out followed by addition of 1 μg of anti-Sp1 antibody (Upstate Biotechnology) for 30 min on ice prior to gel electrophoresis. Anti-p53 antibody (Oncogene Science) was used as a control. Quantitation of binding activity was determined by densitometry. *B,* Sp1 levels were determined by immunoblotting 20 μg of nuclear extract from each sample in *A*. Actin was used as a loading control. *C,* several different lymphoblastoid cells (C3ABR, C28ABR, C35ABR, and C31ABR) were treated with EGF for 16 h prior to preparation of extracts for binding to the Sp1 consensus sequence as described above.

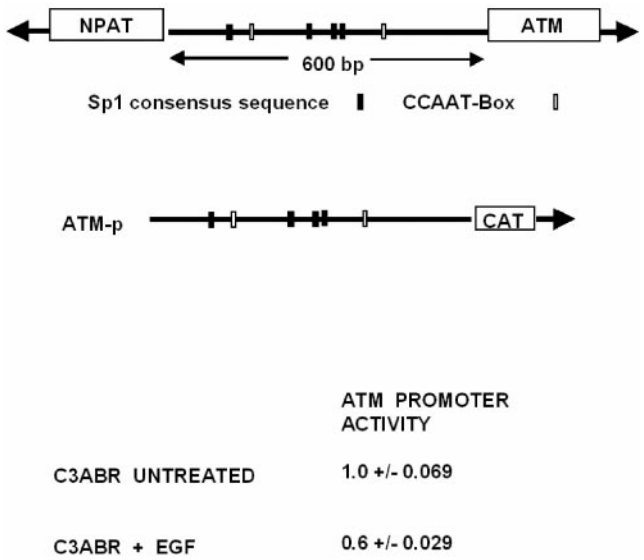

FIG. 5. **Effect of EGF on luciferase activity in cells transfected with the ATM promoter reporter construct.** Common bidirectional promoter for ATM and NPAT. Luciferase-reporter construct for the ATM promoter (pGL3ATM). C3ABR were simultaneously transfected with pGL3-ATM and pRL-CMV (containing the CMV promoter as an internal control) at a ratio of 2:1. 36 h after transfection the cells were incubated with 50 ng/ml EGF for 16 h. The activity of the ATM promoter was calculated as ratio of firefly luciferase (pGL3-ATM) to *Renilla* luciferase (pRL-CMV). The data represent four independent experiments with three samples each. p value was calculated using Student's t test.

pretreated with EGF, at which time ATM protein levels were the same (Fig. 7*B*). These data were further supported by radiation-induced stabilization of p53 and serine 15 phosphorylation of p53 with or without prior incubation with EGF (Fig. 7*B*). The latter provides an *in vivo* measure of ATM kinase activation.

DISCUSSION

Activation of ATM kinase by ionizing radiation and other DNA-damaging agents is critical for modulating its activity in several DNA damage signaling pathways (36, 37). We have demonstrated here that ATM can also be altered at the transcriptional level by continuous incubation with EGF over a 16-h period. Under these conditions EGF-treated cells were more sensitive to subsequent ionizing radiation exposure than untreated, irradiated cells. Evidence exists for both radiosensitizing and radioprotective effects of EGF, which is dependent upon both the cell type and position in the cell cycle (25–29, 38–40). In human squamous carcinoma cells, radiosensitization by EGF appears to be in part due to increasing the magnitude of radiation-induced G_2/M arrest (39). In CaSki cells the enhancement of radiosensitivity was evident from a reduction in the shoulder region of the survival curves (27). The degree of radiosensitization in these cells by EGF was of the same order as that observed in the present study. None of the previous studies have provided a mechanistic explanation for radiosensitization, but the down-regulation of ATM in this study provides a potential explanation. Failure to observe a sensitizing effect of radiation in A-T cells provides further support for an

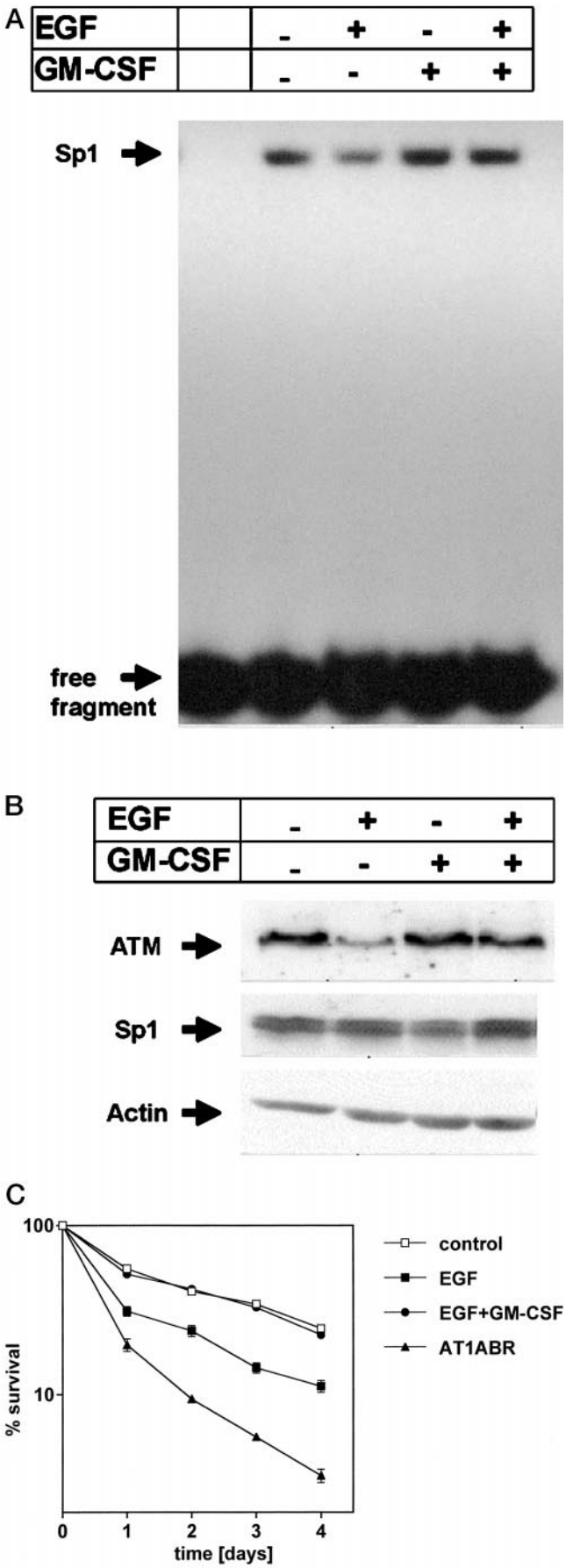

FIG. 6. **Restoration of Sp1 DNA binding activity, amount of ATM, and radiosensitivity GM-CSF after EGF treatment of lymphoblastoid cells.** Cells were treated with EGF (50 ng/ml) for 16 h prior to addition of GM-CSF (10 ng/ml) for 2 h. *A,* Sp1 DNA binding activity was determined by electrophoretic mobility shift assay. *B,* changes in amount of ATM protein detected with anti-ATM antibody. Sp1 protein levels were measured with Sp1 antibody, and actin was used as a loading control. *C,* radiosensitivity was determined as described in the legend to Fig. 1. GM-CSF (10 ng/ml) was added to cells for 1 h after 16 h of EGF treatment.

association between reduction of ATM and radiosensitivity.

The catalytic component (DNA-PKcs) of a second protein involved in DNA damage recognition, DNA-dependent protein kinase (42), was also shown to be down-regulated by EGF, and this would also be expected to increase the radiosensitivity of cells. Down-regulation of both ATM and DNA-PKcs suggests that they may have common regulatory elements responsive to EGF. Indeed this appears to be the case since both genes are organized in head-to-head configuration with other genes, and their putative promoters contain CCAAT boxes and Sp1 consensus sequences. Approximately 0.5 kb separates the 5′ end of the ATM transcript from the 5′ end of another *NPAT*/*E14*/*CAND3* gene, indicating that they share a bidirectional promoter (21, 22, 34). NPAT plays a role in facilitating the entry of cells into S phase (43), and ATM activates the p53 pathway to delay the passage of cells from G_1 to S phase after ionizing radiation exposure (44, 45). DNA-PKcs and Cdc21 (coding for a protein involved in the initiation of DNA replication (46)) also share a bidirectional promoter that contains several CCAAT boxes and Sp1 consensus sites similar to the ATM/NPAT promoter (34, 47). Clearly Sp1 is a candidate for common regulation of these four genes, and its down-regulation or inactivation might account for the effect of EGF in reducing the amounts of ATM and DNA-PKcs. Evidence to support this was provided in a recent report which demonstrated that nitric oxide causes a 4–5-fold increase in expression of DNA-PKcs and protects cells from the toxic effects of a number of agents including ionizing radiation (48). The increased expression of DNA-PKcs was shown to be due to increased binding of Sp1 to the DNA-PKcs promoter. The mechanism by which nitric oxide enhanced Sp1 binding was not elucidated. We have shown here that a decrease in Sp1 DNA binding activity paralleled the EGF-induced decrease in ATM protein, and when the ATM promoter was linked to a reporter gene, transcriptional activation was reduced by EGF. We have established here that GM-CSF which enhances the amount of Sp1 DNA-binding activity rapidly reverses the EGF-induced down-regulation of ATM. Thus there appears to be a causal relationship between the reduction in Sp1 DNA binding activity in EGF-treated cells and the decrease in ATM protein. Evidence for a role for EGF in reducing the amount of Sp1 protein in a rat pituitary cell line (GH_4) has been reported (49). This reduction was accompanied by a 50% reduction in Sp1 binding to a GC-rich element in the gastrin promoter, a 50–60% decrease in Sp1-mediated transactivation of reporter genes and a decrease in cell proliferation. The results obtained here with several normal human fibroblasts and lymphoblastoid cells differ from the data with GH_4 cells, in that Sp1 protein remains unchanged as the DNA binding activity decreases with time after EGF incubation, and in this case also there was no decrease in cell proliferation (results not shown). In our case, it is possible that modification of Sp1 might account for decreased DNA binding activity (35). Several protein kinases alter the DNA binding activity of Sp1 (50), and thus alteration in specific sites of phosphorylation of Sp1 could account for the changes in DNA binding activity reported here.

The human glioblastoma cell line MO59J is characterized by lack of expression of DNA-PKcs and low levels of ATM protein and is extremely sensitive to ionizing radiation (47). Thus it was somewhat surprising in the present study that EGF did

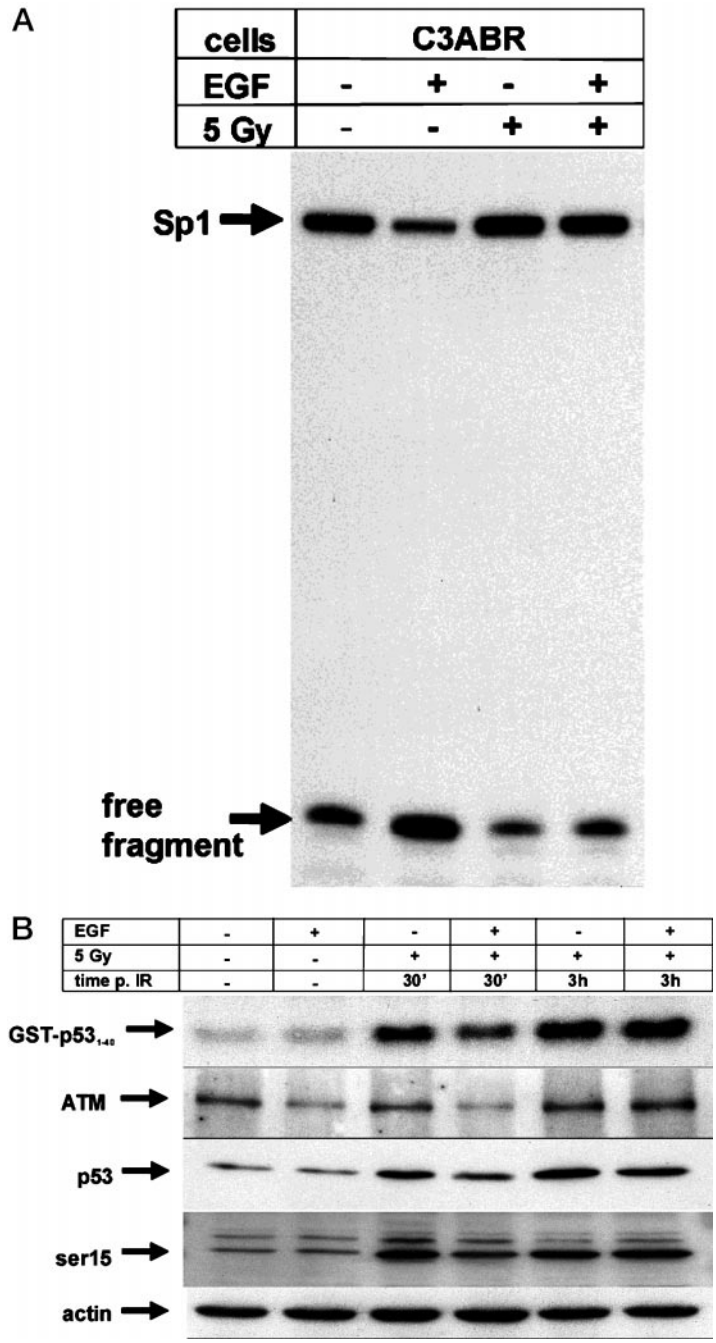

FIG. 7. **Radiation induces Sp1 binding activity and ATM protein/kinase in EGF-pretreated cells.** *A,* effect of radiation on Sp1 DNA binding activity. Cells were either treated with EGF (50 ng/ml, 16 h), radiation (6 Gy, 3 h) alone, or with EGF for 16 h followed by radiation (6 Gy) and subsequent incubation for 3 h. Binding to an Sp1 consensus sequence was as described in the legend to Fig. 4. *B,* effect of radiation on ATM protein and ATM kinase. Untreated or EGF (50 ng/ml, 16 h)-treated cells were subsequently exposed to radiation (6 Gy) and incubated for either 30 min or 3 h prior to preparation of extracts. ATM kinase activity was determined using $p53_{1-40}$ as a substrate (14), and ATM protein was also determined in immunoprecipitates. p53 protein and serine 15 phosphorylated p53 were determined in parallel samples by immunoblotting with specific antibodies. Actin was used as a loading control.

not increase markedly the radiosensitivity of cells in which both ATM and DNA-PKcs were down-regulated. This can be explained by the ability of ionizing radiation to both transcriptionally increase the amount of ATM protein and activate its kinase activity. Compared with activation of pre-existing ATM protein which reaches optimal levels by 20 min post-irradiation (14, 15), EGF-pretreated cells respond more slowly to radiation showing some kinase activity by 30 min but only reaching maximum levels by 3 h post-irradiation. This delay in restoring ATM protein to constitutive levels and in activating ATM kinase appears to account for the reduction in survival in EGF-pretreated cells, post-irradiation, since up-regulation of ATM by GM-CSF restored survival to normal levels. Failure of A-T cells to show any additional radiosensitization also supports this. Although it is likely that EGF operates via Sp1 in reducing the amount of ATM protein, it is apparent that radiation counteracts this. We have shown here that the amount of Sp1 DNA binding activity is rapidly restored when EGF pretreated cells are exposed to radiation, accounting for the increase in ATM. Previous results have revealed that Sp1 DNA binding activity is increased rapidly in human melanoma cells exposed to ionizing radiation (32, 35).

Clarke *et al.* (19) were the first to report alterations of ATM protein in a specific tissue when they failed to detect ATM in normal breast myoepithelial cells, but a significant level of expression was present in the proliferative myoepithelium of sclerosing adenosis. Immunoblotting extracts from freshly isolated PBMCs established that ATM was either absent or present at low levels, but increased ~10-fold in response to mitogenic stimuli over 3–4 days (20). To add to this we have demonstrated here that ATM protein can be down-regulated by EGF to radiosensitize cells, but radiation restores ATM to normal levels and activates its kinase activity, and this is mediated at least in part by the Sp1 transcription factor that has several consensus binding sites in the ATM bidirectional promoter. These observations provide greater insight into the

regulation of ATM that involves not only activation of preexisting protein but also alterations in the amount of ATM protein in response to different stimuli. Determination of ATM expression levels in different tissues/cell types may provide further insight into the response of different tissues to radiation and the factors involved in transcriptional control of the ATM/NPAT promoter. Finally, the results described here raise a fundamental issue in relation to radiosensitization by attenuating ATM protein. Reduction of ATM, in this case by EGF, is countered by radiation exposure that rapidly restores ATM to constitutive levels and would be expected to lessen any therapeutic benefit of radiation. A combination of attenuating ATM, together with inhibitors of its activity, may be a more effective solution.

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Epidermal Growth Factor Sensitizes Cells to Ionizing Radiation by Down-regulating Protein Mutated in Ataxia-Telangiectasia

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