Repression of G₀/G₁ Traverse in Human Fibroblasts Exposed to Low Levels of Ionizing Radiation*

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Quiescent cultures of human fibroblasts were exposed to levels of ionizing radiation sufficient to induce a transient growth delay, while causing only small decreases in long term clonogenicity. Following the mitogenic stimulation of damaged cells, cyclin D-associated kinase activity was induced to levels equivalent to those seen in control cultures. In addition, late G₀/G₁, E2F-dependent transcriptional and translational activity was observed in restimulated irradiated cells. However, cells became arrested prior to entry into S phase in a manner that paralleled the repression of cdk2-associated kinase activity. Cyclin A/cdk2-associated kinase activity was repressed in a biphasic manner following the irradiation of logarithmically growing cells. The initial rapid decline in activity to levels ~50% of those observed in control cultures occurred prior to increases in cellular levels of p21cip1 protein, was not blocked by the addition of cycloheximide, and was not accompanied by alterations in cdk2 phosphoryrosine content. The subsequent repression to undetectable levels was coincident with the induction of p21cip1 and was dependent on de novo protein synthesis. Only a subpopulation of cyclin A complexes were associated with p21cip1 regardless of the magnitude of the repression of catalytic activity, although all cyclin A-cdk2-p21cip1 complexes were inactive. These data suggest that temporally and functionally distinct mechanisms mediate the repression of cyclin-cdk activity in damaged cells. In addition, we present evidence that irradiated cells are competent to traverse S phase and arrest in G₀ in the complete absence of cdk2-associated kinase activity.

Cell cycle traverse in cultured fibroblasts is regulated by the sequential activation of the kinase activity associated with cyclin-cdk1 complexes. The subsequent phosphorylation of members of the retinoblastoma (RB) family of pocket proteins allows for the induction of E2F-dependent transcription and the expression of genes required for the traverse of late G₀/G₁ and entry into S phase (1, 2). Cyclin D-associated kinase activity, induced during G₁ traverse following a mitogen-dependent increase in cyclin D1 mRNA and protein expression (3, 4) and complex formation with cdk4/6 (5), as well as a reduction of p27kip1 protein (6, 7), mediates an initial phosphorylation of p130 and RB resulting in a release of “free” E2F complexes (8). The resulting E2F-dependent transcriptional activity is responsible for an induction of cyclin E protein that, following association with its catalytic partner cdk2, phosphorylates RB on an additional set of residues, resulting in a complete E2F activation and an induction of an additional series of genes including cyclin A (9). A specific role for cyclin A-cdk2 complexes has not been described. However, based on antibody microinjection experiments (10, 11), it has been proposed that cyclin A-associated kinase activity plays a critical role in the maintenance of DNA synthesis. Traverse of cells through G₀ and into mitosis is regulated by the actions of cyclin B-cdc2 complexes (12).

Exposure to agents that inflict damage to DNA causes profound alterations in multiple parameters of cell cycle traverse that appear to be mediated to a large extent by modulations in cyclin/cdk expression or activation (13, 14). The pioneering experiments in this area of research suggested that cells exposed to ionizing radiation largely arrest in G₀ (15). It has been subsequently shown that a number of pathways, including those regulating the expression of cyclin B (16), the phosphorylation of cdc2 (17), or the actions of gadd45 (18), converge on the activation of cyclin B-associated kinase activity to mediate the induction of the G₀/M checkpoint in irradiated cells. While the expression of wild-type p53 is not required for the induction of a G₀ arrest in damaged cells (19), the nature of the molecular phenotype of the arrested cells appears to be p53 dependent (20, 21). A parallel effort has been extended toward an understanding of the molecular basis of the G₀ arrest that is seen in irradiated cells under some circumstances. A critical advance in this arena was the observation that an efficient G₀ arrest after DNA damage was only evident in cells that exhibit wild-type p53 function (22). A potential explanation of the p53 dependence of the damage-induced G₀ arrest was based on the identification of p21cip1, a potent inhibitor of cyclin-dependent kinase activity, as a p53-dependent gene product (23). Based on these data, models have arisen in which it has been proposed that elevated levels of p21cip1, expressed in irradiated cells following the stabilization of p53, inhibit cyclin/cdk-associated kinase activity (predominantly that dependent on cdk2) directly leading to the induction of a G₁ growth arrest (24, 25). While such models have provided a valuable framework for subsequent investigations describing the molecular basis for radiation-induced cell cycle arrests, it has become clear that they remain incomplete. Under some circumstances, there is no direct relationship between the induction of a G₀ arrest and p53 status (26, 27). Furthermore, in cultured MEFs the loss of p53 expression leads to a more severe abrogation of the radiation-induced G₀ arrest than is seen in cells that express wild-type p53 but that are
lacking p21Cip1 (28, 29). In addition, the temporal patterns of alterations in p53 and p21Waf1 levels do not always coincide with the duration of the cell cycle arrest (30). Taken together, these results make it clear that additional, as yet undescribed, molecular events contribute to both damage-induced cell cycle arrests and the underlying modulation of cyclin-CDK activities.

Several reports have described alterations in cyclin A-associated kinase activity following DNA damage. When quiescent human fibroblasts are exposed to high levels of ionizing radiation prior to stimulation with serum, the late G2/M induction of cyclin A-associated kinase activity is not observed. However, the lack of kinase activity under these circumstances is not due to a modification of cyclin A complexes by processes such as the binding of p21Cip1. Instead, it appears to be caused by a marked repression of cyclin A expression (31) that we have proposed is an indirect effect of the absence of cyclin D-associated kinase activity (32). In contrast, the irradiation of logarithmically growing cells that contain cyclin A-cdk2 complexes at the time of damage, causes a rapid loss of cyclin A-associated kinase activity that occurs more rapidly than a loss of cyclin D1 kinase activity (32). While these observations are potentially consistent with a radiation-induced loss of activity by a p53/p21Cip1-dependent process, it has also been reported that cyclin A kinase activity is repressed in irradiated CHO cells that are null for p53 function (33). Based on these results, we have continued our investigation of the of repression of cell cycle traverse in irradiated human fibroblasts and report that: 1) at least two functionally distinct G2/M checkpoints can be invoked in damaged cells depending on the dose of radiation; 2) multiple regulatory networks interact to impinge on cyclin A-cdk2 complexes to repress kinase activity; and 3) cells are able to traverse at least a portion of S phase and enter G0 in the presence of cyclin A-associated kinase activity.

MATERIALS AND METHODS

Cell Culture—Flow 2000 cells, a strain of normal precrisis p53-positive human fibroblasts obtained from Dr. H. L. Moses, were grown in α-Minimal Essential Medium (αMEM) supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere containing 5% CO2. When logarithmically growing cultures were required for experiments, cells were plated in 100-mm tissue culture plates at an initial density of 5 x 10^5 cells/dish, fresh medium containing 10% FCS was added 2 days later, and the experiment was performed after a further 24 h of incubation. When growth-arrested cells were analyzed, plates were seeded with a radiation-induced loss of activity by a p53/p21Cip1-dependent process, it has also been reported that cyclin A kinase activity is repressed in irradiated CHO cells that are null for p53 function (33). Based on these results, we have continued our investigation of the of repression of cell cycle traverse in irradiated human fibroblasts and report that: 1) at least two functionally distinct G2/M checkpoints can be invoked in damaged cells depending on the dose of radiation; 2) multiple regulatory networks interact to impinge on cyclin A-cdk2 complexes to repress kinase activity; and 3) cells are able to traverse at least a portion of S phase and enter G0 in the complete absence of cyclin A-associated kinase activity.

Acute Inhibition of Cell Cycle Traverse by Low Levels of Ionizing Radiation—Serum-starved, G0-arrested cultures of human fibroblasts were exposed to increasing levels of ionizing radiation and immediately treated with fresh medium supplemented with 10 ng/ml PDGF and 10% serum. After a 22-h incubation, samples were harvested and DNA distributions were determined by flow cytometry. At time 0, more than 95% of the cells were arrested with a 2N content of DNA. Stimula-
tion of control cells with mitogens caused over 60% of the cells to exit G0/G1 and initiate DNA synthesis (normalized to 100%). As shown in Fig. 1, exposure of cells to as little as 10 cGy caused a small reduction in the percentage of S phase cells, and 75–100 cGy, levels of radiation that cause only small decreases in long term clonogenicity (34, 35), caused a complete repression of short term cell cycle traverse.

Modulation of Cell Cycle-dependent Pathways in Cells Exposed to Low Level Radiation—We have previously characterized the cell cycle checkpoint induced when quiescent human fibroblasts are exposed to low levels of radiation prior to mitogenic stimulation, inducing what was termed a permanent growth arrest (32). Under these conditions we observed an induction of a checkpoint early in the cell cycle, prior to an increase in cyclin D-associated kinase activity. The next series of experiments were designed to characterize the molecular nature of the checkpoint induced when cells were reversibly arrested following exposure to lower levels of radiation that induce a transient rather than permanent growth arrest. The purpose of these experiments was to determine whether there was a single radiation-induced G0/G1 checkpoint that was reversible under some circumstances and irreversible under others (ultimately dependent on dosage levels), or whether any differences in the temporal nature of the damage-induced
growth arrests might reflect the induction of mechanistically distinct checkpoints.

As seen in Fig. 2A, quiescent Flow 2000 cells contained very low levels of cyclin D1-associated kinase activity. 12 h following the addition of fresh mitogens to control cultures, there was a 3–4-fold increase in the RB kinase activity associated with cyclin D1 immunoprecipitation. Similar to what we had observed before, prior exposure to levels of radiation sufficient to induce a permanent growth arrest completely repressed the mitogen-induced increase in cyclin D1-associated RB kinase activity. Much different results were obtained when cells were exposed to lower levels of ionizing radiation, as shown in Fig. 2B. In this experiment, control cells contained low levels of kinase activity that were increased 12 h following the addition of PDGF and serum, similar to what was seen above. Exposure of cells to lower levels of radiation failed to cause a reduction in cyclin D1-associated kinase activity in mitogen-stimulated damaged cells, even at levels of damage that caused an acute inhibition of G0/G1 traverse. These data suggest the presence of mechanistically distinct checkpoints that mediate the cellular response to either moderate or elevated levels of ionizing radiation.

As shown in Fig. 3A, the late G0/G1 expression of E2F-dependent genes was also observed in cultures transiently arrested following exposure to low levels of ionizing radiation. Quiescent cells expressed undetectable levels of cyclin A, cdk2, and E2F-1 mRNAs. 22 h following the restimulation of control quiescent cells with medium supplemented with PDGF and serum there were dramatic increases in the abundance of each transcript. Prior exposure of cells to levels of radiation that caused only modest decreases in the expression of this series of genes.

The late G0/G1 expression of E2F-2 dependent transcripts in cells exposed to levels of radiation sufficient to cause a complete, albeit transient, inhibition of entry into S phase, was paralleled by increases in the expression of the corresponding proteins, as exemplified by the data shown in Fig. 3B. Cyclin A protein levels were low in quiescent Flow 2000 cells. Restimulation of control cells with fresh medium supplemented with PDGF, and serum caused a robust induction of cyclin A protein. Prior exposure to ionizing radiation up to levels of 100 cGy caused only modest decreases in cyclin A protein expression, even though entry into S phase was systematically repressed over the doses of radiation used in this experiment. Western blots measuring levels of either cdk2 or E2F-1 showed a similar pattern of expression (data not shown).

In stark contrast to the activation of cyclin D1 kinase activity and the expression of late G0/G1 gene expression in cells exposed to low level radiation, the degree of activation of cdk2-associated kinase activity directly paralleled the ability of cells to initiate DNA synthesis as shown in Fig. 3C. Quiescent cells contained very low levels of kinase activity, as expected since cdk protein is not expressed under these conditions. 22 h following mitogenic stimulation of undamaged cells, robust histone kinase activity was observed in cdk2 immunoprecipitates. Exposure to increasing levels of radiation caused a dose-dependent decrease in the activation of cdk2 kinase in proportion to the decrease in DNA synthesis (Fig. 1) despite the fact that both cdk2 and cyclin A protein expression was observed (Fig. 3B). Cyclin E protein, another component of active cdk2 complexes in restimulated cells was also present at equivalent levels in control and growth-arrested cultures (data not shown).

Taken together, the data in Fig. 3 provide conclusive evidence for the presence of functionally distinct radiation-induced G0/G1 checkpoints. As we previously reported, cells exposed to levels of radiation sufficient to cause a marked loss of clonogenicity arrest because of a lack of cyclin D-associated kinase activity, and in the complete absence of late G0/G1 gene expression (32). In marked contrast, cells growth-inhibited following exposure to more modest levels of radiation, contain levels of cyclin D kinase activity equivalent to that seen in control restimulated cultures, and express high levels of cyclin A, cdk2, and E2F-1. Instead, cultures of cells exposed to lower levels of ionizing radiation arrest in a manner that at least parallels, and potentially is mediated by, a repression of cdk2-associated kinase activity. We next carried out a series of experiments that investigated whether the repression of cdk2-associated kinase activity could be entirely accounted for by the actions of p21Cip1. In order to do complete dose response curves and to measure alterations in kinase activity immediately following damage (as opposed to a long term failure to activate kinases), these experiments were performed in logarithmically growing cells.

**Effects of Ionizing Radiation on Cell Cycle Traverse in Logarithmically Growing Flow 2000 Cells—Logarithmically growing Flow 2000 cells were harvested at regular intervals following exposure to 800 cGy of ionizing radiation, and alterations in cell cycle distributions were determined by a flow cytometric analysis (Table I). Over the first 6 h following damage, the number of cells in the G2/M compartment remained constant, reflecting the rapid and complete inhibition of cell division induced in irradiated cells.**
percentage of G1 cells steadily decreased, due to the somewhat delayed repression of G1/S traverse in damaged cultures. The transient repression of ongoing DNA synthesis following exposure to ionizing radiation, together with the continuing exit of cells from the G1 compartment, resulted in approximately a 70% increase in the percentage of S phase cells by 6 h following damage. Between 6 and 12 h following exposure to γ-rays, the percentage of cells in the G1 compartment remained relatively constant while cells in S phase steadily entered G2. By 12 h the entire population was stably arrested in G1 and G2, a pattern that was maintained for several days after exposure to this high level of radiation (data not shown). This pattern of inhibition is essentially identical to what we had seen earlier with other strains of human fibroblasts (35), and was not accompanied by decreases in total cell numbers (data not shown).

**Repression of Cyclin A-associated Kinase Activity in Irradiated Cells**—Logarithmically growing Flow 2000 cells contained readily detectable levels of cyclin A-associated kinase activity, as shown in Fig. 4A. Within 1 h following exposure to 800 cGy, kinase activity decreased by ~50% to a level that was maintained for 2 h following DNA damage. Between 2 and 6 h following exposure to radiation, cyclin A kinase activity decreased a second time, eventually reaching levels approaching those observed in quiescent cultures. These data suggest that the modulation of cyclin A-associated kinase activity was bi-phasic, with an initial rapid decrease followed by a second, more protracted fall. Cellular levels of the cyclin-dependent kinase inhibitor p21<sup>Cip1</sup> were shown in Fig. 4B. p21<sup>Cip1</sup> mRNA levels were low in logarithmically growing Flow 2000 cells. Between 1 and 2 h following exposure to 800 cGy, p21<sup>Cip1</sup> message increased, with peak levels seen at 4 and 6 h following damage. p21<sup>Cip1</sup> protein was also low in quiescent cells and was increased following exposure to ionizing radiation, although with somewhat delayed kinetics compared with the increase in mRNA levels, as would be expected. Significant increases in p21<sup>Cip1</sup> protein were not observed until 2–4 h following damage, after which levels remained relatively constant. Therefore the initial decreases in cyclin A-associated kinase activity following irradiation occurred in the absence of measurable increases in p21<sup>Cip1</sup> protein levels, while the subsequent, complete inhibition of activity was temporally correlated with an increased expression of the CKI.

It has been suggested (36) that an acute repression of cdc25A protein levels regulate a rapid decrease in cyclin E-associated kinase activity in human fibroblasts exposed to ultraviolet radiation. To determine whether the repression of cyclin A-associated kinase activity in damaged cells, such as that seen in Fig. 3A, could be mediated by alterations in cdc25 activity, we measured the phosphotyrosine content of cdk2 immunoprecipitated with cyclin A, as also shown in Fig. 4B. Phosphorylated cdk2 was detected in cells at the time of radiation, and did not systematically vary for at least the first 9 h following exposure to 800 cGy. Cdk2 protein associated with cyclin A was invariant over this time period (data not shown), as we have reported earlier (32). Therefore, even in light of any alterations in cdc25A protein concentrations, the pool of cdk2 potentially...
Radiation-induced Cell Cycle Arrest

Fig. 5. Effects of cycloheximide on the regulation of cyclin A-associated kinase activity in irradiated cells. Logarithmically growing Flow 2000 cells maintained in either control medium or in medium containing 10 μg/ml cycloheximide were harvested at the indicated times following exposure to 800 cGy, and the histone kinase activity of cyclin A immunoprecipitates was determined.

Fig. 6. Dose-dependent effects of ionizing radiation on cyclin A kinase activity. Flow 2000 cells were exposed to the indicated levels of ionizing radiation and harvested 6 h after damage. Histone kinase activity was measured in cyclin A immunoprecipitates as shown in the top panel. Cyclin A or p21Cip1 protein levels were determined by a Western analysis (middle panels). In addition, cyclin A immunoprecipitates were isolated and separated by gel electrophoresis. The amount of p21Cip1 associated with cyclin A was then determined by a Western analysis as shown in the bottom panel.

increased cell cycle arrest. This arrest, however, was not associated with an increase in p21Cip1 protein levels. Instead, the amount of cyclin A-associated kinase activity was decreased following radiation exposure. The decrease in kinase activity was observed in cells exposed to 100 cGy and was more pronounced in cells exposed to 800 cGy. These results suggest that complex formation between p21Cip1 and cyclin A is regulated by processes other than the total cellular levels of the proteins. In addition, these data imply that either all of the cyclin A complexes present in cells exposed to 100 cGy, with some complexes being active and others inhibited, or that only a fraction of the cyclin A complexes are susceptible to association with the CKI. In order to distinguish between these possibilities, we performed the experiment shown in Fig. 7. In this experiment, logarithmically growing cells were exposed to increasing concentrations of ionizing radiation, and harvested following a 6-h incubation. Histone H1 kinase activity was determined in either p21Cip1 immunoprecipitates prepared from cellular extracts (Fig. 7, left lanes), or in cyclin A immunoprecipitates made from p21Cip1-depleted extracts (Fig. 7, right lanes). There was no kinase activity associated with p21Cip1 under any experimental condition. In contrast, cyclin A-associated kinase activity was readily apparent in p21Cip1-depleted extracts, and was repressed following ionizing radiation in a manner identical to that seen in unfractionated extracts (see Fig. 6). These data unequivocally show that all of the cyclin A complexes associated with p21Cip1 are catalytically inactive. In addition, at all levels of ionizing radiation cyclin A was present in a form free of p21Cip1 either as part of an active pool of complexes (for example in cells exposed to 100 cGy), or in an inactive state (in cells exposed to 800 cGy). The second conclusion is based on the fact that both the amount of cyclin A protein and its association with p21Cip1 were equivalent following exposure to all levels of ionizing radiation between 100 and 800 cGy. Therefore, the amount of cyclin A free of p21Cip1 must also be equal in all cases, independent of whether it was present in complexes containing kinase activity.

Relationship between Cyclin A-associated Kinase Activity and the Rate of DNA Synthesis—Following exposure of logarithmically growing cultures to 800 cGy, the peak number of cells in S phase was observed at 6 h. Over the subsequent 6-h period, cells completed their traverse of S, and became arrested in G2 (Table I). Somewhat surprisingly, the final 6 h of ongoing DNA synthesis occurs in the presence of undetectable levels of cyclin A-associated kinase activity (see Fig. 4), given that it has been postulated that cyclin A kinase is absolutely required to maintain ongoing rates of DNA synthesis in S phase cells (10, 11). One possible explanation of these results is that while
Radiation-induced Cell Cycle Arrest

Fig. 7. p21Cip1-associated kinase activity in irradiated cells. Logarithmically growing cultures of Flow 2000 cells were exposed to the indicated levels of ionizing radiation and harvested at 6 h following damage. p21Cip1 immunoprecipitates were isolated from cellular extracts and assayed for histone kinase activity (left lanes). Cyclin A complexes were immunoprecipitated from the supernatants cleared of p21Cip1 and also assayed for kinase activity (right lanes).

Fig. 8. Effects of roscovitine on traverse of S phase. Cultures of Flow 2000 cells were exposed to 800 cGy of ionizing radiation. Cells incubated for 6 h following damage (equivalent to B) were incubated for an additional 6 h in medium containing 25 μM roscovitine prior to harvest (D). Cells were fixed, and cell cycle distributions were determined by a flow cytometric analysis.

cyclin A kinase activity was markedly repressed in irradiated cells, there was a low undetectable level of active complexes sufficient to mediate traverse through the latter stages of S phase. To determine whether this was the case, logarithmically growing cultures of Flow 2000 cells were exposed to 800 cGy of ionizing radiation. Following a 6-h incubation, roscovitine, a specific and potent inhibitor of cdk2 catalytic activity (37), was added to some cultures, and traverse through late S and into G2 was monitored by flow cytometry. As shown in Fig. 8A, at the beginning of the experiment the population of cells was in log growth. 6 h following exposure to ionizing radiation, cells were accumulated in late S phase (Fig. 8B). 12 h after damage, all S phase cells had entered G2 independent of whether they were incubated between 6 and 12 h in drug-free medium (Fig. 8C) or in medium containing the cdk2 inhibitor roscovitine (Fig. 8D). These data strongly support the hypothesis that in cultures exposed to ionizing radiation, cells can maintain high rates of ongoing DNA synthesis even in the absence of cyclin A-associated kinase activity.

DISCUSSION

Investigations of the cellular responses to DNA damage, including those observed following exposure to ionizing radiation, have provided important insights into biological protective pathways as well as molecular processes that are fundamental to the regulation of cell cycle traverse. In particular, studies of radiation-induced cell cycle checkpoints have proven valuable to the understanding of the regulation of cyclin/cdk expression and activation. Several important conclusions can be drawn from the characterization of the cell cycle checkpoints induced in irradiated cells described here. We present clear evidence that at least two mechanistically distinct growth arrests can be invoked in cells following exposure to ionizing radiation as outlined in Fig. 9. As we previously reported (32) exposure of cells to levels of radiation sufficient to induce a permanent growth arrest caused cells to arrest in early G/G1. While the expression of early cell cycle-dependent genes (including both primary response genes directly induced by mitogens even in the absence of protein synthesis, and secondary response genes linked to exit from G and entry into the cell cycle) are observed following the mitogenic stimulation of cells exposed to high levels of radiation, cyclin D activity is not observed, even though p21Cip1 levels are reduced, and the transcription of late G/G1 genes is blocked. In contrast, as described here, when cell cycle traverse is transiently blocked following exposure to lower levels of radiation, cyclin D1 is activated, and a full induction of E2F-dependent gene expression is observed, although cdk2 complexes are not activated and cells do not enter S phase. Only a portion of the repression of cdk kinase could be accounted for by the actions of p21Cip1, while some of the decrease in activity was caused by a yet uncharacterized pathway that was dependent on ongoing protein synthesis, but did not involve cdk2 complex formation or phosphorylation status. While cdk2 activity was potentially required for G1/S traverse, cells could replicate DNA through late S phase and enter G2 in the apparent complete absence of cdk2 activity. The model proposed in Fig. 9 is not inconsistent with the current thoughts concerning ration-induced checkpoints, although it adds to our understanding of the regulatory processes that mediate cellular response to DNA damage.

The activation of E2F-dependent transcription in the complete absence of cdk2-associated kinase activity has not been predicted by earlier models, which propose that a combination of cdk4 and cdk2 mediate full phosphorylation of RB and activation of E2F (9). However, the observations that we report here are entirely consistent with our recent report studying the actions of a JAK inhibitor on G/G1 traverse in BALB/c-3T3 cells (38). Cells growth-arrested in the presence of AG490 contained fully activated cyclin D-associated kinase activity and
expressed control levels of E2F-dependent genes, yet failed to initiate DNA synthesis because of a lack of cdk2-associated kinase activity. Thus, JAK inhibitors induce a phenotype remarkably similar to that observed following the exposure of human fibroblasts to moderate levels of ionizing radiation, confirming the observation that the induction of E2F-dependent transcription can occur in the absence of cdk2-associated kinase activity.

It has been proposed that the lack of cyclin A expression in irradiated cells is due to a p21Cip1-dependent inhibition of cyclin E/cdk2 activity (31). However, we have found that exposure of quiescent cells to high levels of radiation prior to mitogenic stimulation also represses cyclin E and cdk2 expression (32). As we report here, exposure to lower levels of damage allows for cyclin E, cdk2, and cyclin A expression even in the absence of measurable levels of cdk2 kinase activity. The differences in these results well might reflect differences in either the levels of radiation exposure or in the time during G0 traverse at which cells were damaged.

Mайлant et al. (36) reported that a UV radiation-induced degradation of cdc25A protein mediated a rapid fall in cyclin E-associated kinase activity in human fibroblasts. This conclusion was based on the ability of extracts from control or damaged cells to dephosphorylate and activate cyclin E-cdk2 complexes. Whereas these authors did not show the data, they suggested that somewhat different results were obtained when the processes modulating cyclin A-associated kinase were studied. We directly measured the phosphotyrosine content of cdk2 associated with cyclin A in control cells and in cultures exposed to ionizing radiation and did not find any systematic modulation in the phosphorylation of cdk2 that might explain the decrease in kinase activity. It is clearly possible that different biochemical processes are responsible for the modulation of cyclin E versus cyclin A complexes, or that the differences in the types of damage observed following UV or ionizing radiation might well induce distinct inhibitory pathways that separately impinge on the regulation of cdk2-associated kinase activity.

Exposure of cells to increasing levels of ionizing radiation resulted in a dose-dependent increase in the cellular levels of p21Cip1 and decrease in cyclin A-associated kinase activity. Surprisingly, the levels of p21Cip1 associated with cyclin A complexes did not increase in parallel with the radiation dose even at levels of damage where residual activity was observed. There are two potential explanations for this observation. The first possibility is that all cyclin A complexes bind p21Cip1 at each level of radiation, with some difference in the ratio of p21Cip1 to cdk2 determining the presence of catalytic activity. While it has been proposed that cyclin-cdk complexes bound to members of the p21Cip1 family of proteins can, under some circumstances, retain activity (39), this possibility is not universally accepted (40, 41). We failed to find histone kinase activity in p21Cip1 immunoprecipitates, suggesting that all cyclin A complexes bound by the inhibitor were inactive. The presence of cyclin A-associated kinase activity in p21Cip1 immunodepleted extracts suggests that processes other than the cellular level of the inhibitor determine the extent of binding to cdk2 complexes. It therefore appears that a distinct pool of cyclin A complexes are unable to bind p21Cip1 and require a distinct, as yet undescribed, radiation-induced pathway for a full repression of kinase activity.

While the activation of cdk2-associated kinase activity has been postulated to play a major role in the traverse of the G1/S boundary in popular models of cell cycle regulation (1, 2), neither the precise role that cdk2 plays in growth control (other than a putative role in the activation of E2F-dependent transcription that is addressed by the data presented here), nor the absolute requirement for its activity in the traverse of specific portions of the cell cycle, have been conclusively established. Some studies present very clear evidence that repression of cdk2 kinase activity directly induces a cell cycle arrest (42, 43). However knockout mice lacking the structural gene for cdk2 develop and grow normally although a defect in meiosis was identified (44). Equally surprising was the finding that mice lacking the cyclin E gene were viable (45). In addition, it has been shown that cdk2 activity is dispensable in some circumstances for the growth of either human tumor cells (46) or established lines of murine fibroblasts (47). These new results have been taken to indicate a previously anticipated plasticity in the role of cyclin-cdk complexes in the regulation of specific cell cycle transitions (48). The data presented here are consistent with the hypothesis that the repression of cdk2-associated kinase activity following exposure of cells to low levels of ionizing radiation is responsible for the observed G1/G0 checkpoint. However we have not ruled out the possibility that the lack of cdk2 activity parallels the induction of the checkpoint, rather than mediates its induction. Further studies of basic control mechanisms that are involved in the processes that regulate the initiation of DNA syntheses will be required to directly answer this question. We propose that the experimental model described above will be valuable in such an undertaking.

The observation that cells traverse the final 6 h of S phase...
and arrest in G₂ in the absence of measurable cyclin A-associated kinase activity initially suggested that only a minor fraction of activity was responsible for the maintenance of ongoing DNA synthesis, based on the earlier proposal that cyclin A activity was required for traversal of S phase. We were surprised that the addition of roscovitine, a potent cdk2 inhibitor, did not alter the ability of damaged cells to complete S phase and become arrested with a 4N content of DNA. We conclude from these data that there is not an absolute requirement for cyclin A-associated catalytic activity for traversal of cells at least through late S phase. Whether irradiated cells invoke distinct pathways to maintain DNA synthesis until cells can arrest with a stable content of DNA is not known. It is possible that cyclin A activity is actually required only for G₂/S traversal and the initial portions of S phase, if it is required at all (see above). A determination of the molecular targets of cyclin A-cdk2 complexes that mediate the observed effects on DNA synthesis will allow for the determination of the effects of exposure to radiation on specific pathways.

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