An Overactivated ATR/CHK1 Pathway Is Responsible for the Prolonged G₂ Accumulation in Irradiated AT Cells

Xiang Wang†, Jay Khadpe‡, Baocheng Hu‡, George Iliakis§, and Ya Wang‡‡

From the †Department of Radiation Oncology, Kimmel Cancer Center of Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the ‡Institute of Radiation Biology, University of Essen Medical School, 45122 Essen, Germany

Induction of checkpoint responses in G₁, S, and G₂ phases of the cell cycle after exposure of cells to ionizing radiation (IR) is essential for maintaining genomic integrity. Ataxia telangiectasia mutated (ATM) plays a key role in initiating this response in all three phases of the cell cycle. However, cells lacking functional ATM exhibit a prolonged G₂ arrest after IR, suggesting regulation by an ATM-independent checkpoint response. The mechanism for this ataxia telangiectasia (AT)-independent G₂ checkpoint response remains unknown. We report here that the G₂ checkpoint in irradiated human AT cells derives from an overactivation of the ATR/CHK1 pathway. Chk1 small interfering RNA abolishes the IR-induced prolonged G₂ checkpoint and radiosensitizes AT cells to killing. These results link the activation of ATR/CHK1 with the prolonged G₂ arrest in AT cells and show that activation of this G₂ checkpoint contributes to the survival of AT cells.

In response to ionizing radiation (IR), proliferating cells slow their progress through the cell cycle by activating the DNA damage-induced checkpoints, G₁, S, and G₂ phase checkpoints, believed to promote DNA repair and to benefit genomic integrity (1–4). ATM, the protein product of the gene mutated in AT cells, is one such central signal kinase responding to IR (5, 6). Many proteins serving as checkpoint effectors are phosphorylated and activated by ATM kinase. As such, p53, CHK2, BRCA1, and Rad17 are involved in G₁ and G₂/M cell cycle arrests (7–13), while CHK2 and NBS1/SMC1 are involved in the S checkpoint (14–16). AT cells deficient in ATM function show an abnormality in all checkpoint responses including G₁, S, and G₂ phase checkpoints and are sensitive to IR (17–20), indicating that the target of caffeine in AT cells might be ATR which is critical for AT cell survival.

We show here that the prolonged G₂ checkpoint in irradiated human AT cells without ATM function correlates with the overactivated ATR/CHK1 pathway following IR. Like caffeine (a nonspecific inhibitor of ATR) or UCN-01 (a nonspecific inhibitor of CHK1), Chk1 siRNA also abolishes the IR-induced prolonged G₂ checkpoint and radiosensitize AT cells to killing. These results clearly demonstrate that an overactivated ATR/CHK1 pathway is responsible for the IR-induced prolonged G₂ checkpoint in AT cells and that this checkpoint is important for maintaining AT cell survival.

EXPERIMENTAL PROCEDURES

Cell Lines, Chemical Treatment, and Irradiation—Both GM847 (ATM⁻/⁻) and AT5BIVA (ATM⁻/⁻) cells are transformed human fibroblasts. These cells were adapted to growing in Dulbecco’s modified Eagle’s medium supplemented with 10% iron-supplemented calf serum (Sigma). The incubations were at 37 °C in an atmosphere of 5% CO₂ and 95% air. Caffeine (Sigma) or UCN-01 (NCI) was added to the culture 30 min before the cells were exposed to x-rays (310 kV, 10 mA, 2-mm aluminum filter) and remained in the culture after IR until the cells were collected.

One- and Two-parameter Flow Cytometry Assay—For propidium iodide (PI) one-parameter assay, cells were collected at different times following IR and stained with PI solution as described previously (38). For bromodeoxyuridine (BrdUrd, Sigma) and PI, a two-parameter assay, we followed the method described by McKay et al. (39) with a minor modification. Thirty μM BrdUrd was added to the growth medium immediately following IR and was maintained in the medium until cells were collected (24 h following IR). The FITC-conjugated anti-BrdUrd antibody was purchased from Dako Co. After cell collection, the cells were incubated with the antibody according to the manufacturer’s instructions.

This paper is available on line at http://www.jbc.org
Overactivated ATR/CHK1 in Irradiated AT Cells

The cells were assessed by a flow cytometer (Coulter Epics Elite) for PI (DNA content) and FITC (DNA synthesis) measurements. ATR Kinase Activity Assay—ATR activity was examined with a chromatin-bound extract prepared as described previously (40). Briefly, cells were collected and washed in cold phosphate-buffered saline. Proteins were then extracted with cold 0.1% Triton X-100 in CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 20 min at 4 °C. The sample was then pelleted by low speed centrifugation at 3,000 rpm for 5 min at 4 °C. The supernatant was named fraction 1. These pellets were then re-extracted by incubating in CSK buffer and collected by centrifugation at 3,000 rpm for 10 min at 4 °C. This supernatant was named fraction 2. The final pellet fraction (containing chromatin-bound proteins) was solubilized in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 40 mM Tris, pH 7.2, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) and was named fraction 3. For kinase assay, 500 μg of fraction 3 was mixed with 2 μg of ATR antibody (sc-1887, Santa Cruz Biotechnology, Inc.) in the presence of 20 μl of a 50% (v/v) protein G-Sepharose slurry (Invitrogen) in 500 μl of Buffer A (0.5% Nonidet P-40, 1 mM Na3VO4, 5 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline buffer) and gently rotated overnight at 4 °C. Immune complexes were washed twice with Buffer A, then twice with Buffer B (10 mM HEPES, pH 8.0, 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol). The kinase immunoprecipitate was incubated at 30 °C for 30 min with 1 μg of PHAS-1 (Stratagene) in 25 μl of Buffer B containing 10 μCi of [γ-32P]ATP. Samples were analyzed by 12% SDS-PAGE and the kinase activities determined by the incorporation of [32P] into PHAS-1 protein using a PhosphorImager.

Purification of GST-CDC25C290–295–BL21 cells were transformed with plasmid pGEX-2T (Amersham Biosciences) encoding CDC25C290–295. The GST-CDC25C290–295 was purified by using the microspin™ GST purification module (Amersham Biosciences) according to the manufacturer’s instructions.

CHK1 Kinase Activity Assay—Cell extracts were prepared for this purpose by using the NE-PERTM kit (Pierce) according to the manufacturer’s instructions. The nuclear extracts (250 μg) were then mixed with 1 μg of CHK1 antibody (sc-7898, Santa Cruz Biotechnology, Inc.) in the presence of 10 μl of a 50% (v/v) protein A-Sepharose slurry (Repligen). The following procedures are similar to those described previously (41), except that kinase buffer without NaCl was used.

Western Blot—The nuclear extracts were used for Western blot (ATR, CHK1, CDC25A, and CDC2) assay. The whole cell lyses were used for Western blot detecting phospho-CHK1. The ATR antibody (sc-1887), CHK1 antibody (sc-8404), CDC25A antibody (sc-7389), and CDC2 antibody (sc-54) were purchased from Santa Cruz Biotechnology, Inc. Phospho-CHK1 (Ser345) antibody was purchased from Cell Signal Technology.

CDC2 Phosphorylation and Kinase Activity Assay—The CDC2 (also called CDK1) phosphorylation and CDC2 kinase assay are similar to previous reports (40). Cell extracts were prepared using the NE-PERTM kit (Pierce) for the kinase assay according to the manufacturer’s instructions. The nuclear extracts (250 μg) were then mixed with 1 μg of CDC2 antibody (sc-54, Santa Cruz Biotechnology, Inc.) in the presence of 10 μl of a 50% (v/v) protein A-Sepharose slurry (Repligen) for the measurement of CDC2 activity as described before (40), except that kinase buffer without NaCl was used.

Colony-forming Assay—Cellular sensitivity to radiation was determined by the loss of colony-forming ability as described previously (38).

Transfection of Chk1 siRNA—The Chk1 siRNA was designed to specifically target the sequence of 127–147 from the start codon region of the human Chk1 mRNA (5’-AACGCGUGCUAGACUGUCCA-3’) (32). The siRNA was synthesized by Dharmacon Research Inc. The scramble duplex RNA (Dharmacon Research Inc.) was used as the control RNA. The RNAs were delivered to the cells by OligoFECTAMINETM (Invitrogen) according to the manufacturer’s instructions. The cells were analyzed 36 h posttransfection.

RESULTS

Prolonged G2 Accumulation in Irradiated AT Cells Is Abolished by Kinase Inhibitors—Cells from AT patients have been reported to exhibit a prolonged G2 accumulation after exposure

| Table 1 |
| --- |

| ATME and ATME cells |
| --- |

| Total cell population |
| --- |

| G0/M | G0/M |
| --- | --- |

| PI only | 54 | 33 | 51 | 29 |
| PI + BrdU, 0 | 50 | 25 | 50 | 28 |
| PI + BrdU, R | 7 | 15 | 78 | 50 |
| PI + BrdU, UCN-01, 0 | 47 | 22 | 30 | 50 |
| PI + BrdU, UCN-01, R | 38 | 24 | 38 | 29 |
to IR (19, 22–24), suggesting that an overactivated G2 checkpoint exists in irradiated AT cells. To test this hypothesis, we first examined whether this G2 accumulation in irradiated AT cells was a reversible process. We treated AT cells with 4 mM caffeine (a nonspecific inhibitor of ATM and ATR) or with 100 nM UCN-01 (a nonspecific inhibitor of CHK1). We observed that both caffeine (data not shown) and UCN-01 clearly reduced the G2 accumulation in AT cells (Fig. 1A), explaining that the inhibitor blocked the IR-induced G2 checkpoint. To exclude the possibility that the inhibitor held the irradiated cells in S phase resulting in fewer cells entering G2 phase, we observed BrdUrd and PI, the double-labeled signals in both IR and the inhibitor-treated cells (Fig. 1B). The results (Fig. 1 and Table I) showed that there is not much difference of S phase ration between the cells treated and those not treated with the inhibitor. This indicates that the prolonged G2 accumulation in irradiated AT cells reflects a cellular active G2 checkpoint that is sensitive to caffeine or to UCN-01.

Abolishing the Prolonged G2 Accumulation with Kinase Inhibitors Sensitizes AT Cells to IR-induced Killing—It is known that AT cells are very sensitive to IR-induced killing. However, it remains unknown whether the prolonged G2 accumulation observed after irradiation contributes to the survival of AT cells. Checkpoint activation facilitates cell DNA repair; thus, prolonged G2 accumulation should play a protective role for AT cell survival. Previous work provides hints that this might indeed be the case (37). To test this hypothesis, we examined the radiosensitivity in irradiated AT cells under conditions in which the IR-induced G2 arrest was abolished. After the prolonged G2 accumulation was abolished by caffeine or UCN-01, AT cells became much more sensitive to IR-induced killing (Fig. 2), suggesting that this prolonged G2 accumulation in irradiated AT cells is important for cell survival.

A Highly Activated ATR/CHK1 Pathway Exists in Irradiated AT Cells—To test whether the caffeine-sensitive response in AT cells is regulated by the ATR pathway, we measured ATR activity in the wild-type and in AT cells. There was no difference in ATR activity between irradiated and control samples from both cytoplasmic and nuclear extracts (data not shown).
However, ATR activity of the chromatin-bound fraction was higher in irradiated (12 h) than in non-irradiated cells for both cell lines (Fig. 3A), suggesting that this pool of ATR contains the protein activated in response to DNA damage. When compared with the non-irradiated controls, the level of ATR activity increased in irradiated AT cells more than in irradiated wild-type cells (Fig. 3B), indicating an overactivated ATR in irradiated AT cells.

The main downstream target of ATR for regulating the checkpoint is CHK1. CHK1 is an important G\textsubscript{2} checkpoint regulator in mammalian cells exposed to IR (32, 38, 40). Although CHK2 is also implicated in IR-induced G\textsubscript{2} checkpoint (42), the CHK2-regulated response depends on ATM kinase (10, 42, 43). Therefore, the prolonged G\textsubscript{2} accumulation in irradiated AT cells (ATM\textsuperscript{+/+} and ATM\textsuperscript{-/-}) is not likely to be regulated by this kinase. We next examined whether the CHK1 pathway was involved in the prolonged G\textsubscript{2} accumulation in irradiated AT cells. Although the phosphorylation of CHK1 in cells following IR is hard to detect by using one-dimensional gel electrophoresis (30), we observed more phosphorylated CHK1 in irradiated AT cells than the wild-type control cells by increasing the radiation dose (20 Gy) and by using the whole cell lyses (Fig.

**Fig. 4. Chk1 siRNA abolishes the G\textsubscript{2} checkpoint response and sensitizes AT (ATM\textsuperscript{-/-}) cells to IR.** A. The levels of CHK1 expression were measured with the extracts from either Chk1 siRNA or control RNA treated human fibroblast cells (ATM\textsuperscript{+/+} and ATM\textsuperscript{-/-}) cells. Proliferating cell nuclear antigen (PCNA) signal was detected by the antibody (sc-56, Santa Cruz Biotechnology, Inc.) as the internal control. B. The treatments of Chk1 siRNA are as described under “Experimental Procedures.” The cells were treated with Chk1 siRNA for 36 h then were irradiated (2 Gy). The cells were collected at 12 h after IR. The preparation and measurement of flow cytometric profiles of cell cycle distribution are the same as described in the legend to Fig. 1. C, as described under “Experimental Procedures,” the cells were treated with Chk1 siRNA for 36 h and then were irradiated (2 Gy). After IR, the cells were trypsinized and plated to new dishes for colony formation with the medium. Data shown are the average from three independent experiments.
Overactivated ATR/CHK1 in Irradiated AT Cells

3A). Higher CHK1 kinase activities in both irradiated wild-type and AT cells as compared with the non-irradiated controls (Fig. 3A) were observed. The increased ratio of CHK1 activity in irradiated AT cells is much higher than that in irradiated wild-type control cells at 12 h following IR (6 Gy) (Fig. 3, A and B), indicating that CHK1 is more activated in irradiated AT cells.

The G2 checkpoint is believed to be mediated by an inhibition of the CDC25 phosphatase that activates the CDC2 kinase by removing inhibitory phosphates (Thr14 and Tyr15), thus allowing entry into mitosis (20, 32, 44). CHK1 could regulate IR-induced G2 checkpoint by phosphorylating CDC25A, which results in CDC25A degradation (32). To examine whether activation of the CHK1 kinase was associated with CDC25A protein level changes in irradiated AT cells, we examined the CDC25A levels. The results (Fig. 3A) showed that less CDC25A protein was observed in AT cells than in the wild-type control cells following IR (6 Gy). We next measured CDC2 activity. The results are consistent with those of CHK1 activation (Fig. 3) and of G2 arrest (Fig. 1). The CDC2 activities decreased in both wild-type and AT cells at 12 h after IR (6 Gy), but the changes were more apparent in AT cells (Fig. 3, A and B). These observations suggest that CDC25A and CDC2 are the downstream effectors of CHK1 in the regulation of the G2 arrest in AT cells.

Chk1 siRNA Abolishes the Prolonged G2 Accumulation and Radiosensitizes the Cells to IR—To confirm that the ATR/CHK1 pathway is responsible for the prolonged G2 accumulation in irradiated AT cells, we examined the effects of Chk1 siRNA on this checkpoint response. The Chk1 siRNA specifically inhibited CHK1 expression in the transfected cells (Fig. 4A) and reduced the prolonged G2 accumulation in irradiated AT cells (Fig. 4B). By using BrdUrd and PI, the double-labeled method, we observed the effects of Chk1 siRNA on BrdUrd incorporation. Similar with the UCN-01 results, Chk1 siRNA did not hold the irradiated cells in S phase (data not shown), which probably is also because of the role of CHK1 in abolishing the S checkpoint (21, 32). These results provide direct evidence that the ATR/CHK1 pathway plays a key role in the prolonged G2 arrest of irradiated AT cells.

To study the relationship between G2 checkpoint response and radiosensitivity, we examined the radiosensitivity of AT cells after abolishing their G2 checkpoint response by Chk1 siRNA. Chk1 siRNA radiosensitized both wild-type and AT cells, but the sensitization in AT cells is larger than that in wild-type cells (Fig. 4C), indicating that this component of the checkpoint response is more critical in AT cells than in wild-type cells.

DISCUSSION

The AT phenotype of prolonged G2 accumulation following IR was reported by different groups several years ago (19, 22, 23), but the underlying mechanism remained unknown (24). Our results indicate for the first time that the prolonged G2 accumulation in irradiated AT cells is regulated by the ATR/CHK1 pathway.

Although ATM and ATR are two of the most important DNA damage signal transducers in mammalian cells (4, 17, 25, 45–49), it was generally believed that ATM mainly responded to DNA double strand breaks (DSBs) induced by IR or chemical agents, and ATR mainly responded to other types of DNA damage induced by UV or chemical agents. Additional evidence now demonstrates that besides ATM, ATR is also a very important checkpoint regulator in IR-irradiated cells (21, 26, 40). Our results show that both ATR and its substrate, CHK1, are overactivated in AT cells following IR. Furthermore, the IR-induced, prolonged G2 accumulation in AT cells is abolished by blocking the ATR/CHK1 pathway, indicating that the overactivated ATR/CHK1 pathway is responsible for the prolonged G2 accumulation in irradiated AT cells.

Following IR, activation of the ATM pathway is observed almost immediately in mammalian cells (14, 21, 24, 42, 50). Activation of the ATR/CHK1 pathway is observed about 1 h later and reaches a maximum level at about the 3-h time point (21). This observation suggests that ATM and ATR regulate different pathways in response to the induction of DNA DSBs. ATM could be activated from a trans-acting process immediately by changes in the structure of chromatin induced by DSBs (50). Alternately, ATM could be activated from combination of a trans-acting (changes in the structure of chromatin) and a cis-acting (DNA-binding) process that either through ATM directly binding to DNA DSBs (51, 52) or by other DNA-binding protein formed complexes with ATM (53), thus playing a role in the initiating stage of multichannel checkpoints following IR. The fact of ATM-regulated S and G2-M checkpoints in a dose (IR)-dependent manner is supported more by the model of combined trans- and cis-acting processes. ATR is also a DNA binding protein (54, 55). In the absence of ATM, ATR may have a greater opportunity to interact directly with the damaged DNA induced by IR and cause the observed overactivation. However, we cannot exclude the possibility that activated ATM inhibits ATR activity and AT cells without ATM showing an overactivated ATR/CHK1 pathway following IR. These hypotheses require rigorous testing.

Two major DNA DSB repair pathways, non-homologous end joining (NHEJ) and homologous recombination repair (HRR), exist in mammalian cells. NHEJ is a very fast process and HRR is a relatively slow process. HRR is thought to occur mainly during S and G2 phase (56), suggesting that it benefits from a checkpoint that holds the cells in these phases of the cell cycle. The function of ATM is linked with HRR (57), suggesting that the ATM-dependent checkpoint facilitates HRR. Our previous data suggest that NHEJ is a process independent of checkpoints but that HRR is a checkpoint-utilizing process in vertebrate cells (58, 59). When the IR-induced prolonged G2 accumulation is abolished, ATM−/− cells became much more sensitive to killing by IR, suggesting that the ATR/CHK1-dependent checkpoint enhances cell survival by facilitating HRR and NHEJ.

In summary, we show here that IR-induced prolonged G2 accumulation in irradiated AT cells reflects an ATM-independent checkpoint regulated by the ATR/CHK1 pathway. We also show that AT cells become more sensitive to IR-induced killing when this checkpoint is abrogated, indicating that it is important for the survival of irradiated AT cells.

Acknowledgments—We thank Dr. Martin Lavin for reading this manuscript, Nancy Mott for help in the preparation of the manuscript, and Peggy Mammen for help in assisting with laboratory work.

REFERENCES

1. Paulovich, A. G., and Hartwell, L. H. (1995) Cell 82, 841–847
2. Paulovich, A. G., Toczyski, D. P., and Hartwell, L. H. (1997) Cell 88, 315–321
3. Eller, M. S., Ostrom, K., and Gilbreath, B. A. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 1087–1092
4. Zhou, B.-S., and Elledge, S. J. (2006) Nature 440, 435–439
5. Shiloh, Y. (1997) Annu. Rev. Genet. 31, 696–692
6. Lavin, M. F., and Khanna, K. K. (1999) Int. J. Radiat. Biol. 75, 1201–1214
7. Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plo shrink, B. S., Vogelstein, B., and Parmace, A. J., Jr. (1992) Cell 71, 587–597
8. Banin, S., Moyal, L., Shieh, S.-Y., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Perves, C., Reiss, Y., Shiloh, Y., and Zev, Y. (1998) Science 281, 1674–1677
9. Cannam, C. E., Lim, D.-S., Cimprich, K. A., Taya, Y., Tanah, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) Science 281, 1677–1679
10. Matsumoto, S., Huang, M., and Elledge, S. J. (1998) Science 282, 1893–1897
11. Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. (2000) Science 287, 1824–1827
12. Xu, B., Kim, S.-T., and Kastan, M. B. (2001) Mol. Cell. Biol. 21, 3445–3450
13. Bao, S., Tibbetts, R. S., Brumbaugh, K. M., Fang, Y., Richardson, D. A., Ali, A,
An Overactivated ATR/CHK1 Pathway Is Responsible for the Prolonged G2 Accumulation in Irradiated AT Cells
Xiang Wang, Jay Khadpe, Baocheng Hu, George Iliakis and Ya Wang

J. Biol. Chem. 2003, 278:30869-30874.
doi: 10.1074/jbc.M301876200 originally published online June 4, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301876200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 33 of which can be accessed free at http://www.jbc.org/content/278/33/30869.full.html#ref-list-1