Doxorubicin Activates ATM-dependent Phosphorylation of Multiple Downstream Targets in Part through the Generation of Reactive Oxygen Species*

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The requirement for the serine/threonine protein kinase ATM in coordinating the cellular response to DNA damage induced by ionizing radiation has been studied extensively. Many of the anti-tumor chemotherapeutics in clinical use today cause DNA double strand breaks; however, few have been evaluated for their ability to modulate ATM-mediated pathways. We have investigated the requirement for ATM in the cellular response to doxorubicin, a topoisomerase II-stabilizing drug. Using several ATM-proficient and ATM-deficient cell lines, we have observed ATM-dependent nuclear accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues. This was accompanied by an increased binding of p53 to its cognate binding site, suggesting transcriptional competency of p53 to activate its downstream effectors. Treatment of cells with doxorubicin led to the phosphorylation of histone H2AX on serine 139 with dependence on ATM for the initial response. Doxorubicin treatment also stimulated ATM autophosphorylation on serine 181 and the ATM-dependent phosphorylation of numerous effectors in the ATM-signaling pathway, including Nbs1 (Ser245), SMC1 (Ser285), Chk1 (Ser317 and Ser345), and Chk2 (Ser333 and Thr68). Although generally classified as a topoisomerase II-stabilizing drug that induces DNA double strand breaks, doxorubicin can intercalate DNA and generate reactive oxygen species. Pretreatment of cells with the superoxide scavenger ascorbic acid had no effect on the doxorubicin-induced phosphorylation and accumulation of p53. In contrast, preincubation of cells with the hydroxyl radical scavenger, N-acetylcysteine, significantly attenuated the doxorubicin-mediated phosphorylation and accumulation of p53, p53-DNA binding, and the phosphorylation of H2AX, Nbs1, SMC1, Chk1, and Chk2, suggesting that hydroxyl radicals contribute to the doxorubicin-induced activation of ATM-dependent pathways.

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DNA double strand breaks (DSBs) are among the most cytotoxic DNA lesions. They arise through both endogenous (e.g. oxidative respiration) and exogenous (e.g. ionizing radiation (IR)) sources. In response to DSBs, cells must react immediately to repair the lesion, arrest the cell cycle to facilitate repair, or, in cases when damage is too extensive, initiate apoptosis.

Ataxia-telangiectasia mutated (ATM) is a member of the phosphoinositide 3-kinase-like family of serine/threonine protein kinases (reviewed in Refs. 1–3). ATM plays a central role in the cellular response to IR-induced DNA damage, essentially acting as a critical switch controlling whether and when a cell arrests following DNA damage. In response to DNA DSBs induced by IR, ATM, which exists in an unstimulated cell as an inactive homodimer or higher order multimer, autophosphorylates to generate the active, monomeric kinase (4). Activation of ATM results in the phosphorylation of a diverse array of downstream targets that participate in numerous cellular events, including DNA damage recognition and processing, regulation of three cell cycle checkpoints (G1, intra-S, and G2/M), and apoptosis (1–3). Among the most well studied targets are the tumor suppressor protein p53 and the checkpoint kinase Chk2.

To date, most studies have investigated the effects of IR on the activation of ATM and ATM-dependent signaling pathways. IR is a potent DNA-damaging agent, inducing both DNA single strand breaks and DSBs, in large part through the actions of reactive oxygen species (ROS) generated by the ionization of water molecules in the cell and through lipid peroxidation. In addition to IR, many of the anti-tumor chemotherapeutics commonly used in the treatment of cancer induce, either directly or indirectly, DSBs, yet, at present, few DNA-damaging chemotherapeutics have been evaluated for their ability to activate ATM and ATM-dependent signaling pathways. It is well established, however, that numerous anti-cancer drugs induce the nuclear accumulation of p53 (5, 6). The ability of these chemotherapeutics to induce p53 accumulation has been correlated directly with the DNA damaging capacity of the drug (5).

Several key pieces of evidence support a role for ATM in drug-induced DNA damage. Recently, arsenite, a potent human carcinogen that induces DSBs, was reported to induce p53 accumulation in an ATM-dependent manner (7). This increase in p53 was linearly correlated with strand break induction. Hexavalent chromium (Cr(VI)), a broad spectrum DNA-damaging agent, activates ATM kinase activity and induces the...
phosphorylation of p53 on serine 15 (8). Genistein, a tyrosine kinase inhibitor and topoisomerase II poison, activates ATM protein kinase activity and induces phosphorylation of ATM on serine 1981 and the ATM-dependent phosphorylation of histone H2AX on serine 139 and p53 on multiple serine residues (9, 10). Although not classically considered a DNA-damaging chemotherapeutic, the monofunctional DNA-alkylating agent N-methyl-N'-nitro-N-nitosoguanidine stimulates ATM kinase activity and the ATM-dependent phosphorylation of p53 in serum 15, possibly triggered by the strand breaks created during the DNA repair process (11). Given the critical role for ATM in the cellular response to DSBs and the prominent, although not exclusive, role for ATM in the phosphorylation of p53 in response to DNA damage, we sought to examine the effects of the anti-tumor anthracycline, doxorubicin, on ATM and its downstream effectors.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Doxorubicin, wortmannin, ascorbic acid, and N-acetylcysteine (NAC) were purchased from Sigma. Stock solutions of doxorubicin and wortmannin were prepared in dimethyl sulfoxide, protected from light, and stored at −20 °C. Stock solutions of ascorbic acid and NAC were prepared fresh in 0.9% NaCl, with the pH of the NAC stock solution adjusted to pH 7.5.

**Cells**—ATM-proficient (BT and C3ABR) and ATM-deficient (L3 and AT1ABR) human lymphoblastoid cell lines were as previously described (9, 10). Cells were maintained as suspension cultures in either RPMI 1640 (BT and L3) or Dulbecco’s modified Eagle’s medium/P-12 (C3ABR and AT1ABR) media (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. Chemotherapeutics, inhibitors, antibod-317ies, or equivalent volumes of carrier were added directly to the cell medium at the start of each experiment, unless otherwise stated. Where indicated, cells were irradiated in the presence of serum-free medium using a Gammacell 1000 cesium-137 source (MDS Nordion, Ottawa, Canada).

**Antibodies**—The p53-specific monoclonal antibody DO-1, agarose-conjugated DO-1, and agarose-conjugated Pab1801 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphospecific antisera to serines 6, 9, 15, 20, 37, 46, and 392 of human p53, serines 17 and 345 of Chk1, and serines 317 and 345 of Chk2 were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA), as was an antibody reactive for the total pool of Chk1. Phosphospecific antisera to serine 343 of Nbs1 and serine 957 of SMC1 were purchased from Novus Biologicals (Littleton, CO), as were antibodies to histone H2AX (Upstate Biotechnology, Lake Placid, NY). A rabbit polyclonal antibody specific for the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) was raised against a recombinant protein fragment (amino acids 2018–2136) and has been previously described (12).

**Immunoblots and Immunoprecipitation**—Crude nuclear protein extracts (500 μg NaCl extraction) were prepared from logarithmically growing cells, as previously described (9). Protein concentrations were determined using the Bradford-based Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as the standard. For immunoblots, 30 μg of protein were resolved by SDS-PAGE and probed with antibodies to p53 (DO-1), actin, or a phosphospecific antisem to p53 phosphorylated at serine 15.

Detection and analysis of p53 phosphorylation at serines 6, 9, 20, 37, 46, and 392 were performed after immunoprecipitation of p53 using agarose-conjugated DO-1 and Pab1801 antibodies as described previously (13). Immunoprecipitation/immunoblot experiments for the detection of p53 phosphorylation at these sites were carried out as described (10).

For the analysis of ATM phosphorylation at serine 1981 and the phosphorylation of other downstream effectors of ATM, whole cell extracts were prepared from logarithmically growing cells. Briefly, 8–10 × 106 cells were harvested, washed twice in phosphate-buffered saline (PBS), and lysed by sonication in NET-N buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5, 1% (v/v) Nonidet P-40 containing 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin A) and phosphatase inhibitors (1 mM activated Na3VO4, 25 mM NaF, 1 μM microcystin-LR). Protein concentrations of cleared lysates were determined using a Lowry-based, detergent-compatible protein assay (Bio-Rad) using bovine serum albumin as a standard. For immunoblots examining ATM, DNA-PK, or SMC1, 60 μg of protein were separated on 8% SDS-polyacrylamide gels (0.25% acrylamide/bisacrylamide), transferred to nitrocellulose in SDS-electroblot buffer (25 mM Tris, 192 mM glycine, 0.04% (v/v) SDS, 20% (v/v) methanol) at 100 V for 60 min. For all other proteins, 60 μg of protein were separated on 10% SDS-polyacrylamide gels (29:2:0:8 acrylamide/bisacrylamide) and transferred to nitrocellulose as described above, but without the addition of SDS to the electroblot buffer.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assays using crude nuclear extracts (500 μM NaCl extraction) were performed as described previously (9).

**Immunofluorescence Microscopy for Histone H2AX Phosphorylation**—To evaluate the phosphorylation of histone H2AX on serine 139, logarithmically growing ATM-proficient BT and ATM-deficient L3 cells were treated as described above for immunoblots. Cells were harvested and resuspended twice in PBS, washed once in PBS, and 1 × 106 cells in each were centrifuged onto coverslips (800 rpm, 5 min). Cells were fixed in 3.7% (v/v) formaldehyde for 10 min at room temperature, followed by permeabilization in PBS containing 0.5% (v/v) Triton X-100 for 10 min. Samples were blocked in PBS containing 1% (w/v) bovine serum albumin for 30 min prior to incubation with primary antibody (1:400 in blocking buffer) at room temperature for 2 h and extensive washing in PBS containing 0.05% (v/v) Tween 20. Cells were then incubated with Alexa 488-conjugated goat anti-mouse (Molecular Probes, Inc., Eugene, OR) secondary antibody (1:500 in blocking buffer) for 30 min at room temperature, followed by further PBS/Tween 20 washes. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (1 μg/ml in PBS) (Sigma) for 10 min. After extensive washing in PBS, coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA). For detection of immunofluorescence, slides were viewed using a Leica DMRXA2 microscope. For each experimental point, an average of 250 cells was scored for reactivity on serine 139-phosphorylated H2AX as a percentage of the total number of cells within the field. To avoid bias in selecting fields to score, the observer was blinded to the experimental treatment and selected fields and counted cells in the 4,6-diamidino-2-phenylindole channel prior to observing them in the fluorescein isothiocyanate channel. For each field, the percentage value of 4H2AX-positive cells was calculated, and the mean and S.D. of values from multiple fields within an experimental point were determined and represented graphically using Prism (version 3.0) software (GraphPad Software, San Diego, CA). The differences between ATM-proficient and ATM-deficient cells or the effect of pretreatment with NAC was analyzed using an unpaired, one-tailed Student’s t test. p values less than 0.005 were deemed statistically significant.

**Image Analysis**—Image analysis was performed using ImageQuant software (Amersham Biosciences). In the evaluation of specific phosphorylation events, phosphorylation levels were normalized to total protein levels by dividing the intensity of the phosphospecific signal by the intensity of the signal measured from blots using antibodies recognizing the total pool of protein.

**RESULTS**

**Doxorubicin Induces ATM-dependent Stabilization and Phosphorylation of p53 on Serine 15**—Previous studies have shown that phosphorylation of p53 in response to IR is mediated by the ATM protein kinase (14, 15) and that ATM is important for p53 stabilization and for stimulating the transactivation functions of p53 at early times after IR. Doxorubicin has previously been shown to stimulate the nuclear accumulation and phosphorylation of p53 (5, 13), but neither of these studies investigated the requirement for ATM in these events. Here, we demonstrate that doxorubicin-induced stabilization and phosphorylation of p53 on serine 15 at early time points following treatment occur only in the presence of the ATM protein kinase (Fig. 1, A and B). Treatment of ATM-proficient BT cells with doxorubicin (1 μM) induced phosphorylation of
with dimethyl sulfoxide (0.5%) and incubated for 2 h served as a positive control (C). Specific antibody (DO-1) to p53 (p53) and a polyclonal antiserum to actin.

Nuclear extracts were prepared and analyzed by sequential immunoblotting using antibodies to p53 and phosphorylation on serine 15 require ATM and are abrogated by pretreatment with wortmannin.

ATM-proficient (C3ABR) and ATM-deficient (AT1ABR) cell lines (data not shown).

Very similar results were obtained in experiments with a second pair of ATM-proficient (BT) and ATM-deficient (L3) cells and immunoblotted with phosphospecific antisera to p53. Doxorubicin treatment induced the phosphorylation of p53 at serines 6, 9, 20, 37, 46, and 392, and, in all cases, phosphorylation was ATM-dependent (Fig. 2). In contrast, only very weak, if any, phosphorylation was observed at a later time point (4 h) in ATM-deficient cells. Very similar results were obtained in C3ABR (ATM-proficient) and AT1ABR (ATM-deficient) cells (data not shown).

**Doxorubicin Stimulates p53-DNA Binding in ATM-proficient Cells**—To determine whether the doxorubicin also stimulated p53 to bind its cognate DNA binding site, nuclear extracts isolated from doxorubicin-treated ATM-proficient (BT) and ATM-deficient (L3) cells were analyzed by an electrophoretic mobility shift assay. Treatment of ATM-proficient cells with doxorubicin was found to increase dramatically the ability of p53 to bind its cognate DNA binding site (Fig. 3). In contrast, binding of p53 to DNA was significantly reduced in ATM-deficient cells.

**Doxorubicin Induces Phosphorylation of ATM on Serine 1981—Stimulation of ATM kinase activity following irradiation has recently been demonstrated to occur after autophosphorylation of ATM on serine 1981 (4). To examine whether doxorubicin could also induce autophosphorylation of ATM on serine 1981, ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) for 120 min prior to extract preparation and immunoblotting with a phosphospecific antisera to serine 1981 of p53. Doxorubicin-induced phosphorylation of ATM on serine 1981—

Although p53 is an important target of ATM, activation of ATM results in the phosphorylation of a diverse array of downstream targets that participate in multiple cellular processes. Analysis of ATM-dependent phosphorylation of multiple downstream effectors in the ATM-Signaling Pathway—Although p53 is an important target of ATM, activation of ATM results in the phosphorylation of a diverse array of downstream targets that participate in multiple cellular processes. Analysis of ATM-dependent phosphorylation of one substrate cannot provide an accurate picture of the complexity of the cellular response. To gain a broader perspective on the requirement for ATM in the early cellular response to doxorubicin treatment, ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) and incubated for 60 or 120 min prior to extract preparation and immunoblotting with phosphospecific antisera to known downstream effectors of ATM. Exposure to doxorubicin induced the ATM-dependent phosphorylation of all substrates tested. Interestingly, the sub-
strates appear to stratify into two groups, those manifesting in early phosphorylation events (≤60 min) and late phosphorylation events (>60 min). Serine 15 of p53 (Fig. 1A), threonine 68 of Chk2 (Fig. 4B) and serine 343 of Nbs1 (Fig. 4B) were all phosphorylated in an ATM-dependent manner within 60 min of the initiation of doxorubicin treatment. In contrast, ATM-de-
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Fig. 3. Doxorubicin stimulates p53-DNA binding in ATM-proficient (BT) cells. Oligonucleotides containing a consensus p53 binding site were annealed and end-labeled with [γ-32P]ATP. Nuclear extracts (9 μg of protein) from untreated or doxorubicin-treated (1 μM, 2 h) ATM-proficient (BT) or ATM-deficient (L3) cells were assayed for binding activity to the 32P-labeled binding site in the presence of 1 μg of poly(dI-dC)·poly(dI-dC) and 4 μl of the p53 monoclonal antibody Pab421 (to stabilize the binding of p53 to its cognate binding site) (56). The DNA-protein complex (bound) was separated from free probe (free) by electrophoresis through a nondenaturing, 4.5% polyacrylamide gel.

Fig. 4. Doxorubicin induces the autophosphorylation of ATM on serine 1981 and ATM-dependent phosphorylation of multiple downstream effectors in the ATM signaling pathway. A, ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM, 2 h) or exposed to 10-Gy IR and allowed to recover for 2 h prior to harvest. Whole cell extracts were prepared and analyzed by sequential immunoblotting using a phosphospecific antiserum to ATM (4BA), and a polyclonal antiserum to DNA-PKcs (to verify the loading of comparable protein levels in all lanes). B, ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) and harvested at the indicated times. Whole cell extracts were prepared, and the phosphorylation status of downstream effectors within the ATM signaling network was analyzed by immunoblotting with available phosphospecific antisera. An extract from BT cells irradiated with 10-Gy IR and allowed to recover for 2 h served as a positive control (IR).

Chk2 is accompanied by a reduction in the electrophoretic mobility of Chk2. In response to treatment with doxorubicin, phosphorylation of Chk2 at threonine 68 is readily detectable within 60 min and precedes the appearance of an electrophoretically retarded, hyperphosphorylated species of Chk2 (Fig. 4B), suggesting that threonine 68 is one of the first residues in Chk2 to be phosphorylated following exposure to doxorubicin. This is in contrast to the phosphorylation of Chk2 at serine 33 and/or serine 35 that is detectable only after 120 min and in the hyperphosphorylated form of Chk2 with reduced electrophoretic mobility (Fig. 4B). Given the increased abundance of the phosphorylated forms of the protein, this is unlikely to represent protein destabilization but rather may reflect a change in the secondary structure with reduced affinity for the antiserum.
In a manner similar to Chk2, Nbs1 is phosphorylated in an ATM-dependent manner at multiple serine residues, including serine 343 (23–26). This is accompanied by a modest reduction in the electrophoretic mobility of Nbs1. In response to treatment with doxorubicin, phosphorylation of Nbs1 at serine 343 is detectable within 60 min of treatment and precedes the appearance of a reduced mobility form of Nbs1 (Fig. 4), suggesting that serine 343 is one of the first residues in Nbs1 to be phosphorylated following treatment with doxorubicin.

Doxorubicin Induces Phosphorylation of Histone H2AX in Both an ATM-dependent and ATM-independent Manner—A very early and sensitive marker of DSB induction is the phosphorylation of histone H2AX on serine 139 (27, 28). Phosphorylated H2AX (also referred to as γH2AX) can be visualized as foci by immunofluorescence using phosphospecific antibodies. In response to IR, this phosphorylation event has been shown to be mediated in a redundant manner by ATM and DNA-PKcs, with ATM playing a more predominant role in the very early times after treatment and under certain growth conditions (29). To determine whether doxorubicin induces phosphorylation of histone H2AX and the preference for ATM in this process, cytospins were prepared from logarithmically growing, doxorubicin-treated BT (ATM-proficient) and L3 (ATM-deficient) cells and examined by immunofluorescence with a phosphospecific antibody for phosphorylation of histone H2AX at serine 139. Robust phosphorylation of histone H2AX at serine 139 was observed within 30 min of doxorubicin treatment, with modest further increases up to 120 min in the ATM-proficient BT cells (Fig. 5). In contrast, H2AX phosphorylation was delayed in the ATM-deficient L3 cells, rising above background at 60 min and reaching a level similar to ATM-proficient cells at 120 min (Fig. 5). These results, demonstrating that doxorubicin induces phosphorylation, albeit delayed, in ATM-deficient cells, suggest that, similar to other published reports (29), ATM may play an important role at early times after treatment, but protein kinases other than ATM contribute in a complementary or redundant manner to the doxorubicin-induced phosphorylation of histone H2AX.

N-Acetylcysteine Abrogates Doxorubicin-mediated Stabilization and Phosphorylation of p53 and Attenuates p53-DNA Binding—Although generally classified as a topoisomerase II-stabilizing drug that induces DSBs, doxorubicin can intercalate DNA and generate ROS through the reaction of its quinone moiety with cytochrome P450 reductase and NAD(P)H (30). To evaluate a possible role for ROS in doxorubicin-mediated effects on p53, ATM-proficient BT cells were either pretreated with 0.9% NaCl (0, lanes 1 and 3) or increasing concentrations of ascorbic acid (A, lanes 2 and 4–6) or NAC (B, lanes 2 and 4–6) for 30 min prior to the addition of 1 μM doxorubicin (lanes 3–6) and further incubation for 2 h. Nuclear extracts were prepared and analyzed by immunoblotting as for Fig. 1. C, electrophoretic mobility shift assays were carried out with the extracts from lanes 1, 3, and 6 in B as described for Fig. 3.
suggesting that hydroxyl radicals may play a role in doxorubicin-induced activation of ATM-dependent pathways. Consistent with the reduced phosphorylation and accumulation of p53 in doxorubicin-treated cells pretreated with NAC, p53 in extracts prepared from these cells showed a dramatically reduced ability to bind its cognate DNA-binding site (Fig. 6C). Similar results were observed with antioxidant pretreatment in C3ABR (ATM-proficient) cells.

Pretreatment with N-Acetylcysteine Attenuates the Doxorubicin-mediated, ATM-dependent Phosphorylation of Multiple Downstream Effectors in the ATM Signaling Pathway—To gain a broader perspective on the role of hydroxyl radicals in the early cellular response to doxorubicin treatment, ATM-proficient BT cells were pretreated for 30 min with NAC prior to the addition of doxorubicin (1 μM). The incubation was continued for a further 60 or 120 min, and extracts were then prepared and immunoblotted with phosphospecific antisera to known downstream effectors of ATM. Pretreatment of cells with NAC significantly attenuated or delayed the doxorubicin-induced phosphorylation of all substrates tested (Fig. 7, A and B). For ATM, Nbs1 and SMC1, pretreatment with NAC only partially attenuated the observed phosphorylation, whereas for Chk1 and Chk2, NAC pretreatment led to a nearly complete abrogation of the doxorubicin-induced phosphorylation. Similar results were observed with NAC pretreatment in the ATM-proficient C3ABR cell line.

Qualitatively similar results were observed in cells pretreated with pyrrolidinedithiocarbamate (PDTC), another hydroxyl radical scavenging antioxidant. In addition to its antioxidant properties, paradoxically, PDTC can also function as an oxidant, and in cells treated with PDTC alone, ATM, Nbs1, Chk1 (serine 345), and Chk2 (threonine 68) phosphorylation was observed (data not shown). Interestingly, subsequent incubation with doxorubicin did not induce further phosphorylation of ATM or Chk2; nor was SMC1 phosphorylation detectable with sequential incubations of PDTC and doxorubicin (data not shown).

Pretreatment with N-Acetylcysteine Only Partially Attenuates the Early, Doxorubicin-mediated Phosphorylation of Histone H2AX—To gain some insight into the role of hydroxyl radicals in the doxorubicin-mediated phosphorylation of histone H2AX, logarhythmically growing, ATM-proficient BT cells were pretreated for 30 min with NAC prior to the addition of doxorubicin (1 μM) and continued incubation for an additional 60 or 120 min. Cytospins were then prepared and examined by immunofluorescence with a phosphospecific antibody for γH2AX at serine 139. Pretreatment of cells with NAC partially, but significantly, attenuated the phosphorylation of histone H2AX at 60 min (Fig. 8), but this effect was no longer observed at 120 min, a time when redundant protein kinase(s) become engaged to phosphorylate histone H2AX (29). This result may reflect the dual nature of doxorubicin and suggests that some DNA damage is attributable to the ROS generated by doxorubicin, whereas the remainder may represent the DSBs generated through the doxorubicin-mediated stabilization of topoisomerase II-DNA cleavable complexes. It may be these cleavable complexes that signal to the complementary protein kinase(s) that phosphorylates H2AX with slightly delayed kinetics.

DISCUSSION

The serine/threonine protein kinase, ATM, plays a critical role in the cellular response to DNA damage. Exposure to IR generates DSBs, leading to the rapid activation of ATM in the cell. Interestingly, many of the anticancer drugs in active clinical use today also have the capacity to induce DSBs; however, little is known about the role of ATM in response to the damage induced by these drugs. We present here the finding that doxorubicin, a topoisomerase II poison, induces ATM autophosphorylation and the ATM-dependent phosphorylation of multiple downstream effectors within the DNA damage response pathway. We further present evidence that ROS, specifically hydroxyl radicals, participate in the doxorubicin-mediated activation of this complex pathway.

A previous study has shown that p53 is phosphorylated at four serine residues in an ATM-dependent manner in response to IR (13). In contrast, doxorubicin induced the phosphorylation of p53 at serines 6, 9, 15, 20, 37, 46, and 392, and, in all cases, phosphorylation was ATM-dependent. In response to genistein, a plant isoflavonoid, p53 is phosphorylated at six serine residues (serines 6, 9, 15, 20, 46, and 392) in an ATM-dependent manner, whereas the related bioflavonoid quercetin induced phosphorylation at these sites in a strictly ATM-independent manner (10). It is becoming clear that multisite phosphorylation is a dynamic and powerful method of delicately modulating the activity of proteins within the cell. Phosphorylation at different regions within a cell can control localization, stability, protein-protein interaction, DNA binding activity, and enzymatic activity, among others (33). In the case of p53, initial studies demonstrated that casin kinase-I-dependent phosphorylation of threonine 18 is dependent on the prior phosphorylation of serine 15 (34, 35). In addition, acetylation of p53 at lysines 320 and 383 requires the prior phosphorylation of p53 at serine 15, and the phosphorylation of additional amino-terminal sites further stimulates these acetylation events (13). Recent reports have presented evidence for much more extensive interdependence in the phosphorylation of amino-terminal residues in p53 (36). Prior phosphorylation of serine 15 appears to be required for the efficient phosphorylation of serine 9, serine 20, and threonine 18, whereas serines 6 and 9 are dependent upon one another for phosphorylation without affecting the phosphorylation of other residues in the amino terminus of p53 (36). Clearly, the phosphorylation of p53 is regulated in an intricate and dynamic manner. The role of ATM in this process is equally complex, responding to a specific subset of chemotherapeutics and DNA-damaging agents, each triggering a unique pattern of downstream post-translational modifications.

Although primarily regarded as a topoisomerase II poison, numerous cellular effects of doxorubicin are mediated through its generation of ROS. Recently, it has been demonstrated that prolonged treatment of cells with doxorubicin (0.86 μM, 24–120 h) leads to an increase in p53 protein levels, followed by the p53-mediated transcriptional up-regulation of manganese superoxide dismutase and glutathione peroxidase-1 (37). This was associated with an increased production of ROS, and cotreatment with NAC was shown to reduce significantly the number of apoptotic cells. Through the use of chemical antioxidants, we have shown that hydroxyl radicals play a role in the doxorubicin-induced activation of ATM-dependent pathways. It is tempting to speculate that the partial suppression of histone H2AX, ATM, Nbs1, and SMC1 phosphorylation by NAC pretreatment (Figs. 7 and 8) reflects the multifaceted nature of doxorubicin through its generation of ROS. Recently, it has been demonstrated that PARP cleavage by doxorubicin is attributable to the ROS generated by doxorubicin, whereas the balance reflects the DSBs generated by doxorubicin through its stabilization of topoisomerase II-DNA cleavable complexes. Were this the case, it would suggest that the phosphorylation of Chk1 and Chk2 reflects an NAC-sensitive oxidative stress response more than a direct DNA damage response. However, we cannot exclude the possibility that the observed effects of NAC pretreatment on H2AX, Nbs1, and SMC1 phosphorylation are attributable, in part, to the NAC-induced suppression of ATM autophosphorylation.
(minutes to hours) response to DNA damage, whereas other phosphoinositide 3-kinase-like kinases, such as ATR, can complement the response at later time points or, in the case of cells lacking ATM, compensate for its absence. Therefore, time after damage must be an important experimental consideration when studying the role of ATM in any given response (3, 38).

Keeping this in mind, all experiments presented herein were conducted within 4 h of cell treatment. Several previous studies have assessed the role of ATM in the cellular response to doxorubicin. Some of these used very late time points (16–24 h), and hence, interpretation of the data may be hampered by the activation of redundant pathways (39–41). Other studies...
have examined early time points (up to 4 h) and have demonstrated that doxorubicin induces the ATM-dependent phosphorylation of p53 at serine 15 (42) and activates a mitogen-activated protein kinase/extracellular signal-regulated kinase pathway leading to stimulation of IκB kinase activity and activation of the prosurvival transcription factor NF-κB in an ATM-dependent manner (43). Interestingly, it has been well studied that the expression and function of NF-κB are upregulated in response to ROS (44), although the role for ROS in the doxorubicin-induced activation of NF-κB remains to be studied.

Inherited defects in the gene coding for ATM lead to development of ataxia-telangiectasia (A-T). Consistent with the central role of ATM in cell cycle regulation in response to DNA damage, this autosomal recessive disorder is characterized by profound sensitivity to IR, cancer predisposition, immunodeficiency, genomic instability, and a progressive loss of motor control due to cerebellar ataxia (reviewed in Refs. 2 and 45). A multitude of studies have supported a role for ROS in aspects of ATM function as well as the pathogenesis of A-T (reviewed in Refs. 46–48). It has been suggested that ATM could be a sensor of perturbations in redox homeostasis or oxidative damage, triggering the activation of signal transduction pathways responsible for protecting cells from such insults (46, 48). Thus, the absence of functional ATM would result in cells under a continuous state of oxidative stress. Consistent with this are observations that A-T cells and tissues exhibit significantly reduced rates of GSH resynthesis following depletion (49) and show reduced levels of nicotine adenine dinucleotide (50) and elevated levels of numerous biomarkers of oxidative damage (51). We demonstrate here that hydroxyl radicals play a role in the rapid activation of ATM and ATM-dependent signaling pathways, which further supports the hypothesized link between ATM function and ROS. Interestingly, doxorubicin has been demonstrated in vivo to induce an immediate and acute reduction in GSH levels in erythrocytes, liver, and cardiac tissue, and the administration of thiol donors (cysteamine or NAC) prevents this fall (52). It is tempting to speculate, given this and the impaired recovery from GSH depletion in A-T cells (49), that ATM may play a role, either directly or indirectly, in modulating the GSH biosynthesis/recycling pathway.

Although A-T is rare, studies suggest that 1–2% of the general population is heterozygous for mutations in ATM, and clinical and epidemiological evidence points to an increased cancer risk, particularly breast cancer, within this carrier population (53). In addition, these carriers have an intermediate sensitivity to IR (54, 55). Interestingly, many of the anti-tumor chemotherapeutics used in the treatment of breast cancer have the capacity to induce DSBs or generate ROS. For breast cancer patients heterozygous for mutations in ATM, exposure to these drugs or IR could lead to more profound manifestations of side effects or the increased incidence of secondary, treatment-related malignancies. Identification of drugs that do not activate ATM could lead to modified treatment protocols for these patients with the aim of reducing side effects and improving the long term outcome of therapy.

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