Hypoxia-Induced Phosphorylation of Chk2 in an Ataxia Telangiectasia Mutated–Dependent Manner

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Abstract

Chk2 is a serine/threonine kinase that signals to cell cycle arrest, DNA repair, and apoptotic pathways following DNA damage. It is activated by phosphorylation in response to ionizing radiation, UV light, stalled replication forks, and other types of DNA damage. Hypoxia is a common feature of solid tumors and has been shown to affect the regulation of many genes, including several DNA repair factors. We show here that Chk2 is phosphorylated on Thr68 and thereby activated in cells in response to hypoxia, and that this phosphorylation is dependent on the damage response kinase ataxia telangiectasia mutated (ATM) but not on the related kinase ATM and Rad3-related. Moreover, phosphorylation of Chk2 under hypoxia was attenuated in cells deficient in the repair factors MLH1 or NBS1. Finally, Chk2 serves to protect cells from apoptosis under hypoxic growth conditions. These results identify hypoxia as a new stimulus for Chk2 activation in an ATM-, MLH1-, and NBS1-dependent manner, and they suggest a novel pathway by which tumor hypoxia may influence cell survival and DNA repair.

Introduction

Chk2 is a tumor suppressor that is mutated in a variety of human tumor types (reviewed in ref. 1) and in a subset of Li-Fraumeni patients (2–4). Chk2 is a serine/threonine protein kinase that plays a key role in cell cycle arrest following DNA damage. After ionizing radiation or UV damage, the ataxia telangiectasia mutated (ATM) and the ATM and Rad3-related (ATR) kinases are activated, respectively. ATM and ATR both can phosphorylate Chk2 on Thr68 (5, 6), which in turn leads to the phosphorylation of a number of downstream proteins by Chk2, including p53 (on Ser20; refs. 7–9). On phosphorylation by Chk2, p53 is stabilized (10, 11), causing cell cycle arrest and regulating DNA repair.

Other factors that participate in the activation of Chk2 under various conditions include NBS1 (12, 13), 53BP1 (12, 14, 15), BRCA1 (16), and MLH1 (17). In terms of ionizing radiation–induced signaling, NBS1 and 53BP1 have been placed in parallel pathways upstream of Chk2 (12). Both proteins affect the phosphorylation of Chk2 on Thr68. In addition, work by Brown et al. (17) suggests that Chk2 phosphorylation after ionizing radiation is MLH1 dependent. Whereas MLH1 is not suspected to be a kinase, the authors suggest a scaffolding role whereby it facilitates the interaction between ATM and Chk2. In addition to modulation by these upstream factors, Chk2 has been shown to autophosphorylate following exposure of cells to ionizing radiation (14, 18–20).

Solid tumors characteristically exhibit heterogeneity in vascularization, perfusion, and oxygenation, with regions of moderate to severe hypoxia. The pattern of hypoxia in tumors is dynamic with both temporal and spatial variations. Clinical studies indicate that extensive hypoxia in human tumors correlates with poor prognosis (21–24), reflecting not only the radioresistance of hypoxic cancer cells but also a more aggressive phenotype.

We and others have proposed that hypoxia may contribute to tumor progression by promoting genetic instability. Increased levels of mutations have been detected in hypoxic cells (25–27) and hypoxia has been found to cause decreased expression of the DNA repair genes MLH1, MSH2, and Rad51 (28–30).

Because MLH1 has been suggested to play a role in Chk2 activation, we initially hypothesized that the decrease in MLH1 levels under hypoxia might cause an attenuation of Chk2 activation in response to ionizing radiation. Interestingly, however, we found that Chk2 phosphorylation is directly induced by hypoxia itself and that this induction is substantially dependent on ATM. In addition, MLH1 and NBS1 were also found to play roles in Chk2 activation by hypoxia. Furthermore, a key downstream substrate of Chk2, p53, was found to be altered under hypoxic conditions in a Chk2-dependent manner, indicating that hypoxia-induced phosphorylation of Chk2 leads to functional activation and downstream signaling. Finally, Chk2 seems to protect cells from hypoxia-induced apoptosis and, thus, plays a role in cell survival under hypoxic stress. These results identify a new mechanism by which hypoxia can influence cell signaling and DNA damage response pathways.

Materials and Methods

Cell culture. HeLa, MCF-7, RKO, and HCT15 cells were maintained in DMEM that was supplemented with 10% fetal bovine serum (FBS). HCT116/2-3 and HCT116/3-6 cells (31) were grown in McCoy's medium with 10% FBS and 0.4 mg/mL G418. HCT15 stable cell lines overexpressing wild-type (WT) Chk2 were maintained in 0.8 mg/mL G418. LXIN (NBS1 mutant) and NBS1 (LXIN corrected with NBS1 cDNA) cells were cultured in DMEM with 10% FBS and 0.4 mg/mL G418. NBS1 and LXIN cells were obtained from Dr. Patrick Concannon (Molecular Genetics Program, Baranoraya Research Institute at Virginia Mason, Seattle, WA) (ref. 32).

Plasmids and RNA interference. The HA-Chk2 expression vector was obtained from Dr. David F. Stern (Department of Pathology, Yale University, New Haven, CT) (ref. 18). RNA interference (RNAi) target sequences for ATM were ligated into the pSUPER vector (OligoEngine, Seattle, WA). The oligonucleotide sequences are as follows: ATM-1A, GATCCCGCGCCCGTTCCGAGATCTCCTAAGTTTCTCTAAGATCTCAATCGGGCGTG; ATM-1B, GTTCGAGATCCTAGAGAGCTCAGATCTCAATCGGGCGTG; ATM-2A, GCTTCTTCTTTGGA; ATM-2B, AGCTTTCTTTTTGGA; and ATM-2C, AGCTTTCTTTTTGGA. Small interfering RNA (siRNA)—expressing stable cell lines were made by cotransfecting

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©2003 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-05-1160

Cancer Res 2005; 65: (23). December 1, 2005 10734 www.aacrjournals.org
pSUPER constructs with pcDNA3 (Invitrogen, Carlsbad, CA) at a ratio of 10:1 using FuGENE 6 reagent as directed by the manufacturer (Roche, Indianapol, IN). Stable lines were selected and maintained in 0.8 mg/mL G418. RNAi directed against ATG (ATR-2 duplex, Dharmacon, Lafayette, CO), ATM (siGENOME SMARTpool reagent, Dharmacon), or luciferase (Luciferase GL-2 duplex, Dharmacon) was done transiently using Oligofectamine (Invitrogen).

**Hypoxia and desferrioxamine treatments.** Hypoxic cell culture conditions were established as previously described (33) using a continuous flow of a mixture of 95% N2 and 5% CO2 gas certified to contain <10 ppm O2 (Airgas Northeast, Cheshire, CT). Unless otherwise specified, experiments were done with 0.01% O2. Hypoxic cell culture experiments in which in situ lysis was done were carried out in an INVIVO2 400 chamber (Biotrace, Cincinnati, OH) with glove box capabilities to allow manipulation under hypoxia. Desferrioxamine (Sigma, St. Louis, MO) treatments were carried out by media supplementation to a final concentration of 250 μmol/L. Ionizing radiation was done with a 137Cs irradiator at a dose rate of 165 rad/min.

**Phosphatase assay.** Phosphatase treatments of protein samples were carried out using λ-phosphatase as per instructions of the manufacturer (New England Biolabs, Beverly, MA). Briefly, 100 μg of total cell lysate were incubated with 1× reaction buffer, 2 mmol/L MnCl2, and 1 μL of λ-phosphatase. Reactions were carried out for 30 minutes at 30°C. Where indicated, phosphatase inhibitors (50 mmol/L NaF and 10 mmol/L Na3VO4) were added to the reactions before the introduction of enzyme.

**Western blot.** Cells were lysed with radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 0.1% SDS) and 100 μg of total protein per sample were resolved by SDS-PAGE. Proteins were detected by standard immunoblot protocol using the following primary antibodies: phospho-Chk2(Thr68) and p53 (D0-1) from Santa Cruz Biotechnology (Santa Cruz, CA); Chk2 (clone 7; Upstate Biotechnology, Lake Placid, NY); phospho-p53(Ser20) (9287; Cell Signaling Technologies, Beverly, MA); tubulin (clone B-5-1-2; Sigma); Hif-1α (clone 54) and NBS1 (clone 34; BD Transduction Labs, Franklin Lakes, NJ); ATM (2c1) and ATR (clone 2B5; GeneTex, San Antonio, TX); MLH1(clone G168-15; BD PharMingen, San Diego, CA); ATM (2c1) and ATR (clone 2B5; GeneTex, San Antonio, TX); MLH1(clone G168-15; BD PharMingen, San Diego, CA); and Glut-1 (GT12-A; Alpha Diagnostic International, San Antonio, TX) and actin (Research Diagnostics, Inc., Flanders, NJ). Each experiment was carried out at least thrice and representative Western blots are shown. Band intensities were quantitated using the public domain NIH Image program (version 1.63; developed at the U.S. NIH and available online at http://rsb.info.nih.gov/nih-image/). Relative increases in Chk2 phosphorylation were calculated as follows: (ratio of phospho-Chk2 in hypoxia versus normoxia) / (ratio of total Chk2 in hypoxia versus normoxia).

**Northern blot.** Total RNA was isolated using the TRIzol RNA isolation reagent (Life Technologies, Carlsbad, CA). Northern blots were done as previously described (28). The following primer pairs were used to produce probes for Northern blot analysis by reverse transcription-PCR (RT-PCR) amplification of mRNA: Chk2, 5′-GCCGCTCTGATGTTCTCGG-3′ (sense) and 5′-TTCGTTGTTCAAACCACGGA-3′ (antisense); vascular endothelial growth factor, 5′-CCTCAGTCTGATTTGCTGCTG-3′ (sense) and 5′-GCTAGTGCTGTCACCGAGGAGG-3′ (antisense). RT-PCR products were confirmed by sequence analysis before use.

**Cell cycle analysis.** Cells cultured in normoxia or hypoxia were stained with propidium iodide. DNA content was analyzed using a Becton Dickinson FACS-Calibur flow cytometer. Histogram construction was done using BD CellQuest Pro software (Becton Dickinson, San Jose, CA). Experiments were conducted in triplicate.

**Caspase activation assay.** Caspase-3 activity was assayed using the CaspACE Colorimetric Assay System according to the instructions of the manufacturer (Promega Corp., Madison, WI). Absorbance readings for each sample were determined at 405 nm using a SpectraMax Gemini XS microplate reader and SoftMax Pro software (Molecular Devices Corp., Sunnyvale, CA). The caspase specific activity for each sample was calculated using the following formula: specific activity = pmol product per hour / μg protein. P values were calculated based on unpaired two-tailed t-tests using the Microsoft Excel Plug-in Analyze-it (Analyze-it Software Ltd., Leeds, United Kingdom). Caspase activation experiments were conducted in replicates of six.

**Results**

**Hypoxia induces Chk2 phosphorylation.** To examine the effects of hypoxia on Chk2 phosphorylation, we cultured HeLa and MCF-7 cells under normoxic or hypoxic (0.01% O2) conditions for 48 hours. As a positive control, cells were irradiated with 10 Gy of ionizing radiation. In both cell lines, hypoxia induced Chk2 phosphorylation on Thr68 (Fig. 1A). Chk2 contains a number of SQ/TQ motifs, which are generally thought of as ATM/ATR consensus phosphorylation sites (34, 35). Of these sites, the most commonly studied is Thr68, which is known to be phosphorylated and lead to Chk2 activation after ionizing radiation damage. Interestingly, although Chk2 phosphorylation increased on exposure to hypoxia, total Chk2 protein levels decreased. By correcting for the decrease in total Chk2 protein levels, we were able to quantify the relative hypoxia-induced increases in Chk2 phosphorylation in HeLa and MCF-7 cells as 3- and 5-fold, respectively. These relative increases in Chk2 phosphorylation were comparable to changes in Chk2 activation following exposure to ionizing radiation. We also observed similar results on treatment of cells for 48 hours with the iron chelator desferrioxamine, a chemical mimetic of hypoxia (Fig. 1B).

Antibody specificity and Chk2 phosphorylation were confirmed by phosphatase treatment (Fig. 1C). Samples from hypoxic or irradiated MCF-7 cells exhibited Chk2 phosphorylation when either added directly to the gel or when preincubated with reaction buffer alone (Fig. 1C, lanes 5, 6, 9, and 10). However, incubation with λ-phosphatase abolished this signal (Fig. 1C, lanes 7 and 11). The signal was maintained by the presence of phosphatase inhibitors to prevent Chk2 dephosphorylation (Fig. 1C, lanes 8 and 12).

To confirm that Chk2 phosphorylation is induced by hypoxia per se rather than during the brief reoxygenation that can occur on removal of cell culture dishes from a hypoxic chamber before lysis, MCF-7 and RKO cells were incubated in a glove box chamber at 0.1% O2. After 48 hours, the cells were lysed in situ under hypoxia and Chk2 phosphorylation was assessed in the lysates by Western blot (Fig. 1D). As shown, Chk2 is indeed phosphorylated on Thr68 under strictly hypoxic conditions; the phosphorylation cannot be attributed simply to a reoxygenation effect. Similar to previous experiments, the relative increases in Chk2 phosphorylation were determined to be 5-fold in MCF-7 cells and 7-fold in RKO cells. Because of the limitations of the glove box chamber, this experiment was conducted under 0.1% O2, representing less severe hypoxia than the conditions for L4 and C. However, the results are consistent, indicating the reproducibility of the Chk2 phosphorylation response over a range of hypoxic conditions.

To determine the kinetics of the Chk2 phosphorylation and of the decrease in total Chk2 protein, time-course experiments were carried out with MCF-7 cells cultured in hypoxia and harvested in situ within a glove box chamber. Increased phosphorylation on Thr68 could be detected after 12 hours of exposure to hypoxia, with maximum signal achieved at 72 hours (Fig. 2A). No further increase in Chk2 phosphorylation was detected at the 96-hour time point. It is of note that cells harvested at all time points retained good viability despite prolonged growth in low oxygen conditions. The decrease in total Chk2 levels was not observed until the 48-hour time point, indicating that the protein is initially activated, but that there is a subsequent decrease in total Chk2 protein levels after exposure to hypoxia.
To explore the mechanism of Chk2 down-regulation, Northern blot analyses were done using MCF-7 and HeLa cells. The results indicate that Chk2 mRNA levels are decreased on exposure to either 48 hours of hypoxia or 24 hours of desferrioxamine treatment (Fig. 2B).

**Upstream activators involved in Chk2 phosphorylation.** As mentioned above, Chk2 is a substrate of the ATM kinase (5, 6). To investigate the potential role of ATM in hypoxia-induced Chk2 phosphorylation, MCF-7 cells were stably transfected with a vector designed to express siRNA directed against ATM (pSUPER-ATMi) or an empty vector control. The resulting cell lines, ATMi-32 and pSpr-7, respectively, were exposed to hypoxic (0.1% O₂, 48 hours) or normoxic growth conditions. At the time of harvest for analysis, the hypoxic cells were lysed in situ within the glove box chamber. As shown in Fig. 3A, ATMi cells exhibited substantially reduced hypoxia-induced Chk2 phosphorylation when compared with the parental MCF-7 or pSpr-7 control cells. Due to the undetectable levels of Chk2 phosphorylation in normoxic samples, we were unable to quantitate the relative increases in Chk2 activation. However, by comparing the ratio of phospho-Chk2 to total Chk2 under each condition, we were able to calculate a 3-fold decrease in Chk2 phosphorylation in ATM-deficient cells in relation to control MCF-7 and pSpr cells.

Because the substrate targets for ATM can also be targets for the related kinase, ATR, we sought to determine whether ATR plays any role in Chk2 phosphorylation under hypoxia. RKO and MCF-7 cells were transfected with a synthetic siRNA duplex of a sequence previously shown to down-regulate ATR expression (36) or with a duplex targeted to the firefly luciferase gene as a control for nonspecific effects of RNAi. Sixteen hours after transfection, cells were either maintained in normoxic conditions or placed in hypoxia (0.1% O₂) for another 48 hours. Again, hypoxic cells were lysed in situ in the glove box chamber. Western blot analysis...

**Figure 1.** Phosphorylation of Chk2 is induced by hypoxia and the hypoxia mimetic iron chelator desferrioxamine. A, Western blot analysis of phospho-Chk2 (Thr68), total Chk2, Hif-1α (positive control for hypoxia), and tubulin (loading control) levels in HeLa and MCF-7 cells under normoxia (N), hypoxia (0.01% for 48 hours; H), or after ionizing radiation exposure (10 Gy; IR) as indicated. B, Western blot analysis, as in (A), of the protein levels in HeLa and MCF-7 cells with or without desferrioxamine treatment (250 μmol/L for 48 hours; DFX). C, effect of phosphatase treatment of cell lysates on detection of phospho-Chk2 in samples from hypoxic or irradiated cells. MCF-7 cells were exposed to normoxia, hypoxia (48 hours), or ionizing radiation as above, and the respective lysates were treated as indicated with λ-phosphatase or λ-phosphatase plus phosphatase inhibitors. TCL, total cell lysate directly loaded onto the gel. Lanes 2, 6, and 10, samples were treated with reaction buffer only. Phosphatase experiments were carried out four times. Representative Western blot. D, Western blot analysis of samples from MCF-7 and RKO cells lysed in situ under hypoxia (0.1% O₂, 48 hours) within a glove box chamber.

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cells were grown in 0.1% O₂ for 48 hours. Luc (ATR) directed against ATR (transfected with synthetic RNAi duplexes) cells (left) and MCF-7 cells (right) were transfected with synthetic RNAi duplexes directed against ATM (pSpr) or luciferase (Luc) or mock transfected (None). Hypoxic cells were grown in 0.1% O₂ for 48 hours and lysed in situ. Western blot analysis was carried out to visualize levels of the indicated proteins. Equal loading was confirmed by Western blot analysis of tubulin in RKO cells and of actin in MCF-7 cells. Dashed lines, nonadjacent lanes from the same gel. C, MCF-7 cells stably expressing siRNA directed against ATM were transiently transfected with synthetic RNAi duplexes targeting luciferase, ATM, or ATR. Parental MCF-7 cells transfected with siRNA against ATM were used as controls for WT protein levels. Hypoxic cells were grown in 0.1% O₂ for 48 hours and lysed in situ. Cell lysates were analyzed by Western blot for the indicated factors. Dashed lines, separate lanes from the same gel. D, derivatives of the MLH1-deficient colon cancer cell line HCT116, HCT116/3-6 (MLH1-), or HCT116/2-3 (MLH1+), were grown in normoxia or hypoxia (48 hours) and cell lysate samples were analyzed by Western blot for the indicated proteins. E, a matched pair of NBS1-deficient or complemented cell lines (NBS1– and NBS1+) were exposed to normoxia or hypoxia for 48 hours and cell lysates were analyzed by Western blot as above.

(Fig. 3B) revealed that ATR down-regulation by RNAi in either RKO or MCF-7 cells does not reduce Chk2 phosphorylation in response to hypoxia.

To further test the role of ATR in hypoxia-induced Chk2 activation, we examined the effect of ATR knockdown in an ATM-deficient cell background. ATM-i cells (constitutively expressing siRNA directed against ATM) were transfected with synthetic siRNA duplexes directed against either ATM or ATR. siRNA targeted to luciferase was used as a control. As shown in Fig. 3C, additional reduction of ATM expression resulted in even greater suppression of Chk2 phosphorylation under hypoxic conditions. However, knockdown of ATR expression had no effect on Chk2 activation in ATM-deficient cells. Thus, residual Chk2 phosphorylation in ATM-i cells seems to be the result of the incomplete knockdown of ATM and not the result of compensatory ATR function. In fact, these results further indicate that ATR has no apparent role in Chk2 phosphorylation in response to hypoxia.

Because MLH1 has been shown to play a role in Chk2 activation following ionizing radiation exposure (17), we examined MLH1-deficient and complemented cells (31) with regard to Chk2 phosphorylation in response to hypoxia. HCT116/2-3 (MLH1+) and HCT116/3-6 (MLH1–) were grown in normoxia or hypoxia for 48 hours. The MLH1-deficient cells exhibited reduced Chk2 phosphorylation under hypoxia compared with the MLH1-complemented cells, which showed a 6-fold higher ratio of phospho-Chk2 to total Chk2 (Fig. 3D). A similar dependency on MLH1 was also seen after desferrioxamine treatment (data not shown).

NBS1, a factor in DNA double-strand break repair, also signals to Chk2 after ionizing radiation exposure (12, 13). We examined NBS1-deficient and cDNA-complemented human cells (32) to test the NBS1 dependence of Chk2 phosphorylation after exposure to 48 hours of hypoxia. Cells deficient in NBS1 exhibited 3-fold lower Chk2 phosphorylation than their complemented counterparts (Fig. 3E). Under the electrophoresis conditions used, a higher mobility species of Chk2 is visible in hypoxic samples using an antibody to total Chk2 (Fig. 3E, lanes 2 and 4). Such a shift in mobility is indicative of Chk2 hyperphosphorylation. Notably, although hypoxia induces Chk2 down-regulation in both NBS1-proficient and -deficient cells, a greater proportion (~50%) of the remaining Chk2 exists as the higher mobility species in cells expressing NBS1. This observation reinforces the data.
obtained using the phospho-Chk2–specific antibody and further indicates that Chk2 phosphorylation is attenuated in NBS1-deficient cells.

Overall, the data in Fig. 3 indicate that ATM, MLH1, and NBS1 all participate in Chk2 phosphorylation in response to hypoxia. In contrast, hypoxia-induced Chk2 phosphorylation is independent of ATR. Likewise, two other mediators of Chk2 activation, 53BP1 and BRCAl, did not affect Chk2 phosphorylation after exposure to hypoxia (data not shown).

**p53 phosphorylation in response to hypoxia is dependent on Chk2 and ataxia telangiectasia mutated.** To show that hypoxia-induced Chk2 phosphorylation leads to functional activation, we examined the phosphorylation of p53 as a known Chk2 target. MCF-7 cells were grown in normoxia or hypoxia for 48 hours and cell lysates were assayed by Western blot for phospho-p53 (Ser20) and total p53. As shown in Fig. 4A, p53 is phosphorylated on Ser20 after exposure to hypoxia and this phosphorylation correlates with an increase in total p53. This phosphorylation event is known to be mediated by Chk2 in response to ionizing radiation (7–9) and its occurrence in hypoxia indicates that Chk2 is functionally active.

To determine whether Chk2 is, in fact, responsible for p53 phosphorylation, a comparison was made between HCT15 cells (Chk2-deficient) and HCT15 subclones complemented with a vector expressing WT Chk2 cDNA. The parental HCT15 cells exhibited minimal hypoxia-induced changes in p53 phosphorylation (Fig. 4B). However, in HCT15 cells complemented with a WT Chk2 cDNA construct, phosphorylation of p53 on Ser20 was substantially increased on exposure to 48 hours of hypoxia (Fig. 4B).

Because we have shown that ATM contributes to Chk2 phosphorylation, we sought to determine whether ATM also affects p53 phosphorylation on Ser20. MCF-7, MCF-7 and ATMi cell lines were cultured in normoxia or hypoxia for 48 hours. Whereas MCF-7 cells exhibited increased p53 phosphorylation after exposure to either hypoxia or ionizing radiation, ATMi cells displayed attenuated p53 phosphorylation in response to both stimuli (Fig. 4C), consistent with a role for ATM in p53 activation under hypoxia. These data indicate that both ATM and Chk2 can be placed in a pathway leading to p53 phosphorylation in response to hypoxia.

**Chk2 protects cells from hypoxia-induced apoptosis.** Chk2 is known to affect a number of cellular processes, including cell cycle arrest and apoptosis. To address the possibility that Chk2 activation in hypoxia might influence cell cycle progression, Chk2-deficient and Chk2-proficient cells were cultured in normoxia or hypoxia (0.1%) for 24 hours and stained with propidium iodide to assay cellular DNA content. Hypoxic cells were treated and harvested within the hypoxia glove box chamber. We did not observe major differences in cell cycle phase redistribution in response to hypoxia in a comparison of Chk2-deficient and Chk2-proficient cells (Fig. 5A) except for a 30% increase in the sub-G1 population in Chk2-deficient cells that were exposed to hypoxia. Essentially no sub-G1 DNA content cells were seen in the Chk2-proficient cells.

Because sub-G1 DNA content is often associated with apoptosis, we examined caspase-3 activation as a measure of cellular apoptosis. Chk2-deficient HCT15 cells and HCT15 subclones complemented with WT Chk2 cDNA were cultured under normoxic or hypoxic conditions for 48 hours and caspase-3 activation was assayed. As shown in Fig. 5B, Chk2-deficient cells exposed to hypoxia exhibit a 3.7-fold increase in caspase activation ($P < 0.0001$), and thus apoptosis, when compared with their normoxic counterparts. Cells expressing WT Chk2, however, did not show a significant hypoxia-induced increase in caspase activity, indicating that Chk2 serves to protect cells from apoptosis under hypoxic conditions.

**Discussion**

The work reported here shows that Chk2 phosphorylation on Thr68 is induced by hypoxia in a manner dependent on ATM, MLH1, and NBS1. p53, a well-documented target of Chk2, is also phosphorylated under hypoxia, and this was found to occur in a Chk2-dependent manner, consistent with functional Chk2 phosphorylation and activation under these conditions. In addition, the presence of Chk2 was found to suppress hypoxia-induced apoptosis. Taken together, these results delineate a novel signaling pathway in response to hypoxic stress that affects cell survival. Because hypoxia is a common feature of solid tumors, this pathway may play a key role in cancer cell growth regulation and DNA damage response.

This line of investigation was prompted by work implicating MLH1 in the ATM-Chk2 signaling cascade that is initiated by ionizing radiation (17). We had previously shown that MLH1 levels are down-regulated by exposure of human cancer cells to hypoxia, and so we hypothesized that any MLH1-dependent response to ionizing radiation might consequently be compromised by hypoxia.
Instead, we found that cells exposed to hypoxia alone exhibited increased Chk2 phosphorylation on the key Thr68 residue. This result was confirmed in a number of different cell types and could also be obtained by treating cells with the chemical hypoxia mimetic desferrioxamine. It is important to note that in our experiments, cell lysates that were harvested in situ within the hypoxia glove box chamber exhibited Chk2 phosphorylation. Hence, the observation of hypoxia-induced Chk2 phosphorylation reported here is a bona fide response to hypoxia and cannot be attributed to the effect of reoxygenation.

Time-course experiments with hypoxia revealed that Chk2 is initially phosphorylated and that there is a subsequent decrease in total Chk2 protein levels (Fig. 2A). The down-regulation of Chk2 was also seen at the mRNA level (Fig. 2B), suggesting that the changes in Chk2 levels are likely the result of transcriptional regulation. Note that the Chk2-expressing subclones of HCT15 cells do not show any hypoxia-induced changes in Chk2 protein levels (Fig. 4B). Because these cells express Chk2 cDNA from a heterologous promoter, the consistent protein levels support our interpretation of the results in Fig. 2B that Chk2 is transcriptionally regulated. The combined data suggest that Chk2 is likely down-regulated in hypoxia as a result of promoter repression rather than an alteration in protein or mRNA stability. Preliminary data indicate that this transcriptional regulation is not dependent on the hypoxia-inducible transcription factor HIF-1α (data not shown) although further studies will be necessary to determine which transcription regulatory factors are involved.

One recent study has suggested that certain substrates shared by the related kinases, ATM and ATR, are phosphorylated by ATR under hypoxic conditions and by ATM on reoxygenation (37, 38). However, we found that ATM is primarily responsible for Chk2 phosphorylation under hypoxia, with ATR having no detectable role. Because in our experiments the cells were lysed in situ under hypoxia within a glove box chamber, we can attribute the ATM-dependent Chk2 activation solely to hypoxia. Furthermore, the 3-fold decrease in Chk2 phosphorylation observed using ATM RNAi probably underestimates the role of ATM in hypoxia-induced Chk2 activation. Although ATM levels are substantially lower in cells stably expressing siRNA against ATM, the knockdown is not complete and the residual ATM may still mediate some
phosphorylation of Chk2. In this regard, MCF-7 cells constitutively expressing siRNA against ATM, which were transfected with synthetic RNAi duplexes against ATM, showed further reduction in ATM levels and a corresponding additional suppression of Chk2 phosphorylation under hypoxia. In contrast, knockdown of ATR in ATM RNAi–expressing cells showed no further suppression of Chk2 phosphorylation under hypoxia, consistent with the lack of effect of the individual ATR knockdown above.

We also tested the roles of MLH1 and NBS1, both of which have been implicated by other studies in ionizing radiation–induced signaling to Chk2 (12, 13, 17). We found that both can influence Chk2 phosphorylation in response to hypoxia with substantial decreases in hypoxia-induced Chk2 phosphorylation in cells deficient in either factor.

Following activation by exposure to ionizing radiation or other DNA damaging agents, Chk2 phosphorylates the downstream effector p53 (7–9). On phosphorylation by Chk2, p53 is stabilized (10, 11), causing cell cycle arrest and regulating DNA repair. Although hypoxia-induced p53 stabilization has been previously reported (39), we have shown here in a comparison of Chk2–deficient and Chk2-proficient cells that p53 phosphorylation and stabilization under hypoxia are both Chk2 dependent. These data indicate that Chk2 phosphorylation in hypoxia is associated with functional activation and downstream consequences. Furthermore, we have shown that p53 phosphorylation is also dependent on ATM, allowing us to place Chk2 and ATM in a hypoxia-induced pathway leading to p53 phosphorylation.

Chk2 shares many substrates with the functionally related kinase Chk1. Although Chk1 has been implicated in posthypoxia cell survival (40), we have been unable to detect Chk1 phosphorylation in our cells under either hypoxia or desferrioxamine exposure (data not shown).

Chk2 is known to affect several cellular processes, including cell cycle arrest, apoptosis, and DNA repair. We have shown here that cells deficient in Chk2 are especially sensitive to low oxygen levels and are more susceptible to hypoxia-induced apoptosis (Fig. 5). We hypothesize that Chk2 either functions to stabilize stalled replication forks or contributes to the repair of DNA lesions, thereby limiting the accumulation of DNA damage and precluding the induction of apoptosis.

Based on the work reported here, we propose a model in which hypoxia can lead to ATM and/or NBS1 activation, either through altered DNA metabolism or changes in chromatin structure. ATM, which may be activated either directly or as a result of communication with NBS1, then phosphorylates Chk2. Because MLH1 has not been shown to possess kinase activity, it is likely that MLH1 acts as a scaffold to properly orient ATM and Chk2. Such a scaffolding role to position ATM together with Chk2 was previously proposed for MLH1 as a response to ionizing radiation (17).

Chk2 is constitutively phosphorylated on Thr68 in many human tumors, especially those in which p53 is mutated (41). However, the cause of this constitutive phosphorylation is unknown. Our results showing that Chk2 is phosphorylated under hypoxic conditions raise the possibility that prolonged tumor hypoxia and/or cycles of hypoxia and reoxygenation may provide a mechanism that leads to persistent Chk2 activation.

Acknowledgments


Grant support: NIH grant ES05775 (P.M. Glazer).

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We thank Z. Yun, D. Campisi Hegan, C. Redlik, and L. Cabral for their help.

References


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