The ATR kinase phosphorylates both p53 and Chk1 in response to extreme hypoxia (oxygen concentrations of less than 0.02%). In contrast to ATR, loss of ATM does not affect the phosphorylation of these or other targets in response to hypoxia. However, hypoxia within tumors is often transient and is inevitably followed by reoxygenation. We hypothesized that ATR activity is induced under hypoxic conditions because of growth arrest and ATM activity increases in response to the oxidative stress of reoxygenation. Using the comet assay to detect DNA damage, we find that reoxygenation induced significant amounts of DNA damage. Two ATR/ATM targets, p53 serine 15 and histone H2AX, were both phosphorylated in response to hypoxia in an ATR-dependent manner. These phosphorylations were then maintained in response to reoxygenation-induced DNA damage in an ATM-dependent manner. The reoxygenation-induced p53 serine 15 phosphorylation was inhibited by the addition of N-acetyl-L-cysteine (NAC), indicating that free radical-induced DNA damage was mediated by reactive oxygen species. Taken together these data implicate both ATR and ATM as critical roles in the response of hypoxia and reperfusion in solid tumors.

It is has been hypothesized that tumor hypoxia plays a critical role in the malignant progression of solid tumors and represents a poor prognostic indicator for tumor control. The mammalian response to hypoxia is complex and varies at different oxygen tensions (1). These include the induction of hypoxia-responsive genes by the transcription factors early growth response-1 (EGR1), AP-1, and hypoxia-inducible factor (HIF)³ (2, 3). HIF is a heterodimer that consists of Hif1α and β subunits that bind to the sequence 5'-RCGTCG-3' (4). Under normoxic conditions HIF-1α is rapidly degraded when it is bound to the von Hippel-Lindau tumor suppressor protein that targets it for ubiquitination (5, 6). Under hypoxic conditions HIF-1α is hydroxylated at a conserved proline residue, number 564, by a family of highly conserved 4-prolyl hydroxylases (7, 8). Under hypoxic conditions the activity of this oxygen-sensitive hydroxylase is repressed, and HIF-1α is unable to complex with VHL, which in turn leads to its increase in stability. In contrast to HIF-1, which is stabilized at 2% oxygen, the protein product of the p53 tumor suppressor gene also accumulates in hypoxic cells but requires more stringent hypoxic conditions (1). Hypoxia-induced p53 activates a cytochrome c-mediated apoptotic pathway that can act as a selective pressure for the expansion of tumor cells with either inactive or mutant p53 (9, 10). The mechanism by which p53 accumulates under hypoxic conditions has been attributed to both a decrease in mdm2 levels (11) and increased translation. mdm2 acts a negative regulator of p53 by targeting p53 for degradation by the ubiquitin-proteosome pathway. mdm2 is a p53-responsive gene that acts to keep p53 in check through a feedback loop. However, under hypoxic conditions, p53 does not seem to transactivate mdm2, and the decrease in mdm2 protein in hypoxic cells is due to degradation of the protein. It has been reported that p53 protein that accumulates under hypoxic conditions is transcriptionally impaired and is unable to induce p21, Bax, or mdm2. This loss of transactivation potential has been attributed in part to the lack of association between p53 and the co-activator p300 in hypoxic extracts. Instead, p53 that accumulates under hypoxic conditions associates with the co-repressor molecule mSin3a, suggesting that can act as a trans-repressor (12). Both p300 and mSin3a have been shown to bind to the amino terminus of p53 (13). The amino terminus of p53 is also the site of mdm2 binding and specific stress-induced phosphorylations. We have shown previously that p53 is phosphorylated by ATR in response to hypoxia at residue serine 15 (1). Those studies also indicated that without this phosphorylation event, p53 accumulation in response to hypoxia was diminished. Several explanations exist for this finding, including the potential masking of a nuclear export signal by this phosphorylation and the subsequent accumulation of p53 in the nuclear compartment (14). In contrast to ATR, we found that ATM had no role to play in the phosphorylation of p53 at serine 15 in response to hypoxia because of a lack of DNA damage under hypoxic conditions. The link between the ATM kinase and phosphorylation of p53 in response to DNA damage-inducing stresses is well established and may well be responsible for suppressing tumor expansion (15, 16).

Histone H2AX has recently been identified as having a phosphatidylinositol 3-kinase motif (SQ) at serine 139 and is a target for both ATM and ATR (17, 18). Histone H2AX is phosphorylated (γH2AX) in response to genotoxic agents, UV, hydroxyurea-mediated replication arrest, and at physiological sites of recombination during class switching (19). During the initiation of DNA fragmentation that occurs during apoptosis, H2AX is also phosphorylated. This phosphorylation occurs with the appearance of high molecular weight DNA fragments but before the externalization of phosphatidylinositol-
serine or the appearance of internucleosomal DNA fragments (20, 21). Recent studies have provided some insight into the function of H2AX. Homozygous null H2AX knockout mice are born with the expected frequency but are radiation-sensitive, growth-retarded, immune-deficient, and infertile (22, 23). Elegant foci studies have shown that H2AX null cells had impaired recruitment of Nbs1, 53bp1, and Brca1 to the sites of DNA damage. However, the formation of Rad51 foci in response to DNA damage was only slightly affected, if at all, in the absence of H2AX (22, 23). These findings indicate that histone H2AX is needed for genome stability and efficient DNA repair and in particular the assembly of specific DNA repair proteins to DNA damage-induced nuclear foci. We have used γH2AX as a marker of both ATR and ATM activity that can be readily assayed.

In this report we have further investigated the phosphorylation of p53 serine 15 by ATR in response to hypoxia, and we have shown that like p53, histone H2AX is also phosphorylated in an ATM-independent manner (20, 21). Homozygous null H2AX knockout mice are born with the expected frequency but are radiation-sensitive, growth-retarded, immune-deficient, and infertile (22, 23). Elegant foci studies have shown that H2AX null cells had impaired recruitment of Nbs1, 53bp1, and Brca1 to the sites of DNA damage. However, the formation of Rad51 foci in response to DNA damage was only slightly affected, if at all, in the absence of H2AX (22, 23). These findings indicate that histone H2AX is needed for genome stability and efficient DNA repair and in particular the assembly of specific DNA repair proteins to DNA damage-induced nuclear foci. We have used γH2AX as a marker of both ATR and ATM activity that can be readily assayed.

In this report we have further investigated the phosphorylation of p53 serine 15 by ATR in response to hypoxia, and we have shown that like p53, histone H2AX is also phosphorylated in an ATM-independent manner in response to hypoxia. Most importantly, co-localization of p53 serine 15 and γH2AX within hypoxic regions of tumors indicates that oxygen concentrations within tumors are low enough to activate ATR. These data indicate that ATR and ATR-mediated signaling have a physiologically significant role to play in tumor development. We have also demonstrated that in contrast to hypoxia, reoxygenation induces a significant amount of DNA damage that can be detected by comet assays. This damage leads to ATM-dependent phosphorylation of p53 serine 15 and other ATM targets. Because of the poorly developed vasculature of tumors, the tumor microenvironment represents a dynamic situation where tumor cells are exposed to both hypoxia and reoxygenation (24). These studies suggest that ATR is the principal kinase for the phosphorylation of p53 in response to hypoxia and that ATM is activated by DNA damage during reoxygenation. Thus, ATR and ATM are activated by different stimuli in the tumor microenvironment.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfections**—The RKO and H1299 cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. GM1526 and GM0536 were maintained in RPMI supplemented with 15% fetal bovine serum. Both GM1526 (ATM^-/-, p53^-/-, p53^-/-, p53^-/-, p53^-/-) and GM536 (ATM^-/-, p53^-/-, p53^-/-, p53^-/-, p53^-/-) isolated from an ataxia-telangiectasia patient) are Epstein-Barr virus immortalized lymphoblastoid cell lines. The parental HCT116 cell line and the ATR^flox derivative were maintained in McCoy's medium supplemented with 10% fetal calf serum. Prior to infection with adenovirus-cre, 5 x 10^5 cells were plated for 24 h. The cells were then infected for 48 h with fresh medium, and virus was added after 24 h. The medium was replaced before hypoxia treatment (25).

**Hypoxia Treatment**—The cells were plated in glass dishes and treatment carried out in a hypoxia chamber (<0.2% O_2) (Sheldon Corp.,...
were sectioned (14). Mounting reagent (Vectashield, Vector Laboratories). Snap frozen tumors were treated with hypoxia treatment. –

Comet assays were carried out as previously described (28, 29). In brief, 1–3 × 10^6 RKO cells were prepared as a single cell suspension in magnesium/calcium-free PBS. Three volumes of a 1% low melt agarose, 2% MeSO solution were added to the cells followed by mixing. The mixture was plated onto a microscope slide and allowed to set on a cold surface. When completely set the slide was immersed in lysis buffer for 1 h (0.03 M NaOH, 1 M NaCl, 0.1% N-lauroylsarcosine) at room temperature. The propidium iodide-stained cells (comets) were visualized using a Nikon Optiphot microscope attached to an Ikegami 4612 CCD camera and fluorescence image analysis system. Using specially designed software, the tail moment of each cell was calculated as the product of the percentage of DNA in the tail multiplied by the length of the comet tail. 200 comets were scored for each treatment.

RESULTS

The Roles of ATR, ATM, and DNA-PKcs in the Induction of H2AX by Hypoxia—Previous work has indicated that H2AX is a substrate for the phosphatidylinositol 3-kinase family (18). To investigate the phosphorylation of H2AX by hypoxia, RKO cells were grown at both 0.02% and 2% oxygen and harvested at different times over a 24-h period (Fig. 1A). We examined the changes in HIF-1α and p53 protein levels and p53 serine 15 and H2AX, HIF-1α accumulated at both 0.02 and 2% oxygen. In contrast p53 only accumulated at 0.02% oxygen. Histone H2AX was clearly phosphorylated in response to extreme hypoxia but remained unaffected at 2% oxygen. These finding suggest that like p53 histone H2AX might be phosphorylated by a stress-activated phosphatidylinositol 3-kinase. To investigate the kinase responsible for this phosphorylation, we made use of both ATM and DNA-PKcs matched cell lines and a conditional ATR knockout cell line (25, 30). Using isogenic ATM-deficient and reconstituted cell lines (GM1526 and GM0536), we found that both p53 serine 15 and H2AX are phosphorylated in response to hypoxia in an ATM-independent manner (Fig. 1B). We also found this to be true in spontaneously transformed mouse embryonic fibroblasts from ATM−/− animals. Thus, a deficiency in ATM had little affect on H2AX phosphorylation. Similarly, cells that lack DNA-PKcs exhibited similar levels of H2AX phosphorylation as parental wild-type cells (Fig. 1C). Taken together, these results indicate that hypoxia does not activate the DNA damage response kinases ATM or DNA-PKcs. Therefore, we hypothesized that ATR was responsible for histone H2AX and p53 phosphorylation in response to hypoxia. HCT116 ATR−/− cells were treated with adenovirus-cre to knock out the remaining ATR allele from this cell line; the cells were then exposed to hypoxia and harvested.
that ATR is activated by replication arrest under hypoxic conditions. As would be expected for an ATR-mediated event, γH2AX foci were not detected in all cells. γH2AX foci were seen in ∼28% of cells. In contrast, under hypoxic conditions when cells were treated with the DNA damaging agent Adriamycin, foci were seen in all cells.

**p53 Ser**15 and γH2AX Staining Co-localizes with EF5-positive Regions in Tumors—To determine whether these in vitro findings with γH2AX or p53 serine 15 occurred in hypoxic tumor regions, we grew tumors in mice from the H1299 cell line expressing tetracycline-inducible p53, which we have previously described (12). Approximately 107 cells were implanted into the flanks of nude mice and were allowed to grow until they reached a diameter of 1 cm. Doxycycline and sucrose were added to the drinking water of half the mice, whereas the remaining half received sucrose alone before being sacrificed 24 h later. Prior to sacrifice, the mice were injected with EF5 to allow the visualization of hypoxic tumor regions (27, 31). Fig. 3 shows tumor sections stained for total p53 in mice that had been fed doxycycline (lower panel) or sucrose alone (upper panel). There was a clear induction of p53 after the addition of doxycycline. This was also verified by northern blotting and persisted while doxycycline was given to the mice (up to 6 days; data not shown). Generating p53-positive tumors this way results in higher levels of p53 than would normally be seen, which ease detection in vivo. Fig. 3C shows the staining of serial sections for γH2AX, p53 Ser15, and EF5. We chose to use serial sections for these studies because the EF5 stain can bleed through to the fluorescein isothiocyanate channel. The overlays of both γH2AX and p53 ser15 with EF5 are shown. Despite the use of serial sections, there was a clear overlap between staining for EF5 and γH2AX as well as EF5 and p53 serine 15. Perhaps more striking is the overlap between p53 serine 15 and γH2AX. It should be noted that not all of the cells within the EF5-positive region stained for p53 serine 15 or γH2AX, consistent with the S phase-dependent nature of ATR activation. It was not possible to visualize individual foci within stained cells. However, both p53 serine 15 and γH2AX did appear to be nuclei in localization. These data provide direct evidence that oxygen levels within tumors can reach levels low enough to induce a replication arrest and
hypoxia or completely in normoxia. The cells were also treated with 8 Gy of ionizing radiation (IR).

The Western blots shown in Fig. 4 demonstrate that neither p53 serine 15 nor H2AX were substantially phosphorylated in cells in conditions designed to mimic those present in tumors. The Western blots shown in Fig. 4 demonstrate that neither p53 serine 15 nor H2AX were substantially phosphorylated in response to any of the stresses tested unless significant apoptosis was also induced. As previously mentioned, H2AX has been shown to be phosphorylated during the early phases of apoptosis (21). Interestingly, treatment with sodium chloride, to mimic osmotic shock, induced one of the highest levels of apoptosis and yet very little p53 serine 15 or H2AX phosphorylation.

**Reoxygenation Induces DNA Damage and ATM-dependent Phosphorylation of p53 Serine 15**—We have previously demonstrated that hypoxia does not induce any detectable DNA damage using the alkaline comet assay (1). We hypothesized, however, that reoxygenation would induce significant amounts of damage. This is physiologically relevant because hypoxia within tumors is often transient, resulting from transient blockage of poorly developed vasculature or increased interstitial pressure (34). It is hypothesized that tumor cells are exposed to continuous cycles of hypoxia and reoxygenation. To assess the amount of damage associated with reoxygenation, RKO cells were treated with hypoxia for 16 h and then harvested after different times after reoxygenation. The relative amounts of DNA damage were then assessed by comet assay. As a reference point, the cells were also exposed to 8 Gy of ionizing radiation (Fig. 5). When cells were harvested entirely in normoxic conditions and hence fully reoxygenated, a significant amount of DNA damage occurred. Reoxygenation induced DNA damage approximately equivalent to treating cells with 4–5 Gy of ionizing radiation. We proposed that this level of damage would subsequently lead to increased or sustained phosphorylation of proteins that contain ATM recognition sites. To investigate this, we again made use of the GM1526 (ATM+/−) and GM0536 (ATM−/−) cell lines. The cells were exposed to hypoxia for 16 h before being harvested after various periods of reoxygenation (Fig. 6A). The p53 protein was clearly phosphorylated at serine 15 in response to hypoxia in ATM wild-type and ATM-deficient cell lines. However, as we predicted, the levels of phosphorylation were sustained in the ATM+/− cell line, whereas they begin to decrease after 10 min of reoxygenation in the ATM nulls. This suggests that in response to the DNA damage that occurs upon reoxygenation, ATM becomes activated and is responsible for maintaining phosphorylation of targets such as p53 serine 15. Reoxygenation leads to the rapid production of ROS (reactive oxygen species) mostly in the form of superoxide molecules. By pretreating cells exposed to hypoxia with a chemical scavenger for ROS, we hypothesized that cells would be protected from the DNA-damaging effects of these molecules, and consequently reoxygenation-induced phosphorylation of p53 serine 15 would be inhibited. RKO cells were exposed to hypoxia in the presence or absence of NAC and then reoxygenated (Fig. 6B). As was seen in the ATM wild-type cells (GM0536), the levels of p53 serine 15 in RKOs remained high and constant during the 35-min period after removal from hypoxia. However NAC significantly reduced the level of p53 serine 15 during reoxygenation. In the presence of NAC, the hypoxia-induced p53 serine 15 appears identical to that seen in the absence of NAC, indicating that the production of ROS during hypoxia treatment either is minimal or has no role in the phosphorylation of p53 at serine 15.

**DISCUSSION**

Many previous reports have demonstrated that γH2AX is a rapidly induced marker of DNA damage; in some cases phosphorylation has been reported within 10 min of genotoxic insult (35). In contrast, phosphorylation of histone H2AX occurs with much slower kinetics in response to hypoxia treatment. We have shown that this phosphorylation is ATR-dependent. Our data suggest that ATR becomes active, perhaps mediated by a change in cellular localization, in response to extreme levels of hypoxia, which induce replication arrest (1). The induction of this replication arrest is directly proportional to the amount
of oxygen present in the microenvironment. In accordance with this observation, plating cells in glass dishes, which retain less oxygen, can increase the kinetics of H2AX and p53 phosphorylation.

These data provide the first in vivo evidence for a role of ATR in tumors. We have demonstrated that ATR does not phosphorylate target molecules like p53 and H2AX until oxygen levels are low enough to induce a complete stop in DNA synthesis, i.e. replication arrest. Significantly, the finding that both p53 and H2AX are phosphorylated in vivo in the hypoxic regions of tumors indicates that these extreme levels of hypoxia do indeed occur in tumors. We have eliminated many other tumor-physiologically relevant stresses as having a co-operative role in the induction of these phosphorylation events.

We have previously showed that hypoxia did not induce any DNA damage detectable by the comet assay. Here, in contrast, we found that cells taken from severe hypoxia to normoxia had a significant amount of comet-detectable damage. This was particularly striking about these findings was the rapid kinetics of DNA damage induction in response to reoxygenation. We hypothesized that this damage might lead to subsequent ATM activation and also may have been mediated by the formation of ROS. We have presented evidence that both of these hypotheses are indeed valid. In the absence of ATM, the level of p53 phosphorylated at serine 15 slowly decreased, whereas it was maintained in the presence of ATM for at least 60 min. The addition of the ROS scavenger NAC inhibited the reoxygenation-induced phosphorylation of p53 at serine 15 but had no effect on the hypoxia-induced phosphorylation of p53 at serine 15.

We propose that both ATR and ATM have roles to play in tumor progression but that these roles may be distinct. Fig. 7 shows our proposed model. Initially ATR responds to replication arrest induced at severe levels of hypoxia followed by an ATM response to the DNA damage induced when these areas become reoxygenated. Our data suggest that the activation of one phosphatidylinositol 3-kinase over another is based on the presence or absence of DNA damage. We have been unable to detect DNA damage in cells that have undergone a replication arrest in response to hypoxia and therefore conclude that damage is not required for the relocalization of ATR to nuclear foci. We do not exclude the possibility that damage nondetectable by Comet assay does occur but would argue that if present it must be at a very low level and certainly not comparable with the significant damage seen upon reoxygenation. As previously mentioned, ATM has a much more defined role in the response to DNA damage, and it is perhaps therefore not surprising that it has a role to play in the reoxygenation response. Further insight will come from the identification of ATM- or ATR-specific targets. Neither Chk 2 or residue 20 of p53 are phosphorylated in response to extreme hypoxia, but both may be induced in response to reoxygenation in an ATM-dependent manner. The identification of these damage-specific targets will allow us to further elucidate the ATM damage response and the ATR replication response. Hypoxia may well be the ideal if not only model to study this further because it is unique in the induction of replication arrest without detectable concomitant damage.

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ATR Responds to Hypoxia, and ATM Responds to Reoxygenation

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