An ATM-independent S-Phase Checkpoint Response Involves CHK1 Pathway

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Abstract

After exposure to genotoxic stress, proliferating cells actively slow down the DNA replication through a S-phase checkpoint to provide time for repair. We report that in addition to the ataxia-telangiectasia mutated (ATM)-dependent pathway that controls the fast response, there is an ATM-independent pathway that controls the slow response to regulate the S-phase checkpoint after ionizing radiation in mammalian cells. The slow response of S-phase checkpoint, which is resistant to wortmannin, sensitive to caffeine and UCN-01, and related to cyclin-dependent kinase phosphorylation, is much stronger in CHK1 overexpressed cells, and it could be abolished by Chk1 antisense oligonucleotides. These results provide evidence that the ATM-independent slow response of S-phase checkpoint involves CHK1 pathway.

Introduction

In response to DNA damage, eukaryotic cells activate a set of surveillance systems that interrupt cell cycle progression to allow time for repair. These surveillance systems are called checkpoints and have been given an empirical definition. The DNA damage checkpoint acts in three stages in the cell cycle, one at the G1-S phase transition (G1 checkpoint), one at S phase (S-phase checkpoint), and one at the G2-M boundary (G2 checkpoint; Ref. 1). With checkpoint failure, the immediate consequence is that the cells increase their sensitivity to being killed, and the long-term consequence is that the cells increase their susceptibility to tumor genesis. S-phase checkpoint monitors progression through S phase, which slows the rate of ongoing DNA synthesis (2–5). Although the genetic requirements for the S-phase checkpoint have been investigated in detail in yeast (6), the mechanism in mammalian cells is still unclear. The ATM gene is mutated in the autosomal recessive disease AT.3 Cells from the AT patients show defects in cell cycle checkpoints (including G1, S-phase, and G2 checkpoints) and hypersensitivity to DNA DSB inducers such as IR. One hallmark of the AT cells is that they show less inhibition of DNA synthesis immediately after IR when compared with the wild-type cells (7, 8). Until now, the ATM-dependent pathway that includes CHK2, Nijmegen breakage syndrome and BRCA1 (the downstream targets of ATM) was considered to be the only major S-phase checkpoint regulator in mammalian cells after IR (9–13). However, as we report here, AT cells do show increasing inhibition as time passes and reach a maximum inhibition at ~3 h after IR, although they have less inhibition of DNA synthesis immediately (about 5 min) after IR. At 3 h after IR, there is little difference in the rates of DNA synthesis between AT cells and wild-type cells. These observations suggest that, besides ATM, another ATM-independent pathway also regulates the S-phase checkpoint after DNA damage. After systematically characterizing the S-phase checkpoint, we found that the IR-induced response of S-phase checkpoint could be divided into two processes: the fast (from several minutes to ~2 h) and the slow (1.0 h to 6 h. After 6 h there was no testing). The ATM-dependent pathway controls only the fast response of S-phase checkpoint; the slow response is controlled by an ATM-independent pathway. The data shown in this report suggest that this ATM-independent pathway involves CHK1. The ATM-dependent and ATM-independent pathways cooperate together to maintain the intact S-phase checkpoint in mammalian cells after IR.

Materials and Methods

Cell Culture. Human primary fibroblasts, ATM mutant AT5BI (kindly provided by Dr. Malcolm Paterson, MRC Reproductive Biology Unit, Edinburgh, United Kingdom) and wild-type WI38 (kindly provided by Dr. Paul Philips, Drexel-Medical College of Pennsylvania Hahnemann) cells were grown in MEM containing 10% fetal bovine serum. Human transformed fibroblasts, ATM mutant AT5BIVA (Camen Depository), and wild-type MRC5SV1 (kindly provided by Dr. Colin Arlett, Medical Research Council Cell Mutation Unit and Center for Medical Research, University of Sussex, Brighton, United Kingdom) rat transformed embryo fibroblasts, A1–5 and B4 cells (kindly provided by Dr. Arnold J. Levine, Department of Molecular Biology, Princeton University, Princeton, NJ) were grown in DMEM containing 10% bovine calf serum. Human transformed lymphoblasts, ATM mutant AT5ABR, and wild-type C3ABR cells (kindly provided by Dr. Martin F. Lavin, The Queensland Cancer Fund Research Laboratory, Brisbane, Queensland, Australia) were grown in RPMI 1640 containing 10% bovine calf serum.

DNA Synthesis, Chemicals, and Irradiation. The measurement of DNA synthesis was similar to that described previously (14). Briefly, a $10^5$ fibroblasts (AT5BI, WI38, AT5BIVA, MRC5SV1, A1–5, and B4 cells) or $3 \times 10^5$ lymphoblasts (AT5ABR and C3ABR cells) from a growing culture were seeded in 60-mm tissue-culture dishes with 3 ml of medium and allowed to grow for more than one doubling time. Fibroblasts were prelabeled with medium containing 10 nCi of $[^{14}C]$thymidine and 0.5 μM cold thymidine. This prelabeling provides an internal control for cell number by allowing normalization for total DNA content of samples. The suspension-growing lymphoblasts were partially taken from a sample for counting before they were loaded onto the microfiber filters.

Before irradiation, the lymphoblast cultures were directly added with the chemicals, and fibroblast cells were replaced with prewarmed medium (washed off $[^{14}C]$thymidine) containing either caffeine, wortmannin, and UCN-01, or wortmannin plus UCN-01 for 30 min. After the cells were exposed to X-rays (310 kV, 10 mA, 2-mm aluminum filter) at room temperature, they were returned to 37°C. The chemicals were kept in the culture until cells were harvested. $[^{3}H]$thymidine at 1 μCi was added for 15 min at different times thereafter. The cells were then collected. The following procedures as described previously (14) were used. The rate of DNA synthesis for each sample was calculated as $[^{3}H]$dpm/$[^{14}C]$dpm (fibroblasts) or $[^{3}H]$dpm/cell number (lymphoblasts) and is presented as a percentage of the control values obtained from sham-irradiated cells at the same time point.

Alkaline Sucrose Gradient Sedimentation. As described previously (15), this method was used to determine the molecular weight distribution of newly synthesized DNA in the irradiated cells. Linear sucrose gradients (5–20%) were prepared in a buffer containing 0.1 M NaOH, 0.9 M NaCl, and 10 mM

Received 9/28/01; accepted 1/22/02.

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1 Supported by NIH Grants CA76203, CA56706, T32-CA09137, and P30-CA56036, and a grant from RTOG.

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3 The abbreviations used are: AT, ataxia telangiectasia; DSBR, double-strand break; IR, ionizing radiation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; DNA-PK, DNA-dependent protein kinase; ATM, ataxia-telangiectasia mutated; CDK, cyclin-dependent kinase.

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EDTA (pH 12.5) in polyallomer tubes. AT and the wild-type cells were irradiated and returned to 37°C for 3 h; 1H]thymidine at 5 μCi/ml was added to the culture for 15 min. The following procedures were carried out as described previously (15).

Flow Cytometry Measurement. As described previously (16), cells were collected at different times, and fixed in 70% ethanol. Cells were stained with the solution (62 μg/ml RNase A, 40 μg/ml propidium iodide, and 0.1% Triton X-100 in PBS buffer) at room temperature for 1 h. The distribution of cells in the cell cycle was measured in a flow cytometer (Coulter Epics Elite).

Treatment of Cells with Antisense Oligonucleotides. Among human, rat, and mouse, the start codon region of Chk1 mRNA is conserved but that of Chk2 is varied. Therefore, the antisense oligonucleotide of rat Chk1 (5'-GGCAGCTGCAATGACTCCA-3'), designed to specifically target the sequence of the start codon region of rat Chk1 mRNA (16), was used to inhibit Chk1 expression in human cells. And the rat Chk2 antisense oligonucleotide (5'-TACGTCTTCATATTCCGAC-3'; Ref. 16), was used as the control oligonucleotide in human cells. The antisense oligonucleotides were delivered to cells by OligofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. Briefly, antisense oligonucleotides (1.0 μM for AT5BIVA and MRC5SV1 cells, 1.5 μM for A1–5 and B4 cells) were added to serum-free MEM containing 20 μl/ml OligofectAMINE reagent. This preparation (1 ml) was added to 30% confluent cells cultured in 60-mm plate (prelabeled with 10 nCi [14C]thymidine for 24 h) for 4.5 h. Additional DMEM (0.5 ml) with 30% iron-supplemented calf serum was then added to the culture for another 14 h. The cells were either prepared to measure the inhibition of DNA synthesis 3 h after IR, as described above, or were directly collected in 1× protein loading buffer [50 mM Tris-Cl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol] and prepared for Western blot.

Immunoprecipitation and Western Blotting. Whole cell extracts were prepared as described previously (16). Four hundred μg were mixed with 1.5 μg of CDK2 antibody (sc-163; Santa Cruz Biotechnology, Inc.) in the presence of 20 μl of a 50% (v/v) protein A-Sepharose slurry (Life Technologies, Inc.) in 400 μl of buffer (0.5% NP40, 1 mM Na3VO4, 5 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride in PBS buffer) and gently rotated overnight at 4°C. Immune complexes were washed three times with the same buffer, boiled in protein loading buffer and then separated in 12% SDS-PAGE gel. For the antisense oligonucleotide experiments, the treated cells were collected, directly lysed in 1× protein gel-loading buffer and separated in 10% SDS-PAGE gel. Antibodies of PCNA (sc-56), CHK1 (sc-8408) and CHK2 (sc-9064) were purchased from Santa Cruz Biotechnology, Inc.; antibody of p21WAF1/Cip1 was purchased from Cell Signaling Technology; and antibody of GAPDH was purchased from Chemicon International.

Results

A Slow Response of S-Phase Checkpoint Exists in Both AT and Wild-Type Mammalian Cells. DNA damage-induced S-phase checkpoint represents the inhibition of DNA synthesis and is measured as a transit decrease in [3H]thymidine incorporation (7, 13). In previous observations, we found that the inhibition of DNA synthesis in mammalian cells showed increasing inhibition as time passed after IR (14), the strongest inhibition usually occurred at ~3 h after IR. To test whether ATM is involved in the time-dependent inhibition of DNA synthesis, we examined the inhibition of DNA synthesis in AT cells at different times after IR. Fig. 1A shows the dose response of DNA synthesis in AT cells and their wild-type counterpart at 0.5 h or 3 h after IR. Although AT cells showed less inhibition of DNA synthesis at 0.5 h after IR compared with the wild-type cells (Fig. 1A, solid and dotted lines only), which is similar to previous reports by other investigators (7, 8, 12), AT cells did show a strong inhibition of DNA synthesis at 3 h after IR (Fig. 1A, solid lines and symbols), which results in little difference from the wild-type cells. The time-kinetic results of DNA synthesis in AT cells and their wild-type counterpart after IR (Fig. 1B) further confirmed that the strong inhibition of DNA synthesis occurred in AT primary fibroblasts (Fig. 1B, AT5BI), AT transformed fibroblasts (Fig. 1B, AT5BIVA), and AT transformed lymphoblasts (Fig. 1B, AT5ABR) at a later time after IR.

We propose to name the inhibition of DNA synthesis that occurred immediately (at several min after IR) “the fast response of S-phase checkpoint,” and to name the inhibition that occurred late (the maxim at ~3 h after IR) “the slow response of S checkpoint.” The result of the fast response lacking in AT cells indicates that ATM is involved. The result of the slow response found in AT cells suggests that an ATM-independent pathway controls it.

The Slow Response of S-Phase Checkpoint Affects Replication Initiation and Is an Active, Independent Process. The DNA synthesis could be inhibited by the checkpoint regulation either at replication initiation or at chain elongation. The ATM-dependent S-phase checkpoint is mainly involved in replication initiation (7). To examine which component of the DNA synthesis is involved in the ATM-independent slow response of S-phase checkpoint, we carried out the gradient experiments. The results (Fig. 2A) showed that the newly synthesized DNA in AT cells was strongly inhibited at 3 h after IR, which is similar to the time interval observed in wild-type cells, although the newly synthesized DNA was less inhibited in AT cells immediately after IR when compared with that observed in the wild-type cells (data not shown). These results indicate that the ATM-independent slow S-phase checkpoint also mainly affects the replication initiation.

To further characterize the slow response of S-phase checkpoint, we examined the effects of inhibitors (caffeine, wortmannin, or UCN-01) of different kinases on the rates of DNA synthesis at different times after IR (Fig. 2B). Wortmannin, which, at 20 μM, inhibits the phosphoinositide-3 kinase family that includes ATM and DNA-PK but not Ataxia and Rad3 related, did not affect the slow response of S-phase checkpoint in AT cells (Fig. 2B, AT5BIVA, 3 h point), which indicates that DNA-PK is not involved in the slow response. Wort-
Fig. 2. The slow response of S-phase checkpoint is also involved in the replicon initiation but is a different process from the fast response of the S-phase checkpoint. A. alkaline sedimentation profiles of nascent DNA from AT5BIVA and MRC5SV1 cells either sham-irradiated (●) or exposed to 30 Gy X-rays (○) and returned to 37°C for 3 h before analysis. Plotted is the percentage of [3H]activity as a function of fraction number. Shown in the figure for comparison are also the sedimentation distances of T2 (167 kb) and T7 (37 kb) phage DNA. B, the effect of the drugs on radiation-induced inhibition of DNA synthesis in transformed fibroblasts. Similar to Fig. 1, before irradiation, cells were added with 3 mM caffeine (●), or 20 μM wortmannin (○), or UCN-01 (●, 100 μM) or 20 μM wortmannin plus UCN-01 (●) or without drug (○) for 30 min. Then cells were exposed to X-rays (30 Gy) and returned to 37°C. The drugs were kept in the culture until cells were harvested. Data shown are the averages from three independent experiments. C, the effect of the drugs on the percentage of S-phase cells in transformed lymphoblasts at different times after 20 Gy irradiation analyzed by flow cytometry. The cells were treated with 3 mM caffeine (●), or 20 μM wortmannin (○), or 100 μM UCN-01 (●) as described in “Materials and Methods.” —, data from non-drug-treated samples.

mammun, on the other hand, mainly inhibited the fast response of S-phase checkpoint in ATM normal cells (Fig. 2B, MRC5SV1, reversed the inhibition of DNA synthesis at 0.25 h) and in DNA-PK-deficient cells, MO59J (data not shown), which resulted in the ATM-like phenotypes in these cells. These results suggest that DNA-PK is not involved in the fast response either. Caffeine, which at 3 mM inhibits ATM and ATR kinases (17), completely inhibited both the fast and the slow responses of S-phase checkpoint in AT and the wild-type cells (Fig. 2B, AT5BIVA and MRC5SV1, reversed the inhibition of DNA synthesis). This result suggests that the target of caffeine in AT cells that controls the slow response might be ATR. The UCN-01 data (Fig. 2B) support this hypothesis. UCN-01, which at 100 μM could efficiently inhibit CHK1 (18, 19) [the ATR downstream substrate (20)], but did not affect CHK2 (18, 19) [the ATM downstream substrate (21–23)], mainly inhibited the slow response in both AT and wild-type cells (Fig. 2B, after 3 h). Moreover, when wortmannin and UCN-01 were administered together, they induced the caffeine-like effects that deleted both the fast and the slow responses of S-phase checkpoint (Fig. 2B, MRC5SV1). Similar results were also observed in the transformed lymphoblasts, AT5ABR and C3ABR (data not shown in Fig. 2B).

By analyzing the effects of the inhibitors on the S-phase checkpoint, we could clearly separate the slow response of the S-phase checkpoint pathway from the fast one. Although both the fast and the slow responses were sensitive to caffeine, the fast one was sensitive to wortmannin and resistant to UCN-01, whereas the slow one was sensitive to UCN-01 and resistant to wortmannin. These results strongly suggest that the fast response of S-phase checkpoint is controlled mainly by the ATM pathway, which is sensitive to caffeine and wortmannin, and the slow response is mainly controlled by an ATM-independent pathway, which is sensitive to caffeine and UCN-01.

To determine that the slow response of S-phase checkpoint, as well as the effects of the inhibitors reflected an active response of the cells rather than a reduction in the S-phase fraction, we measured the cell cycle distribution at different times after IR by a flow cytometry. Fig. 2C shows the ratio of the cells in S-phase. The percent of cells in S-phase gradually increased both in AT (AT5ABR) and in its wild-type counterpart (C3ABR) after IR (Fig. 2C), indicating that the reduced [3H]thymidine incorporation in irradiated cells is derived from a slowdown of DNA synthesis. In addition, caffeine and UCN-01, but not wortmannin, abolished the increase in the percentage of S-phase cells in both AT5ABR and C3ABR cells at 3 h after IR (Fig. 2C), which was consistent with the data of [3H]thymidine incorporation (Fig. 1B, AT5ABR and C3ABR, other data not shown).

The Slow Response of S-Phase Checkpoint Involves CHK1 Pathway. To further test the hypothesis that the slow response of S-phase checkpoint might be involved in the CHK1 pathway, we examined the slow S-phase checkpoint response in the cells in which CHK1 was either over- or down-expressed.

A1–5 and B4 are a pair of transformed rat embryo fibroblasts derived from the same genetic background (24), but only A1–5 cells showed much higher CHK1 expression (16). We compared the rates of DNA synthesis in irradiated A1–5 and B4 cells. Similar to the other mammalian cells tested in our laboratory, this pair of cells also showed a time-dependent S-phase checkpoint response (Fig. 3A). As we expected, A1–5 cells with overexpression of CHK1 showed much more inhibition of DNA synthesis at 3 h after IR compared with B4 cells, although there was not much difference in the inhibition between the two cell lines at short intervals after IR (Fig. 3A, 15 min and 1 h after IR). The stronger slow response of S-phase checkpoint (inhibition of DNA synthesis observed at 3 h after IR) in A1–5 cells was reduced by caffeine and UCN-01 but was not affected by wortmannin (Fig. 3B). These results provide additional information that CHK1 might be involved in the slow response of S-phase checkpoint.

To confirm that the stronger slow response of S-phase checkpoint in A1–5 cells was because of the higher expression of CHK1, we examined the effects of underexpressed CHK1 on the rate of DNA synthesis at 3 h after IR in A1–5 cells by designed Chk1 antisense oligonucleotide. As we reported previously (16), Chk1 antisense oligonucleotide specifically reduced CHK1 expression in A1–5 cells. We show here that Chk1 antisense oligonucleotide released the inhibition of DNA synthesis significantly at 3 h after IR in A1–5 cells (Fig. 3D, no. 3). Although Chk2 antisense oligonucleotide reduced CHK2 expression (16), it did not affect the inhibition of DNA synthesis at the same time (Fig. 3D, no. 3). Because the sequence of the 18 bp around CHK1 start code, which we chose to design the antisense, is the same among mouse, rat, and human, we used this antisense to treat human cells. CHK1 protein levels were reduced in antisense oligonucleotide-treated AT cells and their wild-type counterparts (Fig. 3C). Under such conditions, Chk1 antisense oligonucleotide released the inhibition of DNA synthesis significantly at 3 h after IR in both AT5BIVA and MRC5SV1 cells (Fig. 3D, nos. 1 and 2). At the same time, as an ideal control, rat Chk2 antisense oligonucleotide did not change CHK1 protein levels (Fig. 3C, oligo) and did not affect the inhibition of DNA synthesis (Fig. 3D, nos. 1 and 2).
Fig. 3. CHK1 is involved in the slow response of S-phase checkpoint. A, kinetics of the rate of DNA synthesis as a function of postirradiation incubation time in A1–5 (●) and B4 (○) cells. The dose of radiation is 20 Gy. As indicated in Fig. 1, the rate of DNA synthesis for each sample was calculated as [3H]dpm/[14C]dpm and is presented as a percentage of the control values obtained from sham-irradiated cells at the same time point. B, the effect of the indicated inhibitors on the slow S-phase checkpoint in irradiated A1–5 and B4 cells. The A1–5 (●) and B4 (○) cells were treated with the inhibitors of kinases (3 mM caffeine, or 20 μM wortmannin, or 100 nM UCN-01) as indicated in Fig. 2. The rates of DNA synthesis were measured at 3 h after different doses of IR. C, the protein levels of CHK1 and GAPDH (as the internal standard) were analyzed by Western blotting after the antisense oligonucleotides treatment. Cells were collected and directly lysed in 1× protein gel-loading buffer. D, the effect of Chk1 antisense oligonucleotide on the slow S-phase checkpoint. The treatment with the designed antisense and irradiation were as described in “Materials and Methods.” The three sets of data are from AT5BIVA (group 1), MRC5SV1 (group 2), and A1–5 cells (group 3) treated with the OligofectAMINE reagent alone or combined with Chk1 or with control oligonucleotide. The data shown in A, B, C, and D, mean values and SDs from three independent experiments.

These results give direct evidence that the slow response of S-phase checkpoint involves the CHK1 pathway.

We have previously reported that, in addition to ATM kinase, there is another ATM-independent kinase that controls the S-phase checkpoint in camptothecin-treated cells (25). By using the antisense oligonucleotide, we observed that Chk1 antisense oligonucleotide could release the inhibition of DNA synthesis in camptothecin-treated AT cells (data not shown), which suggests that the ATM-independent regulation in camptothecin-treated cells is related to CHK1 pathway.

The Slow Response of S-Phase Checkpoint Involves Inactivation of CDK2. CDK2 is highly activated and plays an important role during S phase (26). Recently it was reported that the inhibitory phosphorylation of CDK2 on Tyr-15 is involved in the S-phase checkpoint response regulated by the ATM-dependent pathway (11). We investigated further whether the inactivation of CDK2 was also involved in the slow response of the S-phase checkpoint. Because the antibody of Tyr-15-phosphorylated protein binds to both the phosphorylated forms of CDK2 and CDC2, and to observe only the phosphorylation of CDK2, we used this antibody to directly react to the total CDK2 immunoprecipitated from the whole cell lysates with CDK2 antibody.

The protein levels of total CDK2 did not change in both AT (AT5ABR and AT5BIVA) and their counterpart (MRC5SV1 and C3ABR) cells (Fig. 4). However, the phosphorylation of CDK2 on Tyr-15 became much stronger after IR (Fig. 4). The levels of CDK2 phosphorylation in AT5ABR cells gradually increased and reached a maximum at 3 h after irradiation (Fig. 4A) and maintained high levels of phosphorylation for up to 6 h (data after 3 h not shown). On the other hand, the phosphorylation of CDK2 in C3ABR cells increased to a maximum level immediately after irradiation (Fig. 4A) and maintained high levels for up to 6 h (data after 3 h not shown). Either caffeine or UCN-01 significantly inhibited IR-induced phosphorylation levels of CDK2 in both AT5ABR and C3ABR cells at 1 h, 3 h (Fig. 4A), and 6 h (data not shown) after irradiation. However, wortmannin inhibited IR-induced phosphorylation levels of CDK2 only in C3ABR cells at 1 h after irradiation and had no significant effects on the phosphorylation levels of CDK2 at 3 (Fig. 4A) or 6 h (data not shown) in both AT5ABR and C3ABR cells after irradiation. Furthermore, Chk1 antisense oligonucleotide significantly reduced the phosphorylation of CDK2 in both AT5BIVA and MRC5SV1 cells 3 h after IR (Fig. 4B). Compared with Chk1 antisense, the control oligonucleotide had less effect on the phosphorylation of CDK2 at the same time (Fig. 4B). These results combined with the DNA synthesis data shown in Figs. 1–3 suggest that the inhibitory phosphorylation of CDK2 is related to both the fast and the slow responses of S-phase checkpoint.

Discussion

The molecular mechanisms controlling DNA damage checkpoints have important implications for both cancer genesis and cancer therapy. However, less is known about S-phase checkpoint regulation than about G1 and G2 checkpoint regulations. Our results indicate that there is a time-dependent inhibition of DNA synthesis in IR-irradiated cells.

![Diagram](image-url)
The slow response of S-phase checkpoint starts at ~0.5 h after IR and reaches the maximal level at ~3 h after IR. The fast response of S-phase checkpoint starts immediately after IR and gradually increases and reaches the maximal level at ~3 h after IR. The ATM-dependent pathway regulates the fast response of the S-phase checkpoint and an ATR/CHK1-dependent pathway regulates the slow response of the S-phase checkpoint.

AT and wild-type cells. This IR-induced time-dependent inhibition of DNA synthesis was also observed in all of the proliferating mammalian cell lines that we tested (including human and murine, normal and tumor cell lines, data not shown). Our results demonstrate that the time-dependent inhibition of DNA synthesis is regulated by two independent but cooperating pathways: one is the ATM-dependent pathway, which controls mainly the fast response of S-phase checkpoint; the other is an ATM-independent pathway, which controls mainly the slow response of S-phase checkpoint and may use the ATR/CHK1-dependent pathway. We believe that these two pathways are used universally to control IR-induced S-phase checkpoints in mammalian cells. On the basis of the data of AT5BIVA and MRC5SV1 cells shown in Fig. 2B, we could draw a graph to describe the fast and the slow S-phase checkpoint responses (Fig. 5). We should point out here that because the time and level for the fast or slow response of S-phase checkpoints are dependent on cell type, there is no universal definition for the period and intensity of the fast or slow response of S-phase checkpoints.

The ATM-dependent fast response of S-phase checkpoint is thought to primarily represent an inhibition of replicon initiation (7). Our results show that the newly synthesized DNA is also strongly inhibited in AT and wild-type cells 3 h after irradiation. On the basis of these results plus our previous observation (15), which showed that the strong inhibition of replicon initiation in transformed rat embryo fibroblast cell line 3.7, at 3 h after IR, could be mainly reversed by staurosporine (one derivation of UCN-01), the nonspecific CHK1 inhibitor, we believe that the ATM-independent and CHK1-involved slow response of S-phase checkpoint also mainly affects the replicon initiation. A new publication (27), which reported that CHK1 plays a role in an intra-S-phase checkpoint controlling late-origin firing, confirms our findings.

After DNA damage, both ATM and ATR pathways are activated and, in turn, activate their downstream substrates CHK2 and CHK1, respectively (4). The activation of CHK2 and CHK1 then lead to inactivation of their downstream substrate CDC25, the phosphatase. Therefore, CDC25 could not remove the inhibitory phosphorylation of CDK2 on Tyr-15, which prevents the activation of this kinase (28, 29). Our data show that both fast and slow responses of the S-phase checkpoint involve the inhibitory phosphorylation of CDK2. This model is shown in Fig. 5. The family of CDC25 phosphatase has three members: CDC25A, CDC25B, and CDC25C. Although it has been reported that the CDC25A level is significantly reduced after IR in wild-type cells but not reduced in AT cells (11), we did not find any apparent changes of CDC25A levels in either AT or wild-type cells with the antibody (sc-97; Santa Cruz Biotechnology, Inc.) used in our experiments at different times after IR with different doses (data not shown). More studies are needed to explain the controversial results. One possible explanation is that CDC25A may have different isoforms and only one of them degrades in a ubiquitous manner after IR, as described previously (11). At this moment, we still do not know which member of the CDC25 family plays a role in the inactivation of CDK2 during the slow response of the S-phase checkpoint.

It has been reported that, in response to DNA damage, p21WAF1/Cip1 could inhibit DNA replication through direct binding to CDK2 and through association with PCNA (30). Although p21WAF1/Cip1 with PCNA increased as time passed after IR in both AT and the wild-type cells (data not shown), the changes in drug-treated cells in our experiments did not match the DNA synthesis data, which suggests that the p21WAF1/Cip1 with PCNA did not directly relate to the slow response of the S-phase checkpoint.

The fact that the fast and slow S-phase-checkpoint pathways respond to DNA damage at different times suggests that they are activated through different structures of DNA. DNA DSBs induced by IR (31, 32) could immediately activate the ATM pathway. In general, DNA DSBs are repaired within 2 h by nonhomologous end joining (33). During the repair time, either the rejoined but still not normal structure of DNA, or the ATM-induced replication blocks, or both of them, may in turn activate the ATR pathway and continue to promote the process of homologous recombination repair. As time passes after DNA damage, the slow S-phase-checkpoint pathway may gradually take over the role of the fast S-phase-checkpoint pathway. In this way, the two pathways could cooperate with each other to control the S-phase checkpoint and to maintain genetic integrity in mammalian cells.

Acknowledgments

We thank Drs. Malcolm Paterson, Paul Philips, Colin Arlett, Martin F. Lavin, and Arnold J. Levine for the cell lines. We thank Nancy Mott for help in the preparation of the manuscript.

References

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