The ataxia telangiectasia mutated (ATM) and ATR (ATM and Rad3-related) protein kinases exert cell cycle delay, in part, by phosphorylating checkpoint kinase (Chk) 1, Chk2, and p53. It is well established that ATR is activated following UV light-induced DNA damage such as pyrimidine dimers and the 6-(1,2)-dihydro-2-oxo-4-pyrimidinyl-5-methyl-2,4-(1H,3H)-pyrimidinediones, whereas ATM is activated in response to double strand DNA breaks. Here we clarify the activation of these kinases in cells exposed to IR, UV, and hyperoxia, a condition of chronic oxidative stress resulting in clastogenic DNA damage. Phosphorylation on Chk1(Ser-345), Chk2(Thr-68), and p53(Ser-15) following oxidative damage by IR involved both ATM and ATR. In response to ultraviolet radiation-induced stalled replication forks, phosphorylation on Chk1 and p53 required ATM, whereas Chk2 required ATM. Cells exposed to hyperoxia exhibited growth delay in G1, S, and G2 that was disrupted by wortmannin. Consistent with ATM or ATR activation, hyperoxia induced wortmannin-sensitive growth delay in G1, S, and G2 that was disrupted by wortmannin. Consistent with ATM or ATR activation, hyperoxia induced wortmannin-sensitive phosphorylation of Chk1, Chk2, and p53. By using ATM- and ATR-defective cells, phosphorylation on Chk1, Chk2, and p53 was found to be ATM-dependent, whereas ATR also contributed to Chk1 phosphorylation. These data reveal activated ATM and ATR exhibit selective substrate specificity in response to different genotoxic agents.

Cell cycle checkpoints are initiated in an attempt to prevent the fixation of mutations from one cell generation to the next. These checkpoints become activated when cells are exposed to genotoxic agents or other adverse environmental conditions (1). Although the pathways of halting cell cycle progression are clear, the mechanisms of activating these checkpoints immediately following DNA damage are poorly understood. The ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) phosphoinositide 3-kinase-related kinases (PIKKs) are critical for transducing DNA damage signals to checkpoint control proteins (2). ATM is essential for mediating checkpoint control in cells exposed to ionizing radiation and other agents that produce double strand breaks in DNA. Cells derived from AT patients exhibit increased chromosome breaks, defects in cell cycle checkpoints, and increased cell death when exposed to IR. Although ATM kinase activity increases upon binding DNA ends produced by endonuclease cleavage or IR exposure (3, 4), recent studies have suggested ATM does not directly associate with DNA lesions in vivo. Instead, the auxiliary proteins Mre11, Rad50, and Nbs1 of the MRN complex recruit and activate ATM at sites of DNA lesions (5). ATM has also been shown to be stimulated by changes in chromatin structure caused by damage to DNA (6). In contrast, ATR is activated by stalled replication forks and agents that produce bulky adducts, such as UV irradiation (7, 8). ATR activity is dependent upon its association with the ATR-interacting protein (ATR-IP) (9) that targets the ATR-ATR-IP heterodimer to single-stranded DNA (10). Thus, ATM and ATR are recruited to different complexes following genotoxic stress.

Activation of these kinases by damaged DNA results in the direct or indirect phosphorylation or activation of a number of downstream checkpoint controls, DNA repair, or apoptosis-promoting targets including p53, Chk1, Chk2, Brca-1, Rad9, and Rad17 (7, 11–13). Of major concern to cell cycle control are the checkpoint kinases Chk1 and Cds1 (hereafter referred to as Chk2). Chk1 and Chk2 become phosphorylated at serine 345 (14) and threonine 68 (15) following genotoxic exposure, respectively. The roles of Chk1 and Chk2 in response to DNA damage are essential, as inhibition of the ATM/Chk2 pathway results in a loss of the G2/M checkpoint and increased IR sensitivity (16), whereas Chk2 mutation has been implicated in the cancer Li-Fraumeni syndrome (17). ATM and ATR can also directly phosphorylate p53(Ser-15) (18–20) resulting in G2/M checkpoint initiation and increased cell viability following genotoxic insult. p53 activation is crucial to many cellular processes including DNA repair, cell cycle arrest, and apoptosis (for review see Ref. 21). Thus, activation of the Chk1, Chk2, and p53 checkpoint proteins is necessary for maintaining genome stability and overall cellular viability following genotoxic stress.

Whereas both ATM and ATR are able to target all three of these checkpoint proteins, it remains unclear why different genotoxic stresses result in their specific activation and ability to differentially phosphorylate downstream targets. For exam-
ple, ATM is rapidly activated by IR and phosphorylates p53(Ser-15), whereas ATR is slowly activated and is involved in maintaining p53(Ser-15) phosphorylation (18, 19). However, in response to UV-induced damage, ATR phosphorylates p53(Ser-15), and ATM is not required (19, 20). Original reports indicated that ATM and ATR phosphorylate Chk2 and Chk1, respectively, thereby restricting cross-talk between the PIKKs and their downstream targets (7, 14, 22). Recently, ATR-mediated phosphorylation of Chk1 in response to IR was reported (23) suggesting that these two parallel damage response branches may not be mutually exclusive. Taken together, these findings suggest that the ATM and ATR kinases evolved to respond to unique forms of genotoxic stress.

Although unproven, the activities of ATM and ATR may reside in the ability of these kinases to form functional complexes with auxiliary proteins at specific damaged sites. For example, IR produces single and double strand breaks along with a large number of oxidized base lesions, whereas UV predominantly produces thymine dimers resulting in stalled replication forks during DNA replication. The observation that stalled replication forks activate ATR might explain the delayed response to IR. Thus, ATM would act as the initial signal transducer following double strand break detection, whereas ATR would be activated later when stalled replication forks form during replication of damaged DNA. Once replication becomes stalled by bulky adducts or single/double strand breaks, ATR may be activated to elicit cell cycle arrest. In this scenario, ATM and ATR may have evolved to ensure that DNA replication does not occur under adverse environmental conditions. Unlike IR or UV, exposure to hyperoxia (oxygen concentrations greater than 21%) produces genotoxic reactive oxygen species (ROS) chronically over the course of hours and days (24). Because of the chronic nature of exposure, the growth inhibitory actions of hyperoxia were originally attributed to cellular toxicity and oxidation of critical molecules required for cell division (25). Although it is well known that hyperoxia oxidizes nucleotides and promotes sister chromatid exchanges and DNA strand breaks (26, 27), the involvement of checkpoint control has only recently been appreciated. Like exposure to IR, hyperoxia exerts G1 growth delay by stimulating p53(Ser-15) phosphorylation and p53-dependent induction of p21 (28–30). However, unlike IR, where p21-deficient cells progress through S phase and accumulate in G2, p21-deficient cells exposed to hyperoxia accumulate in S phase due to slowing of DNA initiation and elongation (28). Hypothetically, growth arrest in S phase might be attributed to stalled replication forks that form as cells attempt to bypass oxidative lesions formed during exposure. Indeed, a recent study showing that p53(Ser-15) phosphorylation required ATR after 24 h of hyperoxia (31) supports a response involving recognition of stalled replication forks. Most intriguingly, the same study reported that ATM was not required for p53(Ser15) phosphorylation, even though it is widely accepted to be activated by oxidative stress. Because ATM and ATR are temporally activated following IR, it remains possible that ATM is activated early during exposure and ATR is activated later.

The goal of this study was to determine the roles of the ATM and ATR kinases to phosphorylate the checkpoint proteins Chk1, Chk2, and p53 in response to different genotoxins that produce unique DNA lesions. By using ATM(+/+) and ATM(−/−) lymphoblasts, and U2OS osteosarcoma cells with regulated expression of ATR-DNAGnegative protein, the requirement for ATM and ATR to phosphorylate these checkpoint proteins in response to IR, UV, and hyperoxia was compared. Our studies reveal that ATM and ATR become activated following IR, UV, or hyperoxia but exhibit unique roles in their ability to phosphorylate Chk1, Chk2, and p53.

EXPERIMENTAL PROCEDURES

Cell Culture—A549 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). U2OS (human osteosarcoma) cells stably transfected with a tetracycline-inducible ATR-kd (kinase dead) construct were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum plus 50 units/ml penicillin, 50 μg/ml streptomycin to control bacterial growth, and 50 μg/ml hygromycin and 200 μg/ml geneticin for selective pressure of the transfection construct (32). ATM(+/+) and ATM(−/−) lymphoblasts designated GM05536 and GM01526, respectively, were obtained from the Coriell Cell Repositories (Camden, NJ). These cells were cultured at 37 °C in Roswell Park Memorial Institute medium (RPMI) 1640 with l-glutamine and 15% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen).

Genotoxic Exposures—For genotoxic exposures, cells were counted on a hemocytometer, plated on 100-mm dishes (BD Biosciences), and allowed to adhere overnight. The next day, plates containing adherent cells were washed with 10 ml of Hanks’ balanced salt solution, and the media were replaced before exposure to room air (room air with 5% CO2) or hyperoxia (95% O2, 5% CO2) in a Plexiglas box (Belco Glass, Vineland, NJ). Wortmannin (Sigma) was added to the media prior to hyperoxic exposure. The box was sealed, and gases were delivered with a PROcell model 110 (Remex Instruments Co., Redfield, NY) at a flow rate of 5 liters/min for 10 min. For ionizing radiation exposures, plates were exposed to their respective doses using a Shepherd 1300 Curie radiation source at a dose rate of 3.0 gray/min. For ultraviolet light exposures, plates were exposed to 50 J/m2 UVC light using a UVC-515 Multilinker (Ultra-Lum Inc., Carson, CA). U2OS ATR-kd inducible cells were incubated in media containing 1 μg/ml doxycycline for 48 h prior to genotoxic exposure.

Western Blot Analysis—Adherent cells were lysed with buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 25 mM sodium fluoride, 25 mM sodium (β-glycerophosphate, 0.1 mM sodium vanadate, 0.1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 0.3% IGE-PAL CA-630, 0.1 μg/ml pepstatin A, supplemented with 1.9 μg/ml of aprotinin, and 2 μg/ml of leupeptin. Protein concentrations were determined by the BCA method (BCA Protein Assay, Pierce). The lysates were boiled for 5 min in 3× Laemmli Buffer. Laemmli at 1× contains 50 mM Tris (pH 6.8), 1% β-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol. The extracted protein was separated by SDS-PAGE and transferred to polyvinyllidene difluoride membranes (Millipore, Bedford, MA). The membranes were then incubated in primary antibodies for Chk1(Ser-345) (1:500), Cell Signaling, Beverly, MA), Chk2(Thr-68) (1:500, Cell Signaling), p53(Ser-15) (1:1000, Cell Signaling), p53 Ab-6 (1:1000, Calbiochem), FLAG (1:250, Sigma), or as a loading control β-actin (1:5000; Sigma) per the manufacturer’s instructions. Membranes were then incubated in the appropriate secondary antibody as follows: goat anti-mouse (1:4000, Southern Biotechnology, Birmingham, AL), or goat anti-rabbit (1:5000, The Jackson Laboratory, West Grove, PA). After washing the membranes with TBS-T, specific antibody interactions were visualized by chemiluminescence (Amer sham Biosciences).

Statistical Analysis—Values are means ± S.D. Cell phase percentages were analyzed by analysis of variance using Microsoft Excel software for Macintosh, and p < 0.05 was considered significant.

RESULTS

Hyperoxia Induces Wortmannin-sensitive Cell Cycle Arrest—Hyperoxia has been shown previously to inhibit DNA synthesis and induce growth arrest in all phases of the cell cycle with G1 arrest requiring p53-dependent expression of p21 (28, 30, 33). As shown previously (28), p53-expressing A549 cells arrested in all phases of the cell cycle with a predominant population remaining in G1 (Fig. 1A). In contrast, p53-deficient H1299 cells failed to express p21 during exposure and therefore accumulated in S and G2 (Fig. 1B). To determine whether PIKKs were required for this growth arrest, A549 and H1299 cells were exposed to room air or hyperoxia for 48 h in presence of the PIKK inhibitor wortmannin. As expected, wortmannin disrupted cell cycle distributions during hyperoxia with no detect-
able change of cells exposed to room air (Fig. 1). Quantification of the percentage of cells in each phase of the cell cycle confirmed that wortmannin significantly decreased the proportion of hyperoxic A549 and H1299 cells in G2 (Tables I and II). The reduction of G2 phase cells coincided with an increase in the proportion of cells in G1 phase. This increase in the percentage of cells in G1 phase may be caused by release of cells arrested in G2 phase of the cell cycle and/or by the enhanced expression of other G1 checkpoint proteins such as p27 (34). Nonetheless, wortmannin disrupts pathways involved in arresting cells during hyperoxia.

Hyperoxia Activates Checkpoint Proteins through a Wortmannin-sensitive Kinase—Because growth arrest in G1 has been attributed to p53(Ser-15) phosphorylation and p53-dependent activation of p21, the effects of wortmannin on p53 were investigated in A549 cells exposed to room air or hyperoxia (Fig. 2A). p53(Ser-15) phosphorylation was not detected in room air-treated cells. However, p53(Ser-15) and p53 protein levels increased during hyperoxia, associated with increased expression of p21. Wortmannin inhibited phosphorylation of p53(Ser-15) and overall protein accumulation of both p53 and p21 in a dose-dependent manner. Because the checkpoint kinases 1 and 2 are also downstream targets of PIKK activity, Chk1(Ser-345) and Chk2(Thr-68) phosphorylations were assessed in these samples. Minimal levels of both Chk1(Ser-345) and Chk2(Thr-68) were detected in room air-treated cells. Exposure to hyperoxia resulted in increased levels of both Chk1(Ser-345) and Chk2(Thr-68) that were reduced by wortmannin in a dose-dependent manner. Chk1(Ser-345) and Chk2(Thr-68) phosphorylations were also assessed in H1299 cells exposed to room air or 2 days of hyperoxia (Fig. 2B). Phosphorylated Chk1(Ser-345) and Chk2(Thr-68) were not detected in cells cultured in room air. However, phosphorylated Chk1(Ser-345) and Chk2(Thr-68) were detected in cells exposed to hyperoxia, with levels reduced by wortmannin. Taken together, these data show that chronic oxidative stress caused by hyperoxia induces phosphorylation of the checkpoint proteins p53, Chk1, and Chk2 through a wortmannin-sensitive pathway.

### Table I: Effect of hyperoxia and wortmannin on cell cycle distribution in A549 cells

| Exposure | G1 | S | G2/M |
|----------|----|---|------|
| RA       | 54.90 ± 4.12 | 34.08 ± 4.91 | 11.00 ± 1.23 |
| RA + Wort | 54.97 ± 7.54 | 28.37 ± 3.39 | 16.63 ± 5.60 |
| O2       | 51.83 ± 5.78 | 29.71 ± 3.83 | 18.43 ± 3.65 |
| O2 + Wort | 65.77 ± 3.16 | 24.58 ± 4.99 | 9.67 ± 2.44 |

- Hyperoxia significantly increased the percentage of cells arrested in G2/M phase of the cell cycle compared with room air controls (p < 0.05).
- Wortmannin increased the percentage of cells in G1 phase compared with hyperoxic treated samples alone (p < 0.05).
- Wortmannin significantly decreased the percentage of cells that arrested in G2/M phase during hyperoxia (p < 0.05).

### Table II: Effect of hyperoxia and wortmannin on cell cycle distribution in H1299 cells

| Exposure | G1 | S | G2/M |
|----------|----|---|------|
| RA       | 56.63 ± 1.55 | 30.78 ± 2.40 | 12.60 ± 2.55 |
| RA + Wort | 56.33 ± 2.70 | 30.01 ± 2.70 | 13.67 ± 0.67 |
| O2       | 18.17 ± 9.64 | 57.33 ± 8.26 | 24.47 ± 3.76 |
| O2 + Wort | 31.63 ± 6.78 | 51.62 ± 6.92 | 16.89 ± 2.42 |

- Hyperoxia significantly increased the percentage of cells arrested in G2/M phase of the cell cycle compared with room air controls (p < 0.05).
- Wortmannin significantly decreased the percentage of cells that arrest in G2/M phase of the cell cycle compared with hyperoxic treated samples alone (p < 0.05).

Because ATM is required for checkpoint control following oxidative stress, it was of interest to compare the response of the same ATM null cells to oxidative damage caused by IR. Consistent with previous studies (36), high levels of p53(Ser-15) phosphorylation were detected within 15 min following IR in ATM(+/+) lymphoblasts, whereas phosphorylation was less in ATM(−/−) lymphoblasts (Fig. 3B). Additionally, Chk1(Ser-
345) and Chk2(Thr-68) phosphorylation was diminished in ATM(/H11001/)/H11002/ ATM(/H11001/)/H11002/ cells, indicating that ATM is required for the phosphorylation of these kinases as well. Thus, ATM is necessary for phosphorylation of checkpoint proteins following acute (IR) and chronic (hyperoxia) oxidative stress.

To assess whether stalled replication forks signal through ATM, the same cell lines were exposed to UV radiation. UV exposure stimulated phosphorylation of p53, Chk1, and Chk2; however, only Chk2 phosphorylation occurred in an ATM-dependent manner (Fig. 3B). These data suggest that ATM selectively activates the Chk2 response when stalled replication forks are encountered and/or low levels of ROS are produced in response to ultraviolet light.

**ATR Participates in Checkpoint Protein Phosphorylation Following Genotoxic Stress**—ATR has been shown to maintain p53(Ser-15) phosphorylation following IR-induced damage. Because ATR(−/−) cells are nonviable, U2OS-derived osteosarcoma cells exhibiting doxycycline-inducible expression of a dominant negative form of ATR were used to determine the role of ATR in response to IR, UV, and hyperoxia (32). Upon addition of doxycycline, these cells express a FLAG-tagged ATR-kd protein that depletes native ATR activity. Immunoblotting with anti-FLAG antibody confirmed that cells treated with doxycycline expressed ATR-kd protein (Fig. 4A). In response to hyperoxia, p53(Ser-15) was phosphorylated; however, there was no detectable difference in the level of phosphorylation between cells expressing or not expressing ATR-kd even after 1, 2, or 3 days of hyperoxia. Consistent with enhanced phosphorylation, the overall level of p53 protein increased moderately after 1 day in hyperoxia. Again, total p53 protein abun-
phosphorylation of p53(Ser-15), Chk1(Ser-345), and Chk2(Thr-68) and UV-exposed cells and immunoblotted for FLAG-tagged ATR-kd protein, Chk2(Thr-68).

Dependent phosphorylation of p53(Ser-15), Chk1(Ser-345), and Chk2(Thr-68) and UV-exposed cells and immunoblotted for FLAG-tagged ATR-kd protein, Chk2(Thr-68).

These results indicate that Chk1 but not p53 or Chk2 expression was decreased as measured by anti-FLAG antibody detection. These results confirm previous studies indicating that UV selectively activates ATR-dependent phosphorylation of p53 and Chk1 but not Chk2.

DISCUSSION

Accumulating evidence suggests that different forms of genotoxic stress activate specific DNA damage responses. DNA strand breaks caused by IR activate the ATM/Chk2 pathway, whereas UV lesions and blocks in DNA replication activate ATR/Chk1. Whereas ATM and ATR represent two parallel branches of the DNA damage-response pathway, there is controversy in the literature over whether these pathways overlap through Chk1 and Chk2 activation. By using a model of chronic oxidative stress in which DNA is damaged over days of exposure, and making comparisons with acute IR and UV damage, we were able to confirm that the ATM/ATR pathways overlap with respect to the activation of Chk1 and Chk2 (Fig. 5A). One significant observation made in these studies is that regardless of the genotoxic agent, ATM or ATR always phosphorylated the checkpoint proteins p53, Chk1, and Chk2. Thus for all three sources of damage, phosphorylation of the final downstream targets are not unique, rather it is the requirement for ATM or ATR that is different. Because the DNA lesions produced by these agents are unique, we hypothesize that selective activation of the damage-response kinases dictates the means of target specificity following each genotoxin (Fig. 5, A-C).

A major strength of this study is that the same cell lines were exposed to different but well established models of genotoxic stress, thereby allowing direct comparison of the response to specific types of damage. Our studies revealed that whereas ATM and ATR are always activated, their specificity for downstream targets depends upon the type of lesion produced. We speculate that substrate specificity is caused by the recruitment of ATM and ATR to different complexes formed as a result of specific types of lesions. Indeed, a central question in checkpoint signaling is whether sensors exist for each type of damage or whether all damage is signaled through a common intermediate. In response to IR, the ATM homodimer undergoes autophosphorylation on Ser-1981, and this phosphorylation is required for dimer dissociation and subsequent initiation of ATM activity toward other substrates (6). Most interestingly, extremely low doses of IR or agents that disrupt chromatin without causing DNA strand breaks also stimulate ATM autophosphorylation, resulting in expression throughout the nucleus. In response to damage, ATM undergoes autophosphorylation and translocates to nuclear foci that are presumed
to be sites of DNA damage. The MRN complex formed at double strand breaks is necessary for ATM activity because hypomorphs of Mre1 and Nbs1 result in an AT-like disorder (37, 38). ATM in turn phosphorylates Nbs1, which is essential for phosphorylation of Chk2 at sites of DNA lesions. In contrast, ATM-dependent phosphorylation of p53, a nucleoplasmic substrate, did not require Nbs1. These findings suggest that Nbs1 dictates substrate specificity of ATM. Unlike ATM, ATR activity is not stimulated by DNA damage. Instead, ATR bound to ATR-IP is recruited to replication protein A bound to single-stranded DNA (10). ATR-dependent kinase activity requires the independent loading of the proliferating cell nuclear antigen-like complex Rad9-Rad1-Hus1, claspin, and DNA polymerase-α. Understanding how different types of bulky lesions signal through the common intermediate of single-stranded DNA remains unknown. Hypothetically, auxiliary proteins involved in sensing different lesions could dictate substrate specificity for the ATR-ATR-IP heterodimer bound to replication protein A, much like Nbs1 signals to Chk2 specificity for ATM.

Another important observation in this study was that ATM was required for maximal phosphorylation of all three checkpoint proteins during hyperoxia. This confirms the involvement of ATM in the response to oxidative stress in general. In contrast, ATR only phosphorylated Chk1(Ser-345) during hyperoxia, whereas it stimulated phosphorylation of all three checkpoint proteins following IR. Although it has been argued that IR and hyperoxia are similar in that they both produce DNA damage through ROS (39), several studies suggest that the cellular response to these stresses is different. As examples, hyperoxia-tolerant HeLa cells were sensitive to IR, and IR-sensitive Chinese hamster ovary mutants resisted hyperoxia (40–42). Also, a recent study (31) has suggested that the damage response to hyperoxia may be different from that elicited by IR. These data indicate that the DNA lesions produced by IR and hyperoxia along with their ensuing damage responses are unique. Common substrate specificity could be reflective of initial oxidative DNA strand breaks. In the case of hyperoxia, novel repair intermediates may also form as DNA repair becomes compromised under days of oxidative stress. Further investigations should be performed to determine the specific lesions produced during hyperoxic exposure, because these data may reveal which damage-response kinases are most likely recruited during this genotoxic exposure.

The current observation that ATR is not required for maximal p53 and Chk2 phosphorylation during hyperoxia, but is required following radiation, is also consistent with the hypothesis that hyperoxia and radiations activate at least partly distinct damage-response pathways. This may be due to the fact that IR mainly produces single and double strand DNA breaks along with a plethora of oxidized base lesions, whereas hyperoxia acts mainly as a clastogenic agent producing DNA strand breaks, sister chromatid exchanges, along with oxidized bases. One explanation for why IR and hyperoxia produce overlapping yet distinct DNA lesions may be attributed to the source of ROS production. ROS production by IR involves a direct acute oxidation of water from an extrinsic radiation source (43), whereas hyperoxic exposure induces an overproduction of ROS by the intrinsic process of aerobic respiration (44, 45). Thus, there may be differences in the types of lesions formed that provoke differential activation of ATM and ATR. An alternative hypothesis is that cells exposed to radiation are acutely damaged, whereas cells exposed to hyperoxia are damaged for prolonged periods. Because irradiated cells are no longer under oxidative stress following radiation, they may be more apt to re-initiate cell cycle progression compared with hyperoxic cells. As such, irradiated cells are more likely to encounter stalled replication forks that activate ATR.

Our studies confirm the previously established activation of the ATR/Chk1 and ATR/p53 pathway in the response to UV damage (7, 19). The additional activity of the ATM to phosphorylate Chk2 following UV exposure is a novel finding (Figs. 3B and 5B). There is evidence in the literature that ATM activity following UV light acts upstream of nucleotide excision repair, DNA recombination, replication protein A binding to DNA in double strand break repair, and UV-induced DNA damage repair in human skin fibroblasts (46, 47). The activity of ATM in response to UV light may be due to minimal oxidative damage induced by UV-irradiation (48). Although most studies investigate events in the first 60 min following UV exposure, we examined the damage response after 3 h. During this time we speculate DNA strand breaks may form as a result of interrupted nucleotide excision repair or through continued attempts to replicate cross-linked DNA. Only then would these forms of damaged DNA act as substrates for the binding and activation of the ATM kinase. Our findings therefore agree with previously established models and expand the role of ATM to phosphorylate Chk2 following UV damage.

DNA histograms of A549 and H1299 cells revealed that wortmannin predominantly disrupted G2 phase arrest during hyperoxia (Fig. 1). However, DNA histograms of ATM(+/-) and ATM(−/−) cells showed no significant differences in cell phase populations during hyperoxia (data not shown). Also, ATR-kd expression did not affect cell cycle arrest during hyperoxia (data not shown). As shown by Western analysis of p53, Chk1, and Chk2, ablation of ATM or inhibition of ATR is not sufficient to inhibit completely the checkpoint protein phosphorylation. This suggests an overlap of targets between ATM and ATR during hyperoxia. Alternatively, another wortmannin-sensitive kinase, such as DNA-dependent protein kinase, may be acting to stabilize these checkpoint proteins during expo-
sure. Our findings are consistent with the concept that several wortmannin-sensitive kinases are required to detect the DNA lesions produced during hyperoxia.

In summary, the observation that different genotoxic stresses activate checkpoint controls through distinct yet overlapping PIKKs provides an exciting opportunity to clarify why these kinases are selective for their substrates. It is well known that the downstream targets Chk1, Chk2, and p53 are uniquely phosphorylated by ATM or ATR in response to UV and hyperoxia, whereas IR elicits phosphorylation of all three targets via both ATM and ATR. We speculate that this universal response by IR to act through ATM and ATR may be indicative of the broad spectrum of DNA damage produced by this genotoxin, whereas the unique responses elicited by UV and hyperoxia may reflect more distinctive forms of damage such as stalled replication forks and general cellular oxidation, respectively. We present an hypothesis whereby substrate specificity of these kinases is coordinated by the unique binding and activation of ATM and/or ATR with auxiliary proteins at sites of DNA lesions. Hypothetically, the activation of these kinases toward selective substrates may be critical for activating specific DNA repair mechanisms. Indeed, ATM and ATR are very large proteins whose undefined amino-terminal domains could interact with numerous proteins involved in specifying substrate specificities toward checkpoint control and DNA repair. Future studies should evaluate how different lesions stimulate selective activation of PIKKs and clarify how these substrates regulate the cellular response to DNA damage.

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