Activation of the Kinase Activity of ATM by Retinoic Acid Is Required for CREB-dependent Differentiation of Neuroblastoma Cells*

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The ATM protein kinase is mutated in ataxia telangiectasia, a genetic disease characterized by defective DNA repair, neurodegeneration, and growth factor signaling defects. The activity of ATM kinase is activated by DNA damage, and this activation is required for cells to survive genotoxic events. In addition to this well-characterized role in DNA repair, we now demonstrate a novel role for ATM in the retinoic acid (RA)-induced differentiation of SH-SY5Y neuroblastoma cells into post-mitotic, neuronal-like cells. RA rapidly activates the kinase activity of ATM, leading to the ATM-dependent phosphorylation of the CREB protein, extrusion of neuritic processes, and differentiation of SH-SY5Y cells into neuronal-like cells. When ATM protein expression was suppressed by short hairpin RNA, the ATM-dependent phosphorylation of CREB was blocked. Furthermore, ATM-negative cells failed to differentiate into neuronal-like cells when exposed to retinoic acid; instead, they underwent cell death. Expression of a constitutively active CREBVP16 construct, or exposure to forskolin to induce CREB phosphorylation, rescued ATM negative cells and restored differentiation. Furthermore, when dominant negative CREB proteins with mutations in either the CREB phosphorylation site (CREB(S133A)) or the DNA binding domain (KCREB) were introduced into SH-SY5Y cells, retinoic acid-induced differentiation was blocked and the cells underwent cell death. The results demonstrate that ATM is required for the retinoic acid-induced differentiation of SH-SY5Y cells through the ATM-dependent phosphorylation of serine 133 of CREB. These results therefore define a novel mechanism for activation of the activity of ATM kinase by RA, and implicate ATM in the regulation of CREB function during RA-induced differentiation.

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Ataxia telangiectasia (A-T)⁴ is an inherited disease characterized by immune deficiencies, premature aging, neurodegeneration, susceptibility to cancer, and extreme sensitivity to ionizing radiation (1, 2). The product of the A-T gene, the ATM protein kinase, is a key component of the signal transduction pathway activated by DNA damage (3, 4). The activity of ATM kinase is activated in response to ionizing radiation-induced DNA damage (5), and ATM then phosphorylates multiple proteins involved in the DNA damage response, including nbs1, p53, chk2, and SMC1 (reviewed in Ref. 4). These phosphorylated proteins, in turn, regulate the 2 key responses to DNA damage: the activation of cell cycle checkpoints and the initiation of DNA repair. Consequently, cells lacking functional ATM protein exhibit multiple defects in DNA repair (6–9) and loss of cell cycle checkpoints (9, 10), resulting in increased sensitivity to ionizing radiation.

Following DNA damage, the activity of ATM kinase is rapidly increased, and ATM is autophosphorylated at multiple sites (11), including serine 1981 (5). Autophosphorylation of ATM is thought to initiate dimer-monomer transition, and release of the active ATM monomer (5, 11), although the exact contribution of ATM autophosphorylation to ATM activation is still under debate (12). Several additional proteins are involved in activation of ATM kinase, including the Mre11-Rad50-Nbs1 complex, and the Tip60 histone acetyltransferase (13–16). The Mre11-Rad50-Nbs1 complex contains a specific ATM binding domain that is required for recruitment of ATM to sites of DNA damage (17). Subsequent activation of ATM depends on the Tip60-dependent acetylation of the ATM protein, which is proposed as the trigger for activating the kinase activity of ATM (13, 14).

In addition to activation of ATM by DNA damage, ATM participates in cellular pathways that are not directly linked to the DNA damage response. For example, compounds that alter chromatin structure, such as trichostatin A or chloroquine, can activate the kinase activity of ATM independently of DNA damage (5). Insulin can also up-regulate the kinase activity of ATM (18), leading to the phosphorylation of insulin target proteins, including protein kinase B and the eIF-4e-binding protein (18, 19). The ATM protein can also influence the levels of the

The abbreviations used are: A-T, ataxia telangiectasia; RA, all-trans-retinoic acid; BDNF, brain-derived neurotrophic factor; RARβ, retinoic acid receptor-β; CREB, cAMP-response element-binding protein; shRNA, short hairpin RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ser(P)-1981, phosphoserine 1981; CBP, CREB-binding protein.
insulin-like growth factor-1 receptor in cells (20). These observations are consistent with the clinical pathology of A-T, which includes mild diabetes and endocrine growth defects (1, 2). ATM can therefore participate in multiple growth factor signaling pathways in addition to the DNA damage response.

Here, the role of the ATM protein kinase in non-DNA damage-dependent pathways was investigated. In particular, the function of ATM in growth factor-regulated cellular differentiation was examined. A well-characterized system for studying differentiation is the retinoic acid (RA)-dependent differentiation of neuroblastoma cells into post-mitotic neuronal-like cells. Exposure of SH-SY5Y cells to RA stimulates RA-dependent gene transcription (reviewed in Ref. 21), up-regulating a diverse array of genes, including TrkB, the receptor for brain-derived neurotrophic factor (BDNF), making the cells responsive to BDNF (22, 23). Sequential exposure of SH-SY5Y cells to retinoic acid and BDNF results in growth arrest and differentiation of the SH-SY5Y cells into post-mitotic neuronal-like cells (24). The fully differentiated SH-SY5Y cells exhibit widespread neuritic arborization, and express multiple neuronal proteins, including tau, GAP-43, MAP-2, transglutaminase, β-amyloid and others (22–26). Here, we demonstrate that RA rapidly activates the kinase activity of ATM leading to phosphorylation of the CREB protein and differentiation of the SH-SY5Y cells into post-mitotic neuronal-like cells. Inactivation of either ATM or CREB blocks RA-driven differentiation, and results in cell death. The results demonstrate a key role for ATM in the RA-dependent differentiation of neuroblastoma cells.

EXPERIMENTAL PROCEDURES

Cell Culture—SH-SY5Y cells (American Type Culture Collection, Manassas, VA) were maintained in a 1:1 mixture of Eagle’s minimum essential medium and Ham’s F-12 medium supplemented with fetal bovine serum (10%), non-essential amino acids, sodium pyruvate, and HEPES buffer. For differentiation experiments, cells were plated at 30% confluence and allowed to grow for 24 h. All-trans-retinoic acid (Biomol, PA) was added to 15 μM and cells were maintained in RA for 5 days, with media change every day. Cells were then washed in serum-free Dulbecco’s modified Eagle’s medium to remove serum and RA, and incubated in Dulbecco’s modified Eagle’s medium supplemented with BDNF (50 ng/ml) for 7 days. Phase-contrast photography of cell lines was carried out using a light microscope and a Nikon Coolpix digital camera. For shRNA experiments, plasmid pBS/ U6ATMΔm01 (described in Ref. 27), which contains an shRNA sequence targeting nucleotides 601–618 of ATM (GGGAGCTGATTTGAGCCACAATTAGCTTATGGTGCATTACAGCTCC) was transfected into SH-SY5Y cells using FuGENE 6 reagent (Roche) according to the manufacturer’s protocol and individual clones selected using hygromycin (300 μg/ml). An ATM cDNA with 3 base changes in the shRNA targeting site (nucleotides 601–618 of ATM, base changes underlined: AGCGGATTGCAATACAT), which altered codon usage but did not change the amino acid sequence, was constructed. Individual clones derived from the shRNA experiment were then transfected with shRNA-resistant ATM, and cells selected with G418 (400 μg/ml). pCMV-CREB, pCMV-CREB513A, and pCMV-KCREB vectors were obtained from BD Biosciences, and cell lines selected with G418 (450 μg/ml).

Immunoprecipitation, Western Blot Analysis, Luciferase Assay, and Kinase Assay—Cells were lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween 20, 1.5 mM MgCl2, 1 mM EGTA, 2 mM dithiothreitol, 50 mM NaF, 500 μM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin, 3 μg/ml leupeptin, 1× phosphatase inhibitor mixture set 1 (Calbiochem). All immunoprecipitates were washed five times in lysis buffer. For kinase assays, immunoprecipitates were washed in kinase buffer (10 mM HEPES, pH 7.4, 10 mM MgCl2, 50 mM NaCl, 10 mM MnCl2), and incubated in kinase buffer (50 μl) supplemented with 50 μM ATP, p53 peptide (2 μg: EPPLSQEAFADL-WKK), and 10 μCi of [γ-32P]ATP for 30 min at 30 °C. Reactions were terminated as described by us (14, 28). SH-SY5Y cells were transiently transfected with CRE-luciferase reporter plasmid (3 μg: Stratagene) using FuGENE 6 (Roche) and allowed to recover for 24 h. Following treatment, cell lysates were prepared and analyzed for luciferase activity using the Luciferase Assay Kit (Stratagene, CA). Antibodies used were: ATM antibodies, PC116 (Oncogene Science, CA), SC2 and SC1 (Genetex, TX), and Ser(P)-1981 ATM (Rockland Immunochemicals, PA). β-Actin (Cell Signaling), CREB and phospho-CREB (Upstate Biotech, NY), TrkB and VP-16 (Santa Cruz Biotechnology, CA) were also used.

Immunofluorescence—Cells were seeded onto LabTek II chamber slides (Nunc, NY), fixed in paraformaldehyde (14, 28), incubated with Ser(P)-1981 ATM antibody (Rockland Biochemicals), washed, and incubated with IgG-Texas Red (Santa Cruz Biotechnology). Slides were mounted with Fluormount-G (Southern Biotech, AL) and visualized with a Nikon Eclipse TE 2000 microscope.

Cell Survival Assays—MTT assays were carried out using the CellTiter Cell Proliferation Assay Kit (Promega). 5 × 103 cells were exposed to RA (15 μM) for specified time points and then incubated with MTT at 37 °C in 5% CO2 for 1.75 h. Absorbance was recorded at 490 nm (using 670 nm as the reference filter) using a Benchmark Plus microplate spectrophotometer (Bio-Rad). Background absorbance was subtracted. KU-55933 was purchased from Sigma.

RESULTS

To evaluate the role of ATM in the signaling pathways required for differentiation of SH-SY5Y neuroblastoma cells, ATM expression was silenced by stable expression of an ATM-specific shRNA vector (27). Supplementary Fig. 1A demonstrates robust suppression of ATM in multiple individual SH-SY5Y clonal cell lines expressing ATM shRNA. Two of these clones, SA2 and SA6, were selected for further analysis. To control for clonal variation and off-gene regulation by the shRNA, an ATM cDNA construct was engineered to contain silent point mutations in the region targeted by the ATM-specific shRNA. Fig. 1A demonstrates the successful re-expression of the shRNA-resistant ATM in SA2 and SA6 cells (Fig. 1, lanes 2 and 4), although the reintroduced ATM was expressed at slightly lower levels than the endogenous ATM protein (Fig. 1A).
Exposure of SH-SY5Y cells containing a nonspecific shRNA (Fig. 1B, shVector) to retinoic acid for 5 days induced growth arrest, followed by the extension of neuritic processes. Subsequent removal of RA and incubation in serum-free media supplemented with BDNF results in elongation of these neuritic outgrowths, forming a complex network of neuritic processes (Fig. 1B), as previously reported (24, 29). In contrast, both SA2 and SA6 cells, in which ATM was silenced, detached from the dish and subsequently underwent cell death when exposed to RA (Fig. 1B). When ATM was re-expressed in the SA2 and SA6 cells (using an shRNA resistant version of the ATM gene), the ability of the cells to differentiate into post-mitotic neuronal-like cells was restored, demonstrating that this phenotype was due to loss of ATM protein rather than a nonspecific effect of the shRNA vector. This effect was quantitated in Fig. 1C, where exposure of SH-SY5Y cells to retinoic acid inhibited cell proliferation, but did not result in detectable changes in cell viability (Fig. 1C). In contrast, SA2 cells exhibited >90% loss of viability following 72 h exposure to retinoic acid (Fig. 1C). However, re-expression of ATM in SA2 cells rescued the cells from retinoic acid-induced cell death (Fig. 1C) and allowed the cells to differentiate. Similar results were seen with SA6 cells (data not shown). These results were further confirmed by treating SH-SY5Y cells with the ATM-specific kinase inhibitor KU-55933 (30). KU-55933 on its own had no significant impact on SH-SY5Y cell viability (supplementary Fig. 1B). However, combined treatment of SH-SY5Y cells with the ATM kinase inhibitor KU-55933 and retinoic acid induced rapid cell death and failure to differentiate (supplementary Fig. 1, B and C). Thus, both pharmacological and genetic inactivation of ATM in retinoic acid-treated SH-SY5Y cells leads to cell death rather than differentiation. Furthermore, the ability of KU-55933 to inhibit the differentiation of SH-SY5Y cells indicates that it is the kinase activity of ATM that is essential for this process.

A key step in the differentiation of SH-SY5Y cells is the RA-dependent up-regulation of growth factor receptors, including RARβ and TrkB, the receptor for BDNF (23, 24, 31, 32). Up-regulation of TrkB levels is required for BDNF to induce terminal differentiation of SH-SY5Y cells (23, 24). Western analysis demonstrated that RA increased the levels of RARβ and TrkB receptors in both SH-SY5Y and in the ATM-deficient SA2 and SA6 (supplementary Fig. 2A). The inability of SA2 and SA6 cells to differentiate is therefore not due to a failure to up-regulate RA driven transcription of the RARβ and TrkB genes. Overall, Fig. 1 demonstrates a central role for the ATM protein in the retinoic acid-induced differentiation of SH-SY5Y neuroblastoma cells into post-mitotic neuronal-like cells.

The requirement for ATM in RA signaling implies that RA activates the kinase activity of ATM. The up-regulation of the kinase activity of ATM by DNA damage causes autophosphorylation of serine 1981, which can be monitored by immunofluorescence using a phospho-specific antibody to detect autophosphorylation of serine 1981 of ATM 28 (Fig. 2A). As shown by other groups (5, 16, 17), addition of RA to ATM-negative cells to ionizing radiation (A-T) did not result in phosphorylation of serine 1981 of ATM as detected by immunofluorescence, whereas re-expression of ATM in these cells (A-T/ATM, described by us in Refs. 14 and 28) allows detection of autophosphorylated ATM in irradiated, but not control cells. Thus, the phospho-serine 1981 antibody can be used to monitor ATM autophosphorylation in vivo, as shown by other groups (5, 16, 17). Addition of RA to SH-SY5Y cells resulted in rapid ATM autophosphorylation within 15 min of RA addition, and ATM remained autophosphorylated throughout the 5-day exposure to RA (Fig. 2A). Subsequent withdrawal of RA led to loss of ATM autophosphorylation (Fig. 2A) and the cells subsequently underwent cell death (data not shown). However, addition of BDNF (in the absence of RA) maintained ATM in the active, autophosphorylated form (Fig. 2B), and the cells remained in the growth arrested, differentiated state for up to 2 weeks in culture (Fig. 1B). Supplementary Fig. 2B demonstrates that ATM protein levels do not significantly change during the differentiation of SH-SY5Y cells, indicating that the increased phosphorylase 1981 autophosphorylation signal is due to ATM autophosphorylation rather than increased ATM protein levels. Fig. 2A therefore demonstrates increased ATM autophosphorylation, implying increased ATM kinase activity is maintained throughout the RA/BDNF-induced differentiation of SH-SY5Y cells.

To determine whether RA increased ATM autophosphorylation...
Retinoic Acid Activates ATM

Retinoic acid induces rapid ATM autophosphorylation. Immunofluorescent detection of ATM autophosphorylated on serine 1981 detected 15 min later.

Figures 3A, 3B, and 3C demonstrate that the kinase activity of ATM was increased to the same levels seen following DNA damage (Fig. 3A), consistent with the rapid autophosphorylation of ATM seen in Fig. 2. Figs. 2A, 2D, and 3C therefore demonstrate that the kinase activity of ATM is increased when SH-SY5Y cells are exposed to RA.

Retinoids are potent inducers of neuronal differentiation and activate the CREB transcriptional regulatory protein (31, 36). CREB plays a key role in cellular differentiation, with a particularly important function in regulating neuronal function, including neuronal survival (37). The ability of CREB to regulate neuronal survival suggested that ATM may promote differentiation of SH-SY5Y cells through activation of the CREB transcriptional regulator. CREB activation involves the phosphorylation of serine 133 of CREB, which creates a binding site for the CBP histone acetyltransferase, which in turn regulates CREB transcriptional activity (38).
CREB phosphorylation was monitored using a phospho-specific antibody that recognizes phosphoserine 133 of CREB. In Fig. 3B, SH-SY5Y cells exhibited increased phosphorylation of serine 133 of CREB within 5 min of RA exposure (Fig. 3B). Furthermore, phosphorylation of CREB was still detected at 6 and 24 h post-RA treatment. In addition, the autophosphorylation of ATM on serine 1981 (detected by Western blot; Fig. 3C) was also increased 6 and 24 h post-RA exposure. RA therefore induces both activation of the kinase activity of ATM (Figs. 2A and 3A) and phosphorylation of serine 133 of CREB within 15 min of RA addition (Fig. 3, B and C). Furthermore, phosphorylation of CREB and autophosphorylation of ATM are maintained throughout the differentiation of SH-SY5Y cells (Figs. 2A and 3C).

The activation of the kinase activity of ATM and phosphorylation of CREB suggests that ATM may be required for CREB phosphorylation. To test this, the ability of RA to increase the phosphorylation of CREB in the ATM-deficient SH-SY5Y cells was examined. In Fig. 3D, RA increased phosphorylation of CREB on serine 133 in SH-SY5Y cells, but not in the ATM-deficient SA2 or SA6 cell lines, despite the presence of normal levels of CREB protein in these cells (Fig. 3D). Because CREB can be phosphorylated and activated by a diverse array of protein kinases (38), we examined if loss of CREB activation in the SA2 and SA6 cells was specific for RA or if it extended to other potential activators. Cells were treated with forskolin, an activator of adenylyl cyclase, which increases cAMP levels and induces phosphorylation of serine 133 of CREB (39). In Fig. 3E, forskolin treatment stimulated phosphorylation of CREB in both control and ATM-deficient SA2 and SA6 cells. Fig. 3 therefore demonstrates that ATM is required for the phosphorylation of serine 133 of CREB in response to RA. However, CREB phosphorylation in response to other stimuli, including elevated cAMP levels, was independent of ATM.

Phosphorylation of serine 133 of CREB creates a binding site for the CBP histone acetyltransferase, which in turn regulates CREB transcriptional activity (38). To determine whether the RA-dependent phosphorylation of CREB leads to increased transcriptional activity, SH-SY5Y cells were transfected with a luciferase reporter gene linked to a CREB DNA binding element to monitor CREB transcriptional activity. Cells transfected with a promoterless vector had minimal luciferase activity. Both forskolin and RA increased the transcriptional activity of the CRE-Luciferase reporter construct (Fig. 3F), consistent with the ability of both of these agents to increase phosphorylation of serine 133 of CREB (Fig. 3). To determine whether this increased CREB transcriptional activity required ATM, cells were preincubated in the ATM-specific inhibitor KU-55933. KU-55933 had no significant effect on basal transcriptional activity, or on forskolin-stimulated CREB activity. However, the ATM inhibitor KU-55933 specifically inhibited RA-dependent CREB transcriptional activity. These results are consistent with the results from Fig. 3, in which RA-dependent CREB phosphorylation required ATM, whereas forskolin-dependent CREB phosphorylation did not. Overall, Fig. 3 demonstrates that RA activates both ATM kinase activity and CREB transcriptional activity, and that ATM is required for this increased CREB transcriptional activity.

A key question is whether the ATM-dependent activation of CREBs transcriptional activity involves the direct phosphorylation of CREB by ATM. Serine 133 of CREB has no homology to other sequences phosphorylated by ATM (which requires the sequence (S/T)Q), making it unlikely that ATM directly phosphorylates CREB. Indeed, previous work demonstrated that ATM phosphorylates CREB in a DNA damage-dependent manner on several serines, including serine 121, but not serine 133 (40). However, although we can detect increased phosphorylation of serine 121 of CREB in response to DNA damage, RA-dependent ATM activation did not lead to any increased phosphorylation of serine 121 of CREB (supplementary Fig. 2C). This indicates that RA and DNA damage signals activate CREB through distinct ATM-dependent pathways. We have further shown that ATM and RARβ exists as a complex in the cell (supplementary Fig. 2D), implicating an ATM/RARβ complex in the RA-dependent activation of CREB.

CREB is required for neuronal survival (37), implying that the ATM-dependent activation of CREB is essential for the retinoic acid-induced differentiation of SH-SY5Y cells. To determine whether CREB is required for RA-dependent differentiation, two dominant negative CREB constructs, CREBS133A (with a mutation in serine 133, which blocks phosphorylation and transactivation (38)) and KCREB (with mutations in the DNA binding domain that abolish transcriptional activity (38)) were used. Fig. 4A demonstrates stable expression of CREB, KCREB, and CREBS133A in SH-SY5Y cells. When SH-SY5Y cells expressing either vector or CREB were exposed to RA, they underwent growth arrest (Fig. 4B) and began to extrude neuritic processes (Fig. 4C). Furthermore, treatment with BDNF resulted in full differentiation of SH-SY5Y cells expressing either vector or wild type CREB, and was marked by the appearance of an extensive network of neuritic processes (Fig. 4C, magnified image). In contrast, expression of either KCREB or CREBS133A in SH-SY5Y cells was accompanied by a significant decrease in cell viability following exposure to RA (Fig. 4B), similar to that seen in ATM-deficient SH-SY5Y cells (Fig. 1C). However, unlike the ATM-deficient cells, a small fraction (~20%) of the KCREB and CREBS133A cells remained viable, and could be followed throughout the normal differentiation protocol. These surviving cells may reflect residual CREB activity in these cells, as well as the presence of ATM signaling pathways that do not converge on CREB. These surviving KCREB and CREBS133A cells exhibited shorter neuritic processes than the parental SH-SY5Y cells, and these processes did not extend across the vacant regions of the dish (Fig. 4C, magnified image). Fig. 4 therefore demonstrates that inhibition of CREB function replicates many of the properties of the ATM-deficient cells, including increased RA-induced cell death and incomplete differentiation. This demonstrates that the ATM-dependent phosphorylation of serine 133 of CREB and the transcriptional activity of CREB are essential for differentiation of SH-SY5Y cells in response to RA (Fig. 3). Furthermore, this implies that the failure to activate CREB in the ATM-deficient cells is a critical factor that leads to the increased cell death.

To test if activation of CREB by ATM is essential for cell survival, we determined if activation of CREB in the ATM-
deficient cells could bypass the requirement for ATM. For this, we used CREBVP16, in which the transactivation domain of VP16 is fused to the DNA binding domain of CREB. CREBVP16 exhibits constitutive CREB activity, and has been shown by several groups to function independently of upstream regulatory signals (38, 41–43). In addition, the adenyate cyclase agonist forskolin was used to activate the cAMP-dependent protein kinase, leading to phosphorylation and activation of CREB (44).

Supplementary Fig. 2 demonstrates overexpression of CREBVP16 in SA2 cells, and Fig. 3E demonstrates the ability of forskolin to induce phosphorylation of serine 133 of CREB in SH-SY5Y, SA2, and SA6 cells. In Fig. 5A, ATM-deficient SA2 cells exposed to RA underwent rapid cell death. However, incubation of SA2 cells with forskolin or transfection with CREBVP16 rescued the SA2 cells and they survived exposure to RA. Furthermore, Fig. 5, B and C, demonstrate that CREBVP16 and forskolin both restored the ability of RA to stimulate the extrusion of short neuritic processes in the ATM-deficient SA2 cells. However, when RA was withdrawn from these cells, and the cells switched to minimal media supplemented with BDNF, both the forskolin and CREBVP16 complemented cells underwent cell death, and failed to achieve the fully differentiated state seen in Fig. 1 (data not shown). Activating CREB in ATM-deficient cells therefore bypasses the requirement for ATM during the early stages of differentiation of SH-SY5Y cells by RA. However, constitutive CREB activation by forskolin and CREBVP16 was insufficient for full BDNF driven differentiation, presumably due to either loss of additional ATM-dependent pathways, or due to incomplete spatio-temporal regulation of CREB activation in the experimental system.

Retinoic Acid Activates ATM

FIGURE 4. Dominant negative CREB proteins block RA-induced differentiation. A, expression of CREB, CREB{S133A}, or KCREB in SH-SY5Y cells was detected by Western blot (WB) analysis. B, SH-SY5Y cells expressing the indicated CREB construct were incubated with RA and cell viability measured by MTT assay. Results ± S.D. (n = 6). C, SH-SY5Y cells expressing the indicated construct were differentiated by sequential exposure to RA and BDNF. Asyn, cells growing asynchronously in 10% fetal calf serum. RA, cells cultured with RA for 5 days in the presence of 10% fetal calf serum. RA/BDNF, following retinoic acid exposure, cells were switched into serum-free medium supplemented with BDNF for a further 5 days. Regions containing neuritic processes are shown in magnified format.

FIGURE 5. Activation of CREB bypasses the requirement for ATM. A, SH-SY5Y cells (○), ATM-deficient SA2 cells (●), SA2VP16CREB cells (□), or SA2 cells plus forskolin (△) were incubated with retinoic acid for the indicated times. Results ± S.D. (n = 6). B, asynchronous SA2 cells (Asyn) were incubated with RA (15 μM) or RA plus forskolin (50 μM) for 4 days. C, asynchronously growing SA2 cells (Asyn) stably transfected with VP16 CREB (SA2VP16CREB) were exposed to RA for 4 days.
DISCUSSION

These results indicate that RA activates the intrinsic kinase activity of ATM. This conclusion is based on several observations, including the ability of RA to increase the autophosphorylation of ATM on serine 1981 in vivo, and direct measurement of increased ATM kinase activity in vitro. In addition, the ATM kinase inhibitor KU-55933 was able to block ATM-dependent differentiation of SH-SY5Y cells, further confirming an essential role for the kinase activity of ATM in this process. Activation of ATM by genotoxic events leads to the recruitment of ATM to discrete nuclear foci that form at DNA strand breaks (Fig. 2) (5, 14). However, activation of the kinase activity of ATM by RA results in a diffuse nuclear distribution of activated ATM, similar to that observed when ATM is activated through non-DNA damage pathways (5). We interpret this to mean that ATM activation by RA does not involve DNA damage. Previous studies have also shown that ATM can be activated by insulin (18, 19), indicating that ATM can participate in multiple receptor-linked signaling pathways in the cell. Interestingly, ATM does not appear to be required for RA-regulated gene transcription, because loss of ATM did not affect the transcriptional up-regulation of either TrKB or RARβ by RA (supplementary Fig. 1C). This implies that activation of ATM by RA is dispensable for RAR-dependent transcription of RA-responsive genes.

The mechanism by which retinoic acid activates ATM is not clear. A potential explanation is that RA alters the transcription of genes that regulate ATM activity. However, the rapidity of ATM activation (<15 min after RA exposure; Fig. 2) argues against a role for RA-dependent gene transcription in ATM activation. An alternative mechanism for ATM activation is suggested by reports that agents that induce an open, relaxed chromatin structure activate the activity of ATM kinase independently of DNA damage (5). In the unliganded state, RARs bind to target genes in a corepressor complex, repressing transcription and maintaining the chromatin in a compacted form. RA binding by RAR causes dissociation of repressor complexes and recruitment of coactivator proteins, including the p300/CBP histone acetyltransferase and SWI/SNF chromatin remodeling complexes, leading to acetylation and structural changes in the chromatin that promote transcription (reviewed in Ref. 21). The overall effect is to convert the repressed, compacted chromatin structure to a more open, transcriptionally active form. The RA-induced remodeling of the local chromatin structure adjacent to the liganded RAR complexes may therefore provide the signal to activate the ATM protein. Furthermore, ATM and RARβ were shown to co-precipitate from cell extracts, indicating the presence of an ATM-RARβ complex in SH-SY5Y cells. Given the wide distribution of RA responsive genes in eukaryotic cells (21), localized changes in chromatin structure at 100s of sites in response to RA binding to the ATM-RARβ complex may be sufficient to trigger ATM activation.

The results demonstrated a key role for ATM in the RA-dependent phosphorylation of serine 133 of CREB. In the absence of ATM, RA-induced phosphorylation of serine 133 of CREB was lost, indicating that the kinase activity of ATM is upstream of CREB phosphorylation. It is unlikely that ATM directly phosphorylates serine 133 of CREB because it is not contained within an ATM consensus phosphorylation site, and ATM does not directly phosphorylate serine 133 in vitro (40). Furthermore, in response to DNA damage, ATM phosphorylates CREB at several sites, including serine 121, but not serine 133 (40). This phosphorylation of CREB on serine 121 by ATM after DNA damage was associated with decreased CREB transcriptional activity (40). In our experiments, RA did not induce any detectable phosphorylation of CREB at serine 121. ATM therefore appears to regulate distinct patterns of CREB phosphorylation in response to DNA damage (phosphorylation of serine 121 of CREB and transcriptional repression (40)) and RA (phosphorylation of serine 133 of CREB and transcriptional activation). The activation of ATM by RA does not, therefore, lead directly to the phosphorylation of serine 133 of CREB. Instead, ATM may modulate the activity of one or more CREB kinases. Multiple CREB kinases have been identified (reviewed in Ref. 38), and RA can activate several of these, including protein kinase B and the extracellular signal-regulated kinase (ERK) and RSK kinases (31, 32, 45). Furthermore, the ability of RA to activate these kinases was independent of RA-Rs transcriptional regulatory functions (31, 32). ATM has also been shown to be upstream of protein kinase B (19), and RSK kinases (46, 47). ATM may therefore function as the intermediary between RA and the activation of upstream CREB kinases that directly phosphorylate serine 133 of CREB and activate its transcriptional activity. ATM may therefore be a key component of a non-transcriptional RA signaling pathway that mediates early signaling events in the cell following RA exposure.

These results have revealed a novel role for the ATM protein kinase in the RA-dependent differentiation of SH-SY5Y cells. The differentiation of SH-SY5Y cells into neuronal-like cells requires the ATM-dependent phosphorylation of serine 133 of CREB and the subsequent activation of CREB transcriptional activity. CREB plays a key role in neural development, memory, plasticity, and in neuronal survival (36, 38). Previous work in transgenic mouse models has shown that CREB is a survival factor for differentiating neurons (36–38, 48). The results presented here are consistent with this model, in which ATM activates a CREB-regulated survival pathway during differentiation of neuroblastoma cells. These results also have implications for the neurodegeneration seen in A-T patients. Neurodegeneration in A-T patients occurs through the cumulative loss of purkinje cells, leading to the characteristic ataxia and cerebellar atrophy (1, 2). Accumulating evidence suggests that defective DNA repair, due to loss of ATM, underlies this progressive neurodegeneration in A-T patients (49–51). The results presented here indicate that ATM may also play an additional role in mediating the survival of neurons through regulating the activation of CREB in response to RA. Furthermore, ATM can phosphorylate and inactivate CREB transcriptional activity in response to ionizing radiation (40). ATM may therefore play a dual role in regulating CREB, increasing CREB activity in neuronal cells in response to growth signals from retinoic acid or BDNF, and decreasing CREB activity in response to DNA damage through direct phosphorylation of unique CREB residues. The ability of ATM to tightly regulate CREB activity may then control the
balance between cell survival and cell death in neuronal cells exposed to DNA damage. Consequently, in A-T patients, the loss of functional ATM would loosen the control on CREB activity, particularly in response to DNA damage, and this may lead to the eventual death of the neuron. In conclusion, these results have revealed a novel role for the ATM protein kinase in the retinoic acid-induced differentiation of SH-SY5Y cells. The ability of RA to activate the kinase activity of ATM independently of DNA damage, and the ability of ATM to activate CREB indicate that ATM may have important cellular functions in addition to its role as a sensor of DNA damage.

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