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14. ABSTRACT

Ataxia-telangiectasia mutated (ATM) is a serine/threonine protein kinase that acts as a master switch controlling the cell cycle in response to ionizing radiation-induced DNA double-strand breaks (DSBs). Carriers of *ATM* mutations are at increased risk for breast cancer. Since many anti-tumor chemotherapeutics used in breast cancer treatment have the capacity to induce DSBs, I have investigated the requirement for ATM in the cellular response to these agents. I have previously identified doxorubicin as an agent that stimu lates ATM autophosphorylation and the ATM-dependent phosphorylation of multiple downstream effectors of ATM in part through the generation of hydroxyl radicals . I have now expanded these studies to examine the functionally related topoisomerase II poison, etoposide. In contrast to doxorubicin, etoposide induces the phosphorylation of several downstream effectors in an ATM-independent manner despite stimulating ATM autophosphorylation, and etoposide appears to stimulate a signaling cascade initiated by the ATM-related protein kinase, ATR. Studies are now underway to identify proteins that interact with ATM following drug treatment. Characterization of a role for ATM in the cellular response to anti-tumor chemotherapeutics could have significant implications for the treatment of breast cancer patients harboring mutations in *ATM*.

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Introduction

ATM is a nuclear protein kinase required for the arrest of the cell cycle at G_1/S, S and G_2/M in response to ionizing radiation (IR)-induced DNA damage (reviewed in (Kurz and Lees-Miller, 2004)). Inherited defects in ATM lead to the development of ataxia telangiectasia (A-T), a progressive neurodegenerative disorder characterized by profound sensitivity to IR, cancer predisposition, immunodeficiency, and a progressive loss of motor control due to cerebellar ataxia (reviewed in (Lavin and Shiloh, 1997). Although A-T is relatively rare, studies suggest that 1% of the normal population is heterozygous for *ATM* mutations and that *ATM* heterozygosity could play a more significant role than BRCA1 and BRCA2 in breast cancer (Khanna, 2000).

Exposure to IR causes DNA double-strand breaks leading to the activation of ATM in the cell (Kurz and Lees-Miller, 2004). Interestingly, many of the anticancer drugs used in the treatment of breast cancer also have the capacity to induce DNA double-strand breaks (Fritsche et al., 1993; Vock et al., 1998), however little has been known about the role of ATM in response to damage induced by these drugs. The aims of my training grant from the US Army Breast Cancer Research Program have been to examine the effects of DNA damaging chemotherapeutics on the activation of ATM *in vivo* and to identify proteins that interact with ATM following treatment with ionizing radiation or ATM activating chemotherapeutics. The continued experimental efforts of the previous year towards this aim are summarized in this report.

Body (Detailed Research Accomplishments)

The requirement for the serine/threonine protein kinase ATM has been extensively studied in the cellular response to IR-induced DNA damage; comparatively little has been known about the role of ATM in response to DNA-damaging anti-tumor chemotherapeutics. I have previously identified doxorubicin, a commonly used topoisomerase II poison, as an agent that stimulates ATM autophosphorylation on serine 1981 and the ATM-dependent phosphorylation of p53 and numerous other downstream effectors of ATM and have shown that these phosphorylation events are, in part, dependent on the generation of hydroxyl radicals. In this past year, a manuscript describing this work was published in the *Journal of Biological Chemistry* (Kurz et al., 2004)

While conducting experiments evaluating the role of ATM in response to DNA damaging anti-tumor chemotherapeutics, an intriguing observation was made. While the cellular response to doxorubicin was entirely dependent on the presence of ATM, treatment of cells with etoposide, a closely related topoisomerase II poison, appeared to be ATM independent. This could have significant clinical implications, as these agents have always been thought to have comparable mechanisms of action. Since many data had been obtained while conducting the doxorubicin experiments, a few additional experiments were conducted to understand this observation further. In ATM-negative cell lines, the etoposide-induced phosphorylation of p53 and Nbs1 was attenuated by pretreatment with the replication inhibitor aphidicolin, suggesting a requirement for cells to progress through S-phase and leading to the speculation that the related protein kinase, ATR, might mediate these events. Studies using ATR-deficient Seckel cells and cells overexpressing a dominant-negative, kinase dead ATR support a role for ATR in the

etoposide-induced phosphorylation of p53 and Nbs1, but also suggest that, in its presence, ATM can initiate an etoposide-induced phosphorylation cascade. To investigate further the differences between doxorubicin and etoposide, comet assays were performed under both neutral and alkaline conditions to compare the DNA damage induced by these chemotherapeutics. Despite their functional similarity and the long-held belief that they both predominantly induce DNA double strand breaks, evaluation of the damage induced by etoposide using the alkaline comet assay revealed an abundance of DNA single strand breaks, an observation not seen in cells treated with doxorubicin. This may be a key observation in explaining the biological differences seen in the cellular response to these agents. This finding of divergent cellular responses to related chemotherapeutics is novel and an important observation in our field. In addition to a manuscript in preparation that describes these results, the experiments characterizing the differences in the requirement for ATM in the cellular response to the topoisomerase II poisons were described in four abstracts, two from meetings I attended (1 regional, 1 international), one from a meeting attended by my mentor (Dr. Susan Lees-Miller), and one written for the Era of Hope meeting which I, unfortunately, was unable to attend because of travel restrictions due to advanced pregnancy. This work was also presented at the annual research retreat of the Southern Alberta Cancer Research Institute and in a seminar I was invited to give in the Department of Pharmacology and Therapeutics at the University of Calgary as part of a faculty recruitment process. In addition to the work described above, I made further progress towards the completion of Aim 1, Task 2. In this regard, the data describing the effects of doxorubicin on the activation of ATM in breast cancer cells have been obtained.

Following the completion of Aim 1, which was expanded in scope to evaluate a broader panel of downstream effectors in ATM signaling pathways, required the work-up of additional techniques for the publication of these important findings and led to the significant findings of divergent cellular responses to the topoisomerase II poisons, I shifted my focus to Aim 2 of the grant. This aim, to identify proteins that interact with ATM following treatment with IR or ATM-activating chemotherapeutics (doxorubicin) in breast cancer cells, first required the optimization of immunoprecipitation and electrophoresis conditions in ATM proficient and ATM deficient cells. During the time that I was establishing these conditions, several other members of our laboratory were working on similar techniques (with different endpoints). Although ATM immunoprecipitation is an established technique in our laboratory, frustratingly no one in the lab was able to immunoprecipitate ATM. Working together, we systematically troubleshooted the protocol, modifying salt, detergent and washing conditions as well as using different batches of Protein A Sepharose to no avail. After a period of more than two months, it was determined that several independent lots of the antibody used for ATM immunoprecipitation had been frozen during shipping from the supplier, resulting in damage to the protein and rendering it useless for immunoprecipitation. These problems have now been resolved. However, their resolution coincided with the commencement of my maternity leave from the lab. As a result, limited progress has been made towards the goals of Aim 2.

The expansion of the project to evaluate a broader panel of downstream effectors in ATM-signaling pathways, the work-up of additional techniques for publication of these important findings, the intriguing observation of divergent cellular responses to two similar chemotherapeutics and the technical difficulties encountered by our laboratory with the immunoprecipitation of ATM combined with the suspension of my research activities when I commenced my maternity leave in June have limited the productivity of experiments to identify proteins that interact with ATM following treatment with IR or doxorubicin. Should I receive approval for a no-cost extension of my award, I am poised to make significant gains towards the goals of Aim 2 of the grant.

In addition to conducting the research described herein, my training continues to be enriched through my interactions with other members of the Southern Alberta Cancer Research

Institute at the University of Calgary. As a member of the Institute, I continue to attend and participate in a weekly journal club and a weekly Work in Progress seminar series. I also continue to participate in regular meetings with the Radiation Oncology group at the Tom Baker Cancer Centre.

Key Research Accomplishments

- I published my research findings describing the activation of ATM and ATM-dependent signaling pathways by doxorubicin in the *Journal of Biological Chemistry* (Kurz et al., 2004).
- In contrast to doxorubicin, I determined that the functionally related topoisomerase II poison, etoposide, induces phosphorylation of p53, Nbs1 and Chk1 in an ATM-independent manner, despite stimulating ATM autophosphorylation.
- I established, using ATM-negative cell lines, that the etoposide-induced phosphorylation of p53 and Nbs1 were attenuated by pretreatment with the replication inhibitor aphidicolin, suggesting a requirement of cells for progression through S-phase.
- I determined, using ATR-deficient Seckel cells and cells overexpressing a dominant-negative, kinase dead ATR, that ATR plays a significant role in the etoposide-induced phosphorylation of p53 and Nbs1, but also that, in its presence, ATM can initiate an etoposide-induced phosphorylation cascade.
- Performing the comet assay under neutral and alkaline conditions, I determined that etoposide induces an abundance of DNA single strand breaks, an observation not seen in cells treated with doxorubicin.
- My work was described in four meeting abstracts and was presented in one research presentation and one research seminar.

Reportable Outcomes

1. My work was presented at the Alberta Cancer Board 2004 Annual Research Meeting held in Banff, Alberta, Canada November 8-10, 2004. The abstract was entitled: Kurz, E.U., Siponen, M.I., Douglas, P. and Lees-Miller S.P. DNA damage responses to the topoisomerase II poisons: How similar chemotherapeutic agents induce different cellular responses.
2. My studies are described in an article published in the Journal of Biological Chemistry: Kurz, E.U., Douglas P, and Lees-Miller, S.P. Doxorubicin activates ATM-dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species. Journal of Biological Chemistry 279, 53272-53281.
3. My work was presented at the Gordon Research Conference on Mammalian DNA Repair held in Ventura, California January 14-20, 2005. The abstract was entitled: Kurz, E.U., Douglas, P., Siponen, M.I. and Lees-Miller, S.P. DNA damage responses to the topoisomerase II poisons: To ATM or not to ATM.
4. I was invited to give an oral presentation at the Southern Alberta Cancer Research Institute Annual Research Retreat on May 30, 2005. My presentation was entitled "DNA damage signaling by the topoisomerase II poisons: How similar chemotherapeutic agents induce divergent cellular responses".
5. My work contributed to a presentation by my mentor, Dr. Susan Lees-Miller, at the 2005 International Workshop on Ataxia Telangiectasia "ATM and the DNA damage response" held in Lake Maggiore, Italy June 8-11, 2005. The abstract was entitled:

Lees-Miller, S.P., Kurz, E.U., Douglas, P., Yu, Y., Ye, R., Goodarzi, A.A. and Povirk L.F. Activation of ATM by DNA damaging and non-DNA damaging agents.

6. My work was accepted for a platform presentation at the Era of Hope 2005 Department of Defense Breast Cancer Research Program meeting held in Philadelphia, PA June 8-11, 2005. I was unable to travel to this meeting due to pregnancy. The abstract was entitled: Kurz, E.U., Siponen, M.I., Douglas, P. and Lees-Miller S.P. DNA damage responses to the topoisomerase II poisons. How similar chemotherapeutic agents induce different cellular responses.
7. In part due to the training obtained with this fellowship, I was invited to apply for a faculty position in the Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary. As part of the recruitment process, I presented my research findings in a seminar entitled "DNA damage signaling by the topoisomerase II poisons: How similar chemotherapeutics induce divergent cellular responses" on May 9, 2005.
8. Due to my involvement in breast cancer research, I was invited to sit on the task force providing direction to the Alberta Cancer Board on programs to distribute funds raised through the "Weekend to End Breast Cancer" charity walk.
9. The comprehensive review I co-authored with Susan Lees-Miller last year and which was published in *DNA Repair* (Vol. 3, pp 889-900) was the fourth most frequently downloaded article from the Journal in 2004. The comprehensive ATM signaling pathway figure I created for the article (Figure 2) has been frequently requested by researchers around the world for teaching and presentation purposes.

Conclusions

It has been reported that mutations in *ATM* could account for up to 5% of breast cancers, thus ATM could play a more significant role in breast cancer than BRCA1 and BRCA2. Characterization of a role for ATM in the cellular response to anti-tumor chemotherapeutics could have significant implications leading to modified treatment protocols with fewer side effects for breast cancer patients who carry mutations in *ATM*.

The research conducted in the abbreviated third year of my training grant from the U.S. Army Breast Cancer Research Program expanded upon the findings of Years 1 and 2, leading to the publication of a manuscript in the *Journal of Biological Chemistry* (Kurz, et al., 2004). Observations made during this study have been expanded to characterize the role of ATM in the cellular response to the functionally related topoisomerase II poison, etoposide. Due to unforeseen technical issues, limited progress was made in the second aim of the grant, to identify proteins that interact with ATM in breast cancer cells following IR or ATM-activating drug treatment. However, with these issues now resolved, I am poised to make significant gains towards the goals of Aim 2 should my request for a one-year, no-cost extension (to August 31, 2006) be granted.

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Appendices

Appendix 1: Abstract from the 2004 Alberta Cancer Board Annual Research Meeting
Appendix 2: Kurz, E.U., Douglas, P. and Lees-Miller, S.P. (2004) Doxorubicin activates ATM dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species. *Journal of Biological Chemistry* 279:53272-53281.
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Appendix 5: Abstract from the 2005 International Workshop on Ataxia Telangiectasia
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Appendix 1

Abstract from the 2004 Alberta Cancer Board Annual Research Meeting

DNA damage responses to the topoisomerase II poisons: How similar chemotherapeutic agents induce different cellular responses.

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Ataxia telangiectasia mutated (ATM) is a cellular protein kinase that acts as a master switch controlling if and when cell cycle progression arrests in response to ionizing radiation (IR). Exposure to IR results in an increase in the kinase activity of ATM, ATM-dependent up-regulation of p53 protein, and the direct and indirect phosphorylation of p53, as well as numerous other downstream effectors of ATM. IR-induced DNA double-strand breaks (DSBs) are thought to be an important trigger leading to ATM activation. Since many anti-tumour chemotherapeutics also have the capacity to induce DNA DSBs, we have investigated the requirement for ATM in the cellular response to two topoisomerase II-stabilizing drugs, doxorubicin and etoposide. Using several human ATM-positive and ATM-negative cell lines, we have observed ATM-dependent accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues in response to doxorubicin. Treatment of cells with doxorubicin also stimulated ATM autophosphorylation on serine 1981 and the ATM-dependent phosphorylation of histone H2AX, SMC1, Nbs1, Chk1 and Chk2. In contrast, the functionally related topoisomerase II poison, etoposide, induced phosphorylation of p53, Nbs1 and Chk1 in an ATM-*independent* manner, despite stimulating ATM autophosphorylation. Studies examining the roles of reactive oxygen species, the ATR protein kinase and the catalytic differences in these agents will be discussed.

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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 1981 and the ATM-dependent phosphorylation of numerous effectors in the ATM-signaling pathway, including Nbs1 (Ser^{343}), SMC1 (Ser^{957}), Chk1 (Ser^{317} and Ser^{345}), and Chk2 ($Ser^{33/35}$ and Thr^{68}). 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.

DNA double strand breaks (DSBs)[1] 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 (G_1, intra-S, and G_2/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 anticancer 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

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[1] The abbreviations used are: DSB, DNA double strand break; A-T, ataxia-telangiectasia; ATM, ataxia-telangiectasia mutated; DNA-PKcs, DNA-dependent protein kinase, catalytic subunit; Gy, gray(s); IR, ionizing radiation; NAC, *N*-acetylcysteine; PBS, phosphate-buffered saline; PDTC, pyrrolidinedithiocarbamate; ROS, reactive oxygen species.

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*-nitrosoguanidine stimulates ATM kinase activity and the ATM-dependent phosphorylation of p53 on serine 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/F-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% CO_2. Chemotherapeutics, inhibitors, antioxidants, 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-containing 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 317 and 345 of Chk1, and serines 33 and 35 and threonine 68 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 antisera reactive to the total pools of Chk2, Nbs1, and SMC1. A polyclonal antibody to actin (A2066) was acquired from Sigma. A phosphospecific antiserum to serine 1981 of human ATM was purchased from Rockland Immunochemicals (Gilbertsville, PA). A phosphospecific mouse monoclonal antibody to serine 139 of human histone H2AX was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). A rabbit polyclonal antibody specific for human ATM (4BA) was a generous gift from Dr. Martin Lavin (Queensland Institute for Medical Research). DPK1, a 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 mM 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 antiserum to p53 phosphorylated at serine 15.

Detection and analysis of p53 phosphorylation at serines 6, 9, 20, 37, 46, and/or 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, $8–10 \times 10^6$ 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 protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A) and phosphatase inhibitors (1 mM activated Na_3VO_4, 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 (30:0.25 acrylamide/bisacrylamide) and transferred to nitrocellulose in SDS-electroblot buffer (25 mM Tris, 192 mM glycine, 0.04% (w/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 mM 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 washed twice in PBS, and 5×10^4 cells in a volume of 200 μl were centrifuged onto coverslips (800 rpm, 5 min). Cells were fixed in 3.7% (w/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 for 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 γH2AX-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 for significance 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 *trans*-activation 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

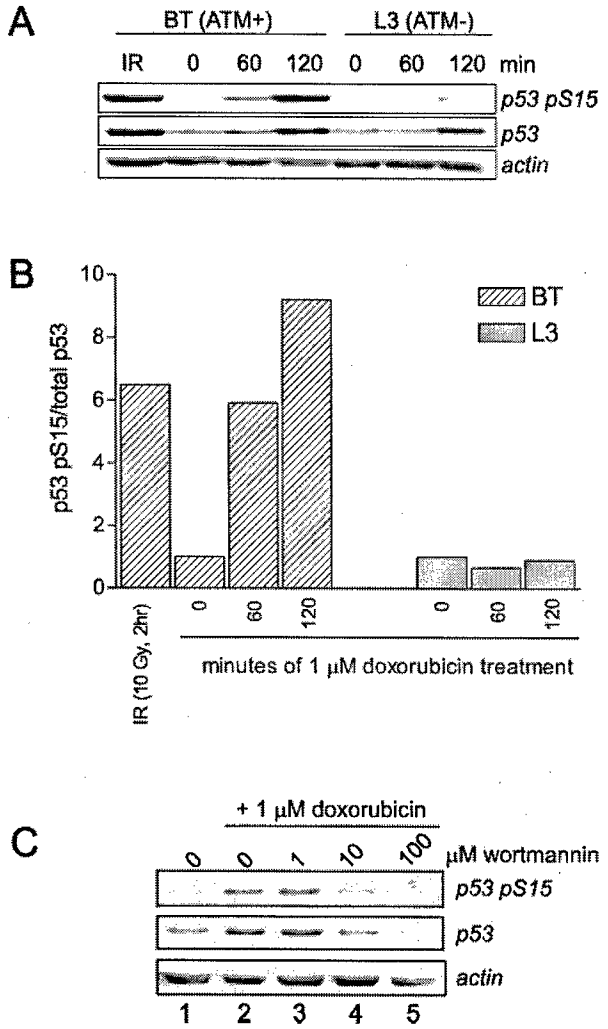

FIG. 1. **Doxorubicin-induced accumulation of p53 and p53 phosphorylation on serine 15 require ATM and are abrogated by pretreatment with wortmannin.** *A*, ATM-proficient (BT) and ATM-deficient (L3) human lymphoblastoid cells were treated with doxorubicin (1 μM) and harvested at the indicated times. Nuclear extracts were prepared and analyzed by sequential immunoblotting using a phosphospecific antiserum to serine 15 of p53 (p53 pS15), a panspecific antibody (DO-1) to p53 (p53), and a polyclonal antiserum to actin. An extract from BT cells irradiated with 10-Gy IR and allowed to recover for 2 h served as a positive control (*IR*). *B*, the immunoblots shown in *A* were scanned and quantitated, and serine 15 phosphorylation was normalized to total levels of p53 (as judged by immunoreactivity with the DO-1 antibody and described under "Experimental Procedures"). *Hatched bars*, ATM-proficient BT cells; *solid bars*, ATM-deficient L3 cells. *C*, ATM-proficient cells (BT) were either pretreated with dimethyl sulfoxide (0 μM wortmannin, *lanes 1* and *2*) or increasing concentrations of wortmannin (*lanes 3–5*) for 30 min prior to the addition of 1 μM doxorubicin (*lanes 2–5*) and further incubation for 2 h. Nuclear extracts were prepared and analyzed by immunoblotting as for *A*.

p53 on serine 15 within 60 min, further increasing at 120 min to a level comparable with that observed with exposure to IR (10 Gy, 2 h). The phosphorylation was absent in the ATM-deficient L3 cells at the times examined; however, in a manner similar to IR (16), doxorubicin induced a modest accumulation of p53 and phosphorylation at serine 15 in ATM-deficient cells at later time points (4 h and longer) (data not shown). Similar results were obtained in experiments with a second pair of ATM-proficient (C3ABR) and ATM-deficient (AT1ABR) cell lines (data not shown).

To confirm further the role of ATM in the phosphorylation and accumulation of p53 in response to doxorubicin, cells were pretreated with wortmannin, a fungal metabolite that binds irreversibly to the ATP binding site of phosphoinositide 3-kinase-like kinases (17, 18). ATM-proficient BT cells were pretreated for 30 min with increasing concentrations of wortmannin prior to the addition of doxorubicin, and incubations were continued for a further 120 min. As shown in Fig. 1*C*, pretreatment of cells with 10 μM wortmannin reduced both the accumulation and serine 15 phosphorylation of p53 to basal levels. Similar results were observed with a second ATM-proficient human lymphoblastoid cell line (C3ABR; data not shown).

Doxorubicin Induces ATM-dependent Phosphorylation of p53 on Serines 6, 9, 15, 20, 37, 46, and 392—In response to IR, p53 becomes phosphorylated on at least eight serine residues (located at amino acids 6, 9, 15, 20, 33, 46, 315, and 392), and it has been demonstrated that ATM is required for the early phosphorylation of the amino-terminal serines at positions 9, 15, 20, and 46, with only weak phosphorylation evident in ATM-deficient cells at later time points (13). To evaluate whether p53 is also phosphorylated at multiple serine residues in response to doxorubicin and to evaluate the ATM-dependence of any such post-translational modifications, p53 was immunoprecipitated from doxorubicin-treated 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-competent 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 antiserum to serine 1981 of ATM. Exposure to doxorubicin induced marked phosphorylation of ATM at serine 1981, to a level comparable with that induced by exposure to IR (10 Gy, 2 h) (Fig. 4*A*).

Doxorubicin Induces 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-

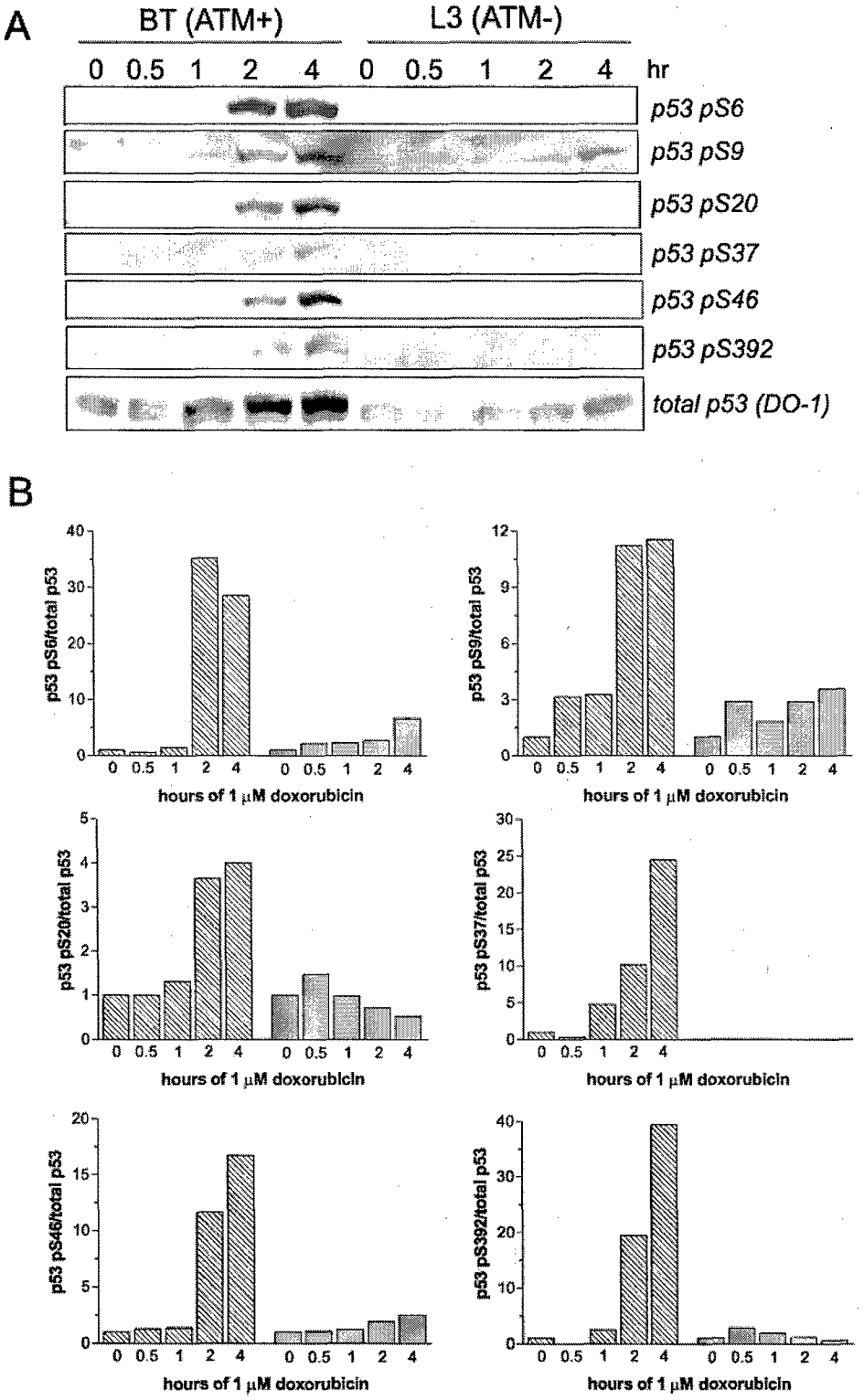

FIG. 2. **Doxorubicin induces ATM-dependent phosphorylation of p53 on serines 6, 9, 20, 37, 46, and 392.** *A*, ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) and harvested at the indicated times. To determine the phosphorylation state of p53 at the indicated serines, p53 was immunoprecipitated from whole cell extracts and analyzed by immunoblotting, as described under "Experimental Procedures," using phosphospecific antisera, followed by incubation with the monoclonal antibody DO-1 for total p53. *B*, the immunoblots shown in *A* were scanned and quantitated, and p53 phosphorylation at individual sites was normalized to total p53 (as judged by immunoreactivity with the DO-1 antibody). *Hatched bars*, ATM-proficient BT cells; *solid bars*, ATM-deficient L3 cells. In the case of phosphorylation of p53 at serine 37, an elevated, uneven background on the immunoblot precluded an accurate quantitative assessment of p53 phosphorylation at this residue in the ATM-deficient L3 cells. Hence, data for this are not included in the graph.

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. 1*A*), threonine 68 of Chk2 (Fig. 4*B*) and serine 343 of Nbs1 (Fig. 4*B*) were all phosphorylated in an ATM-dependent manner within 60 min of the initiation of doxorubicin treatment. In contrast, ATM-de-

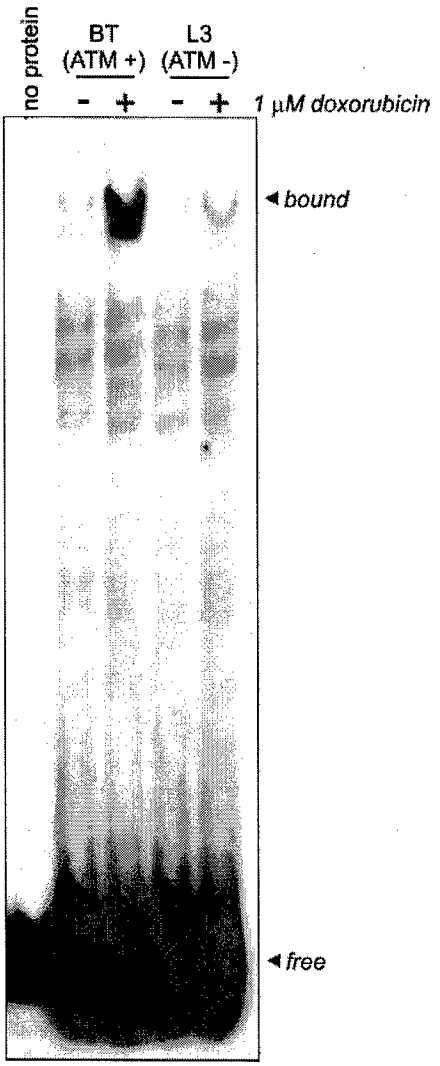

FIG. 3. **Doxorubicin stimulates p53-DNA binding in ATM-proficient (BT) cells.** Oligonucleotides containing a consensus p53 binding site were annealed and end-labeled with [γ-^{32}P]ATP. Nuclear extracts (9 μg of protein) from untreated or doxorubicin-treated (1 μM, 2 h) ATM-proficient (BT) or ATM-deficient (L3) cells were assayed for binding activity to the ^{32}P-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.

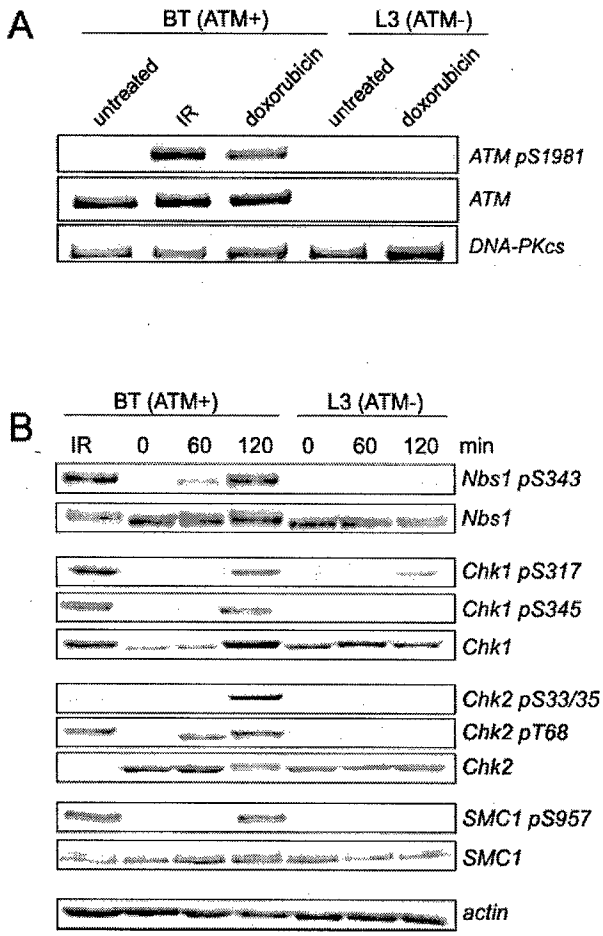

FIG. 4. **Doxorubicin induces the autophosphorylation of ATM on serine 1981 and ATM-dependent phosphorylation of multiple downstream effectors in the ATM signaling pathway.** *A*, ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM, 2 h) or exposed to 10-Gy IR and allowed to recover for 2 h prior to harvest. Whole cell extracts were prepared and analyzed by sequential immunoblotting using a phosphospecific antiserum to serine 1981 of ATM, a panspecific 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*).

pendent phosphorylation of Chk1 on serines 317 and 345, Chk2 on serines 33 and 35, and SMC1 on serine 957 were only detectable 120 min after initiating doxorubicin treatment (Fig. 4*B*). Interestingly, ATM-dependent phosphorylation of Chk1 at serines 317 and 345 in response to either doxorubicin or IR was also accompanied by increased immunoreactivity with the antibody for the total cellular pool of Chk1 (Chk1; Fig. 4*B*). Whether this, in a manner similar to p53, represents protein stabilization and accumulation or whether the phosphorylated protein takes on a secondary structure with higher affinity for the antiserum remains to be determined.

Within its amino-terminal domain, Chk2 contains a cluster of seven potential ATM phosphorylation sites (19–21). Whereas threonine 68 of Chk2 is the primary site of ATM-directed phosphorylation, other sites within this cluster are phosphorylated to a lesser extent, and the amino-terminal 57 amino acids are required, at least *in vitro*, for the efficient phosphorylation of Chk2 by ATM (22). Phosphorylation of 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. 4*B*), 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 only in the hyperphosphorylated form of Chk2 with reduced electrophoretic mobility (Fig. 4*B*). In an inverse manner to that of Chk1, the appearance of this hyperphosphorylated form of Chk2 is accompanied by reduced immunoreactivity with the antibody for the total cellular pool of Chk2 (Chk2; Fig. 4*B*). 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.

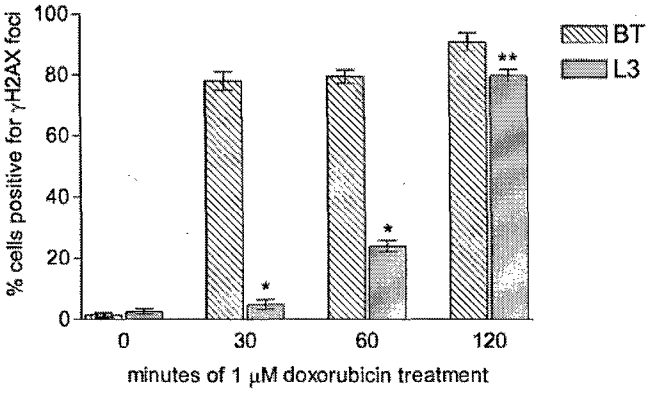

FIG. 5. **Doxorubicin induces the phosphorylation of histone H2AX on serine 139 in both an ATM-dependent and ATM-independent manner.** ATM-proficient (BT) and ATM-deficient (L3) cells were treated with doxorubicin (1 μM) for 30, 60, or 120 min, as indicated. Cytospins were prepared for immunofluorescence as described under "Experimental Procedures." For each experimental point, an average of 250 cells was scored for reactivity for γH2AX as a percentage of the total. Data shown represent the mean and S.D. of values from multiple fields within an experimental point. *Hatched bars*, ATM-proficient BT cells; *solid bars*, ATM-deficient L3 cells. The difference between ATM-proficient and ATM-deficient cells at each time point was analyzed for significance using an unpaired, one-tailed Student's *t* test (*, $p < 0.0001$; **, $p < 0.005$).

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*B*), 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 pretreated with antioxidants prior to treatment with doxorubicin. Pretreatment of cells with the superoxide scavenger (31) ascorbic acid had no effect on the doxorubicin-induced stabilization and phosphorylation of p53 on serine 15 (Fig. 6*A*). In contrast, preincubation of cells with the hydroxyl radical scavenger (31, 32) NAC significantly attenuated the doxorubicin-mediated stabilization and phosphorylation of p53 on serine 15 (Fig. 6*B*),

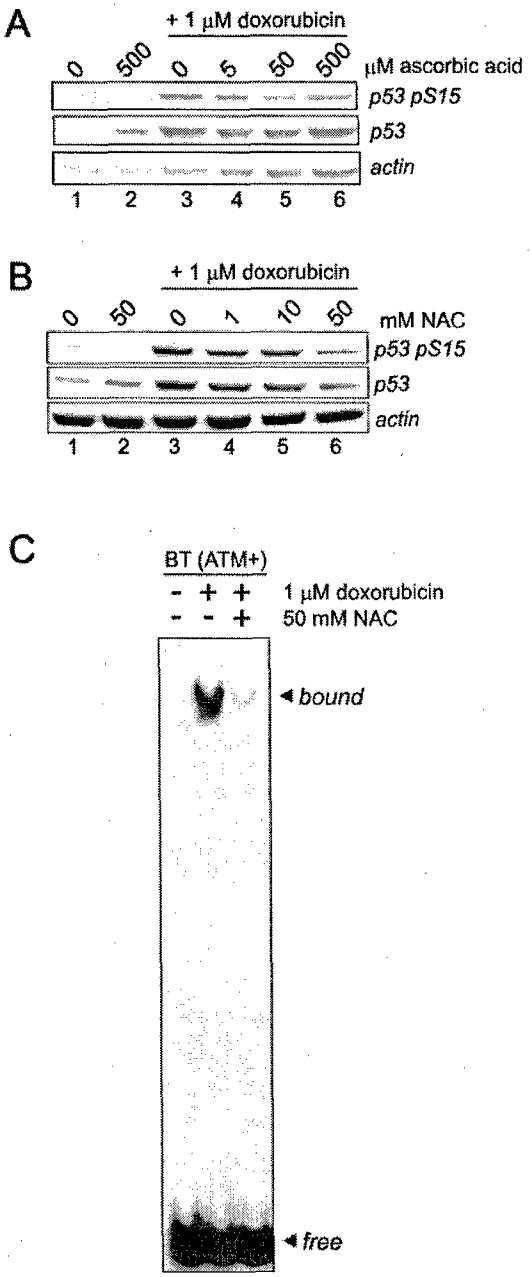

FIG. 6. **NAC attenuates the doxorubicin-mediated induction of p53 phosphorylation and accumulation and p53-DNA binding.** *A* and *B*, ATM-proficient (BT) cells were either pretreated with 0.9% NaCl (*0*, *lanes 1* and *3*) or increasing concentrations of ascorbic acid (*A*, *lanes 2* and *4–6*) or 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. 6*C*). 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, logarithmically 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 casein kinase-1-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, that some level of phosphorylation 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.

In the cell, ATM is predominantly required for the early

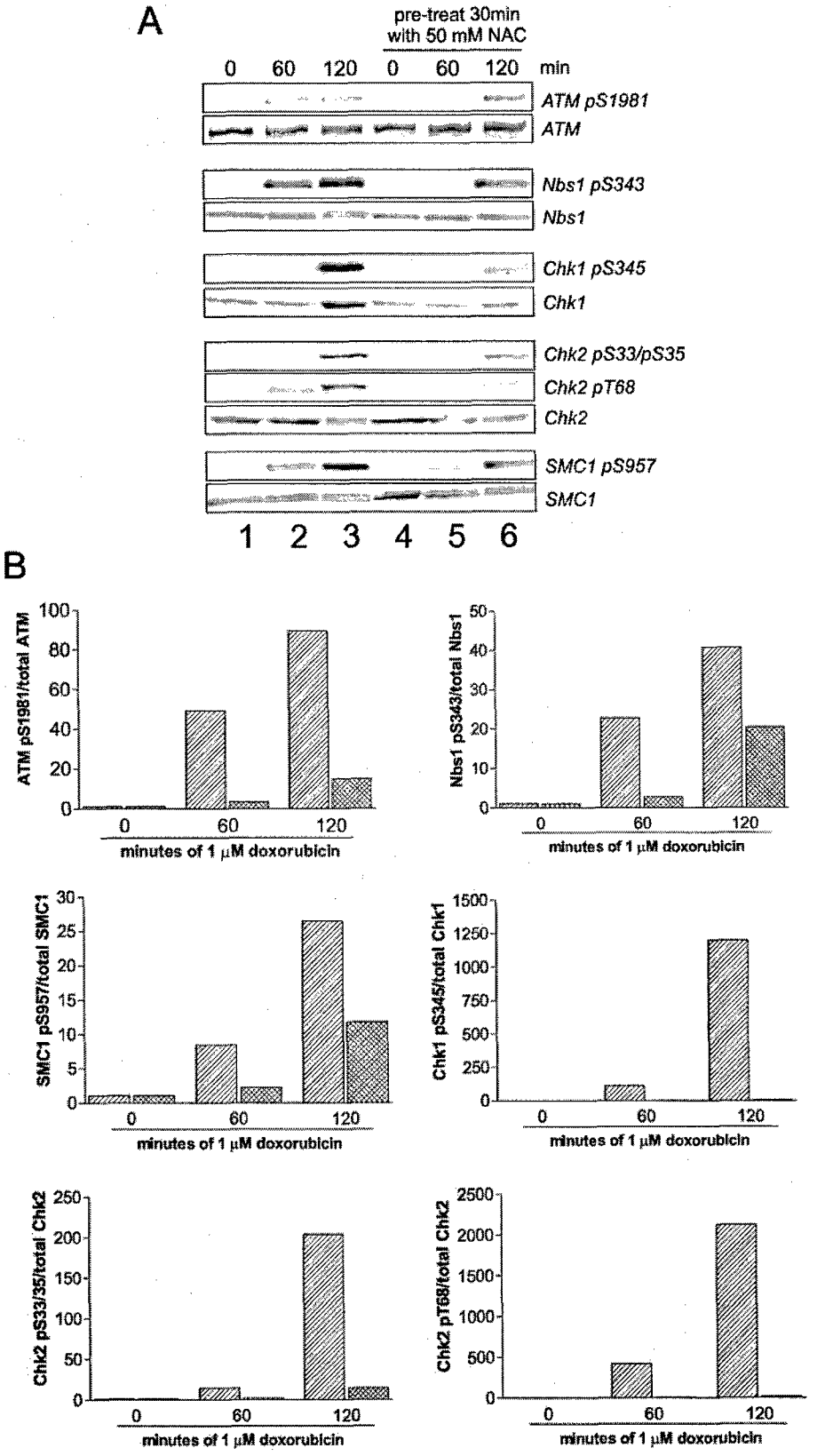

FIG. 7. **The hydroxyl radical scavenger, NAC, attenuates the doxorubicin-mediated, ATM-dependent phosphorylation of multiple downstream effectors in the ATM-signaling pathway.** *A*, ATM-proficient (BT) cells were either pretreated with 0.9% NaCl (*lanes 1–3*) or 50 mM NAC (*lanes 4–6*) for 30 min prior to the addition of 1 μM doxorubicin and further incubation for 60 or 120 min. Whole cell extracts were prepared, and the effect of antioxidant pretreatment on the phosphorylation status of downstream effectors within the ATM signaling network was analyzed by immunoblotting with phosphospecific antisera. *B*, the immunoblots shown in *A* were scanned and quantitated, and phosphorylation at each site was normalized to total levels of each respective protein analyzed. To account for any basal effect of the pretreatment alone, data for each condition (doxorubicin alone or NAC pretreatment) were then expressed as the fold induction of phosphorylation over the doxorubicin-untreated control within each set (*lanes 1* and *4*). *Hatched bars*, treatment with doxorubicin alone (no pretreatment); *cross-hatched bars*, pretreatment with NAC (50 mM, 30 min).

(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

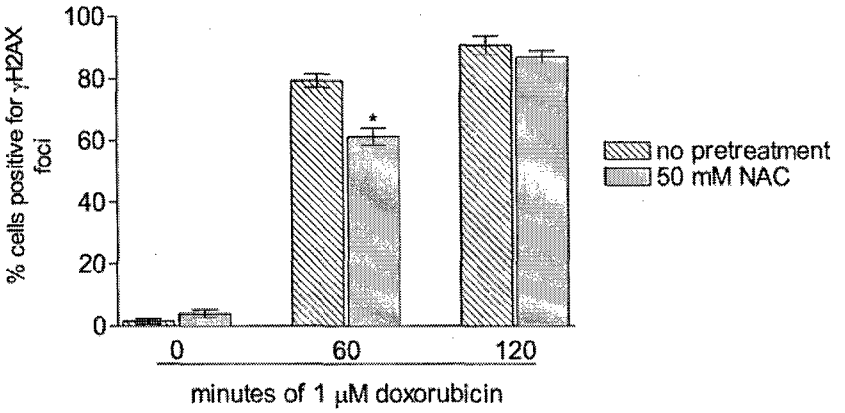

FIG. 8. **The hydroxyl radical scavenger, NAC, only partially attenuates the doxorubicin-mediated phosphorylation of histone H2AX on serine 139.** ATM-proficient (BT) cells were pretreated with either 0.9% NaCl (*no pretreatment*) or 50 mM NAC for 30 min prior to the addition of 1 μM doxorubicin and further incubation for 60 or 120 min. Cytospins were prepared for immunofluorescence as described under "Experimental Procedures." For each experimental point, an average of 250 cells were scored for reactivity for γH2AX as a percentage of the total. Data shown represent the mean and S.D. of values from multiple fields within an experimental point. *Hatched bars*, no pretreatment; *solid bars*, NAC pretreatment. The effect of pretreatment with NAC at each time point was analyzed for significance using an unpaired, one-tailed Student's *t* test (*, $p < 0.0001$).

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 up-regulated 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 mice 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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Appendix 3

Abstract from the 2005 Gordon Research Conference on Mammalian DNA Repair

DNA damage responses to the topoisomerase II poisons: To ATM or not to ATM?

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Ataxia telangiectasia mutated (ATM) is a cellular protein kinase that acts as a master switch controlling if and when cell cycle progression arrests in response to ionizing radiation (IR). Exposure to IR results in an increase in the protein kinase activity of ATM, ATM-dependent upregulation of p53 protein, and the direct and indirect phosphorylation of p53 and numerous other downstream effectors of ATM. IR-induced DNA double-strand breaks (DSBs) are thought to be an important trigger leading to ATM activation. Since many anti-tumour chemotherapeutics also have the capacity to induce DNA DSBs, we have investigated the requirement for ATM in the cellular response to two topoisomerase II-stabilizing drugs, doxorubicin and etoposide. Using several human ATM-positive and ATM-negative cell lines, we have observed ATM-dependent accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues in response to doxorubicin. Treatment of cells with doxorubicin also stimulated ATM autophosphorylation on serine 1981 and the ATM-dependent phosphorylation of histone H2AX, SMC1, Nbs1, Chk1 and Chk2. In contrast, the functionally related topoisomerase II poison, etoposide, induced phosphorylation of p53, Nbs1 and Chk1 in an ATM-independent manner, despite stimulating ATM autophosphorylation. Studies using ATR-deficient Seckel cells suggest that the ATR protein kinase may be mediating these ATM-independent events. Studies examining the role of reactive oxygen species and the catalytic differences between these agents will be discussed.

Appendix 4

Abstract from the 2005 Southern Alberta Cancer Research Institute Annual Research Retreat

DNA damage signaling by the topoisomerase II poisons: How similar chemotherapeutic agents induce divergent cellular responses.

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Ataxia telangiectasia mutated (ATM) is a cellular protein kinase that acts as a master switch controlling if and when cell cycle progression arrests in response to ionizing radiation (IR) induced DNA damage. Exposure to IR results in an increase in the kinase activity of ATM, ATM-dependent upregulation of p53 protein, and the direct and indirect phosphorylation of p53, as well as numerous other downstream effectors of ATM. IR-induced DNA double-strand breaks (DSBs) are thought to be an important trigger leading to ATM activation. Since many anti-tumour chemotherapeutics also have the capacity to induce DNA DSBs, we have investigated the requirement for ATM in the cellular response to two topoisomerase II-stabilizing drugs, doxorubicin and etoposide. Using several ATM-positive and ATM-negative cell lines, we have observed ATM-dependent accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues in response to doxorubicin. Treatment of cells with doxorubicin also stimulated ATM autophosphorylation on serine 1981 and the ATM-dependent phosphorylation of histone H2AX, SMC1, Nbs1, Chk1 and Chk2. In contrast, the functionally related topoisomerase II poison, etoposide, induced phosphorylation of p53, Nbs1 and Chk1 in an ATM-independent manner, despite stimulating ATM autophosphorylation. In ATM-negative cell lines, the etoposide-induced phosphorylation of p53 and Nbs1 was attenuated by pretreatment with the replication inhibitor aphidicolin, suggesting a requirement for cells to progress through S-phase and leading to the speculation that the related protein kinase, ATR, might mediate these events. Studies using ATR-deficient Seckel cells and cells overexpressing a dominant-negative, kinase dead ATR support a role for ATR in the etoposide-induced phosphorylation of p53 and Nbs1, but also suggest that, in its presence, ATM can initiate an etoposide-induced phosphorylation cascade. To investigate further the differences between doxorubicin and etoposide, comet assays were performed under both neutral and alkaline conditions to compare the DNA damage induced by these chemotherapeutics. Despite their functional similarity and the long-held belief that they both predominantly induce DNA DSBs, evaluation of the damage induced by etoposide using the alkaline comet assay revealed an abundance of DNA single strand breaks, an observation not seen in cells treated with doxorubicin. This may be a key observation in explaining the biological differences seen in the cellular response to these agents.

Appendix 5

Abstract from the 2005 International Workshop on Ataxia Telangiectasia

Activation of ATM by DNA damaging and non-damaging agents

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Exposure to ionizing radiation (IR) results in the activation of ATM and ATM-dependent signaling pathways, however, the effects of other DNA damaging and non-DNA damaging agents on ATM and ATM-dependent signaling pathways are less well understood. Previously, we surveyed a series of DNA damaging agents and other biologically active compounds for their ability to induce phosphorylation of ATM on serine 1981. In addition to IR, several topoisomerase (topo) poisons, including doxorubicin and etoposide, were found to induce robust ATM serine 1981 phosphorylation (Kurz and Lees-Miller, 2004). Doxorubicin is a topo II poison that induces DNA double-strand breaks but can also intercalate DNA and undergo redox cycling. We recently showed that doxorubicin induces ATM-dependent phosphorylation of p53 and other substrates in part through the generation of reactive oxygen species (ROS) (Kurz et al, 2004). Here, we show that etoposide, which is also a topo II poison but does not intercalate DNA or generate ROS, appears to stimulate an ATR-dependent signaling cascade. Interestingly, the protein phosphatase inhibitor, okadaic acid (OA), also induces phosphorylation of ATM not through induction of DNA damage, but by inhibition of the activity of a protein phosphatase 2A (PP2A)-like protein phosphatase, and, moreover, that PP2A interacts with ATM in untreated cells but dissociates after IR (Goodarzi et al, 2004). Here, we show that etoposide and doxorubicin also induce dissociation of ATM and PP2A. Preliminary results to characterize further the interaction between ATM and PP2A will also be reported. Finally, we demonstrate that IR and other DNA damaging agents induce the phosphorylation of tyrosyl-DNA phosphodiesterase (Tdp1) on SQ/TQ sites and that this phosphorylation is due to a PIKK family protein kinase, suggesting that Tdp1 may be a target of the ATM signaling pathway.

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Appendix 6

Abstract from Era of Hope 2005

DNA damage responses to the topoisomerase II poisons. How similar chemotherapeutic agents induce different cellular response.

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Ataxia telangiectasia mutated (ATM) is a cellular protein kinase that acts as a master switch controlling if and when cell cycle progression arrests in response to ionizing radiation (IR) induced DNA damage. Inherited defects in ATM lead to the development of ataxia telangiectasia (A-T), a progressive neurodegenerative disorder characterized by profound sensitivity to IR, cancer predisposition, immunodeficiency and a progressive loss of motor control due to cerebellar ataxia. Although A-T is relatively rare, studies suggest that 1% of the normal population is heterozygous for ATM mutations and that ATM heterozygosity could account for up to 5% of all breast cancers, thereby playing a more significant role than BRCA1 and BRCA2 in the genetic predisposition to breast cancer.

Exposure to IR results in an increase in the protein kinase activity of ATM, ATM autophosphorylation, ATM-dependent upregulation of p53 protein, and the direct and indirect phosphorylation of p53 and numerous other downstream effectors of ATM. IR-induced DNA double-strand breaks (DSBs) are thought to be an important trigger leading to ATM activation. Since many anti-tumor chemotherapeutics used in the treatment of breast cancer also have the capacity to induce DNA DSBs, we have investigated the requirement for ATM in the cellular response to two topoisomerase II-stabilizing drugs, doxorubicin and etoposide. Using several human ATM-positive and ATM-negative cell lines, we have observed ATM-dependent accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues in response to doxorubicin. This was accompanied by an increased binding of p53 to its cognate DNA binding site, suggesting a transcriptional competency of p53 to activate downstream targets. Treatment of cells with doxorubicin also stimulated ATM autophosphorylation on serine 1981 and the ATM-dependent phosphorylation of histone H2AX, SMC1, Nbs1, Chk1 and Chk2. In contrast, the functionally related topoisomerase II poison, etoposide, induced phosphorylation of p53, Nbs1 and Chk1 in an ATM-independent manner, despite stimulating ATM autophosphorylation. Studies using ATR-deficient Seckel cells suggest that the closely related ATR protein kinase may be mediating these ATM-independent events. We have further examined the biological differences between these functionally related chemotherapeutic agents. Significantly, pretreatment of cells with a hydroxyl radical scavenger, N-acetyl cysteine, attenuated the doxorubicin-mediated phosphorylation of multiple downstream effectors of ATM, suggesting that hydroxyl radicals may play an important role in the doxorubicin-induced activation of ATM-dependent pathways. This may be a key observation in understanding the biological differences in the cellular response to these agents.

Characterization of a role for ATM in the cellular response to anti-tumor chemotherapeutics could have significant implications, leading to modified treatment protocols with fewer side effects for breast cancer patients harboring mutations in the ATM gene.