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Genetics

Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G_1 arrest after γ-irradiation

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ABSTRACT **In mammalian cells, activation of certain checkpoint pathways as a result of exposure to genotoxic agents results in cell cycle arrest. The integrity of these arrest pathways is critical to the ability of the cell to repair mutations that otherwise might compromise viability or contribute to deregulation of cellular growth and proliferation. Here we examine the mechanism through which DNA damaging agents result in a G_1 arrest that depends on the tumor suppressor p53 and its transcriptional target p21. By using primary cell lines lacking specific cell cycle regulators, we demonstrate that this pathway functions through the growth suppressive properties of the retinoblastoma protein (pRB) tumor suppressor. Specifically, γ-irradiation inhibits the phosphorylation of pRB at cyclin-dependent kinase 2-specific, but not cyclin-dependent kinase 4-specific, sites in a p21-dependent manner. Most importantly, we show that pRB is a critical component of this DNA damage checkpoint. These data indicate that the p53 → p21 checkpoint pathway uses the normal cell cycle regulatory machinery to induce the accumulation of the growth suppressive form of pRB and suggest that loss of pRB during the course of tumorigenesis disrupts the function of an important DNA damage checkpoint.**

The retinoblastoma (*Rb*) gene originally was cloned by virtue of its absence in retinoblastomas (reviewed in ref. 1). Subsequent studies showed that *Rb* gene mutations exist in approximately one-third of all human tumors (reviewed in ref. 1). These mutations result in either complete loss or functional inactivation of the retinoblastoma protein (pRB) and reintroduction of the wild-type gene is sufficient to reverse the tumorigenicity of several *Rb* negative cell lines.

The growth suppressive properties of the pRB are thought to depend on its ability to regulate the cellular transcription factor E2F (reviewed in ref. 2). pRB binds to E2F *in vivo,* and this association is sufficient to inhibit its transcriptional activity. Moreover, the resulting pRB·E2F complex is capable of mediating the transcriptional repression of E2F-responsive genes (3–5). Many E2F-responsive genes have been identified, and each of them plays a critical role in the control of cellular proliferation (reviewed in ref. 2). In addition, E2F binding maps to the "growth suppression" domain of pRB and mutant, tumor derived-forms of pRB all lack the ability to bind to E2F (reviewed in ref. 2).

pRB is phosphorylated in a cell cycle-dependent manner, and these modifications are sufficient to inactivate its ability to bind to E2F and to block cell division (reviewed in ref. 6). Several pRB phosphorylation sites have been identified, and each of them matches the consensus recognition sequence of the cyclin-dependent kinases (cdks; ref. 7). The G_1 cdks, cyclin D·cdk4/6 and cyclin E·cdk2, both can phosphorylate pRB *in vitro* (8–11). In these *in vitro* assays, cyclin D·cdk4 and cyclin E·cdk2 preferentially phosphorylate distinct, but overlapping, pRB sites (12, 13). Cyclin D·cdk4 is the first cdk to be activated in response to growth factors (10) and *in vivo* studies confirm that it is essential for pRB inactivation (14, 15). Indeed, pRB appears to be the only essential target of this kinase (14, 15). Studies addressing the role of cyclin E·cdk2 in pRB regulation have yielded conflicting conclusions (12, 16, 17). One study showed that cyclin D1/cdk4 was sufficient to inactivate both the E2F binding and growth suppressive properties of pRB (12). However, two other labs have reported that inhibition of cdk2 (by either treatment with transforming growth factor β or overexpression of dominant negative cdk2) resulted in the accumulation of an underphosphorylated form of pRB that still can bind to E2F (16, 17). These latter studies suggest that cyclin E·cdk2 contributes to inactivation of the growth-suppressive properties of pRB. In contrast to the D-type kinases, cyclin E·cdk2 is known to have at least one other substrate whose phosphorylation is essential for S-phase entry (18).

Superimposed on normal cell cycle regulation are a number of checkpoint mechanisms, which are not required for normal cell cycle progression but are critical for the cellular response to stress (reviewed in ref. 19). One of the best characterized of the mammalian checkpoint pathways is the DNA damage-induced G_1 arrest. This checkpoint depends on the tumor suppressor p53 (20). The loss of p53 abrogates the DNA damage response, and this loss is thought to contribute to tumorigenesis by permitting the propagation of mutations (reviewed in ref. 21). The mechanism by which p53 imposes the DNA damage-induced G_1 arrest has been partially elucidated. In response to irradiation, p53 induces the transcription of the *p21* gene (22), which encodes an inhibitor of cdks (23–25). Analysis of *p21*-deficient cells confirms that the p21 protein is essential for the integrity of the DNA damage-induced G_1 arrest (26–28). In normal cells, this p21-dependent arrest correlates with the accumulation of hypophosphorylated pRB (29, 30). Although this change in pRB phosphorylation could arise as an indirect consequence of the G_1 arrest, the presence of the pRB-binding papilloma virus E7 protein has been shown to abrogate the DNA damage checkpoint (30, 31). These data suggest that pRB is involved in the DNA damage response; however, the multifunctional nature of E7 makes it difficult to eliminate other possible mechanisms (32, 33).

In this study, we investigate how the p53 → p21 DNA damage checkpoint pathway interfaces with the normal cell cycle machinery. By using cell lines derived from mutant

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Abbreviations: Rb, retinoblastoma; pRB, retinoblastoma protein; cdk, cyclin-dependent kinase; MEF, mouse embryo fibroblast.

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mouse strains, we demonstrate that the p21-mediated arrest depends on the presence of active, growth-suppressive pRB. Induction of the checkpoint by low doses of γ-irradiation results in the down-regulation of cdk2, but not cdk4, activity and the accumulation of partially phosphorylated pRB. Phosphopeptide-specific antibodies confirm that this form of pRB has been phosphorylated on cdk4-specific, but not cdk2-specific, sites. Thus, p21 arrests cells by blocking the inactivation of pRB that normally occurs as cells progress through the G_1 phase of the cell cycle. Our data supports a model whereby this checkpoint blocks cell cycle progression by co-opting normal cell cycle regulatory mechanisms.

MATERIALS AND METHODS

Irradiation and Cell Cycle Analysis. Sparse cultures of mouse embryo fibroblasts (MEFs) were irradiated with 5.5 Gy by using a γ-cell irradiator with a *Cs* source. Untreated and irradiated cultures were harvested for cell cycle analysis 18 hr after γ-irradiation. Cell cycle analysis was performed as described (26).

***In Vitro* Kinase Assays.** Cdk2 and cdk4 *in vitro* kinase assays were performed as described (34). Briefly, cell lysates were precleared with equilibrated protein A beads (Pierce) and incubated with anti-cdk4 (Santa Cruz Biotechnology, C-22) or anti-cdk2 antibody (kindly provided by G. J. Hannon, Cold Spring Harbor Laboratories, Plainview, NY) for 4 hr. Immune complexes were precipitated with protein A beads (Pierce) and incubated in the kinase buffer containing 4 mM ATP, 20 μCi [γ-^{32}P] ATP (NEN/DuPont), and 6 μg of glutathione *S*-transferase-RB (glutathione *S*-transferase fusion with amino acids 792–928 from the C terminus of pRB) or 2 μg of histone H1 (Sigma), for 30–60 min at 30°C. Quantitation was performed by PhosphorImager analysis.

^{32}P-Labeling and pRB Immunoprecipitation. Subconfluent MEF cultures were labeled with 5 mCi/ml of HCl-free ^{32}P-orthophosphate (NEN/DuPont) in DMEM supplemented with 5% dialyzed fetal bovine serum. Labeling proceeded for 4 hr, starting 14 hr after γ-irradiation. Protein extracts were prepared as described (34), normalized for ^{32}P incorporation, and used for pRB immunoprecipitation by using mAbs XZ104, XZ133, and 21C9 (kindly provided by E. Harlow, Massachusetts General Hospital Cancer Center, Charlestown). pRB then was resolved in a 6% polyacrylamide SDS gel, blotted onto Immobilon-P (Millipore), and visualized by autoradiography.

Antiphospho pRB Antibodies. α-Phospho-Ser-870 and α-phospho-Ser-811 have been described (35). These antisera were raised against phosphopeptides derived from human pRB that are absolutely conserved in the mouse protein (Ser-773 and Ser-804, respectively). α-Phospho-Thr-350 was raised against the murine pRB sequence. The phosphopeptide SFETERT(PO_3)PRKNNPC was chemically synthesized, conjugated with keyhole limpet hemocyanin, and then injected into rabbits as described (35). The resulting polyclonal antibodies were purified by column chromatography with the same phosphopeptide linked to Sepharose CL-4B followed by a column of Sepharose CL-4B coupled to the corresponding unphosphorylated peptide SFETERTPRKNNP. Purified antibodies specifically recognized the phosphopeptide in ELISAs (data not shown).

Immunoblotting. Membranes were blocked in TBST buffer (10 mM Tris, pH 7.5/150 mM NaCl/0.03% Tween-20) containing 5% nonfat dry milk. pRB was detected by using mouse mAb G3–245 (PharMingen) at a dilution 1:175 and a three-step protocol using a rabbit anti-mouse secondary antibody and an anti-rabbit tertiary antibody conjugated to horseradish peroxidase (HRP). Phosphoserine 780, phosphoserine 811, and phosphothreonine 350 (35) were detected by using rabbit polyclonal antibodies at 1:300 and 1:100 dilution, respectively and a secondary anti-rabbit antibody conjugated to HRP. Detection was performed by enhanced chemiluminescence.

Two-Dimensional Phosphopeptide Mapping. [^{32}P]pRB was immunoprecipitated, blotted, and visualized as described above. Both band A and band B were excised and subjected to two-dimensional tryptic phosphopeptide mapping as described (7).

RESULTS

γ-Irradiation of fibroblasts results in the activation of the DNA damage checkpoint pathway and thereby induces cell cycle arrest (36). It is well documented that p21 acts as an important downstream target of this p53-dependent radiation response, but the mechanism of p21 action is unknown (26–29). Because the pRB-binding papilloma virus E7 protein is sufficient to override the DNA damage checkpoint (30, 31), we tested whether pRB is required for the p53 → p21-mediated arrest. To address this question, we generated primary MEFs from wild-type, *p53−/−*, *p21−/−*, and *Rb−/−* mouse strains and tested their response to DNA damage. Wild-type and mutant cells were exposed to γ-irradiation, and the degree of G_1 arrest was assessed by comparing the proportion of S-phase cells in irradiated versus unirradiated populations (Fig. 1). Consistent with previous studies, loss of p53 dramatically impairs the G_1 cell cycle block. In contrast, loss of p21 significantly reduced, but did not abolish, this γ-irradiation-induced arrest. This finding supports previous conclusions that p21 is a critical downstream target of p53, but it is not the only mechanism by which p53 can mediate the DNA damage-induced G_1 arrest (26, 27).

When tested in this assay, *Rb*-deficient fibroblasts also were impaired in their ability to arrest in G_1 in response to DNA damage. Significantly, the magnitude of this defect in γ-irradiated *Rb−/−* cells was similar to that observed in the *p21−/−* cells, suggesting that p21 and pRB act in the same DNA damage response pathway. To test this hypothesis, we generated mouse embryos that were deficient for both *p21* and *Rb* and then compared the irradiation response of single and double mutant MEFs derived from littermate embryos (Fig. 1). The G_1 arrest response of the *p21−/−;Rb−/−* cells was indistinguishable from that of either of the single mutant

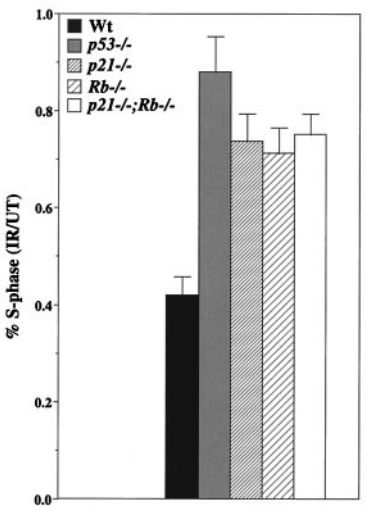

FIG. 1. Analysis of the genetic requirements of the DNA damage-induced G_1 arrest response. Asynchronous cultures of wild-type, *p53−/−*, *p21−/−*, *Rb−/−* and *p21−/−;Rb−/−* fibroblasts were irradiated with a dose of 5.5 Gy and labeled with BrdUrd for 4 hr beginning 14 hr after irradiation. Histogram shows the S-phase fraction of irradiated versus untreated samples with the mean and SD (error bars) from four independent experiments.

MEFs. We therefore conclude that p21 and pRB likely act in the same p53-dependent checkpoint pathway.

To investigate the mechanism by which p21 and pRB participate in the p53-dependent G_1 arrest, we first tested whether the absence of these proteins affects the regulation of the cdks that control the G_1/S-transition. p21 is known to inhibit the activity of both cyclin D·cdk4 and cyclin E·cdk2 (23–25), and these G_1 kinases are down-regulated in response to a variety of DNA damaging agents (37, 38). We therefore compared the level of cdk4 and cdk2 activity in wild-type and *p21*-deficient cells either with or without γ-irradiation treatment (Fig. 2*A*). We did not detect any effect of γ-irradiation on the level of cdk4 activity in either cell type. In contrast, γ-irradiation significantly reduced the level of cdk2 activity in wild-type, but not *p21−/−*, cells. These data suggest that the observed p21-dependent, radiation-induced G_1 arrest is mediated through the specific inhibition of cdk2, but not cdk4, activity.

As pRB is known to control the expression of the cdk2 regulatory subunit, cyclin E, it seemed possible that the loss of pRB could impair the DNA damage response by altering the regulation of cdk2 activity. To address this possibility, we directly compared the level of cdk2 activity in wild-type and *pRB−/−* cells both before and after γ-irradiation (Fig. 2*A*). Although the radiation response was significantly impaired in the *Rb−/−* fibroblasts (Fig. 1), both the basal level of cdk2 activity and the degree of its down-regulation in response to γ-irradiation were similar to those observed in the wild-type cells (Fig. 2*A*). Moreover, the radiation induced inhibition of cdk2 activity was completely abolished in the *p21−/−;Rb−/−* MEFs, confirming that down-regulation of this kinase in the absence of pRB still depends on p21 (Fig. 2*A*). We therefore conclude that loss of pRB does not interfere with either the induction of p21 or the specific inhibition of cdk2 activity in response to γ-irradiation. These observations strongly suggest that pRB acts downstream of p21 in the p53-dependent checkpoint pathway.

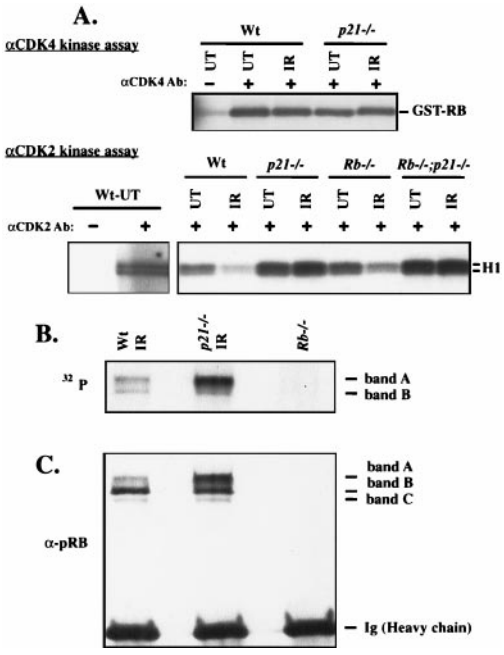

FIG. 2. Effect of γ-irradiation on G_1 cdk activity and pRB phosphorylation. (*A*) Normalized protein extracts from untreated or γ-irradiated (18 hr) cells were precleared with protein A beads, immunoprecipitated with the indicated antibodies, and then assayed for kinase activity by incubation with an excess of [γ-^{32}P]ATP and substrate (histone H1 or a C-terminal fragment of pRB). (*B*) SDS/PAGE analysis of [^{32}P]pRB from wild-type and *p21−/−* cells 18 hr after irradiation. (*C*) Western blot analysis of [^{32}P]pRB with an α-pRB mAb.

There is good evidence to suggest that cdk2 contributes to the phosphorylation of the pRB and that this phosphorylation is required to inactive its growth suppressive properties (16, 17). To test the role of pRB phosphorylation in the DNA damage response, we immunoprecipitated pRB from wild-type (down-regulated cdk2) and *p21−/−* (normal cdk2) irradiated cells that had been metabolically labeled with $^{32}P_i$ (Fig. 2*B*). Consistent with the continued presence of cdk4 activity (see Fig. 2*A*), we detected phosphorylated pRB in extracts of wild-type irradiated cells (Fig. 2*B*). This phosphorylated pRB was present in two distinct forms, a slower migrating species (labeled band A) and a faster migrating species (labeled band B), which were detected at roughly equal levels. Both of these pRB species also were present in the irradiated p21-deficient cells. In these cells, however, the slower migrating form of pRB (band A) was present at much higher levels than the faster migrating form (band B). This finding suggested that γ-irradiation results in a change in the degree of pRB phosphorylation in a p21-dependent manner. The p21-dependence of this change may reflect a direct link between p21 and pRB phosphorylation, or it may arise indirectly because of differences in the cell cycle staging of wild-type and *p21−/−* cells. Our genetic data, which clearly demonstrate a requirement for pRB in DNA damage-induced G_1 arrest, supports a model in which the observed changes in pRB phosphorylation and cdk2 activity are causal, and not consequential, for the arrest.

To assess the relative levels of each pRB species (instead of the degree of ^{32}P incorporation), we also performed immunoprecipitation followed by immunoblotting of whole-cell extracts of wild-type and *p21−/−* irradiated cells (Fig. 2*C*). In this assay, we were able to detect three distinct pRB species. The fastest migrating form (band C) was not detected by ^{32}P labeling even on long exposures (data not shown), indicating that it represents unphosphorylated pRB. Band C was present at a similar low level in both the wild-type and *p21−/−* irradiated cells. The remaining bands comigrated with the ^{32}P-labeled bands A and B. Comparison of the ^{32}P and immunoblotting signals for bands A and B suggested that band A corresponds to the hyperphosphorylated form of pRB whereas band B results from the partial phosphorylation of the pRB protein. Significantly, whereas the protein levels of partially phosphorylated pRB (band B) were similar in the wild-type and mutant irradiated cells, the fully phosphorylated form of pRB was significantly reduced in wild-type cells. We therefore conclude that γ-irradiation blocks the conversion of partially to hyperphosphorylated pRB in a p21-dependent manner.

The difference between the partially and hyperphosphorylated forms of pRB could be caused by differences in either the specific sites of phosphorylation or the extent to which pRB is phosphorylated. To distinguish between these two possibilities, we compared the two-dimensional tryptic phosphopeptide maps of pRB species isolated from either wild-type (predominantly band B) or *p21−/−* (bands A and B) irradiated cells. In wild-type irradiated cells, we detected six prominent pRB tryptic phosphopeptides (labeled 1–6 in Fig. 3). Thus, pRB is phosphorylated at multiple sites in wild-type cells after γ-irradiation. The two-dimensional phosphopeptide map of pRB from *p21−/−* cells was considerably more complex, containing more than 15 major phosphopeptides (labeled 1–15 in Fig. 3). Six of these phosphopeptides (nos. 1–6) were identical to those detected in the map of pRB from wild-type cells. The remainder (nos. 7–15) were either greatly underrepresented or completely absent from the two-dimensional map of pRB derived from wild-type cells (Fig. 3). The simplest interpretation of these data is that the novel phosphopeptides are derived from the *p21−/−*-specific species of pRB, band A, and that transition from partially to hyperphosphorylated pRB

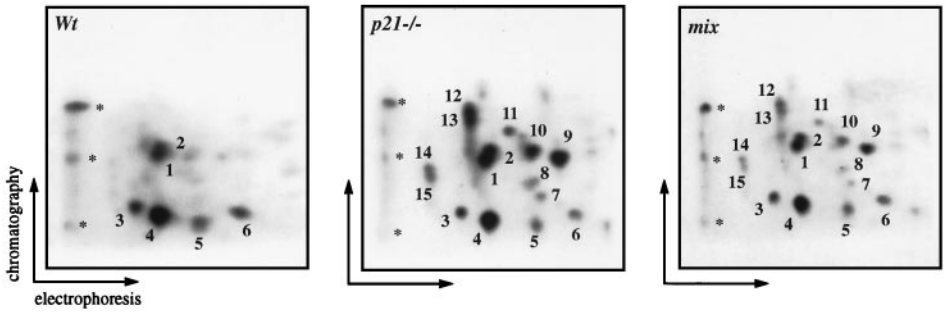

FIG. 3. Two-dimensional phosphotryptic mapping of pRB derived from wild-type or *p21−/−* irradiated cells. pRB was immunoprecipitated from either wild-type or *p21−/−* irradiated cells and subjected to trypsin digestion. The resulting phosphopeptides were resolved by electrophoresis and ascending chromatography and visualized by autoradiography. * denotes phosphopeptides that were present in maps from *Rb−/−* cells and therefore are not derived from pRB.

therefore must involve the phosphorylation of a novel set of sites. By extension of this logic, we conclude that the *p21−/−* cells must contain at least two pRB kinases with different site specificities: one that is similarly active in wild-type and *p21−/−* irradiated cells and results in phosphorylation of a subset of pRB sites (phosphopeptides 1–6) and one that is exclusively active in *p21−/−* irradiated cells and results in the hyperphosphorylation of pRB through the specific modification of a distinct set of sites (phosphopeptides 7–15).

Our kinase assays (Fig. 2*A*) showed that cdk4 activity was present at similar levels in both wild-type and *p21−/−* irradiated cells, but cdk2 activity was present only in *p21−/−* irradiated cells. This finding suggested that cdk4 accounts for the partial phosphorylation of pRB (phosphopeptides 1–6), whereas cdk2 is responsible for phosphorylating the second set of sites that switch pRB from the partially to the hyperphosphorylated form (phosphopeptides 7–15). To test this hypothesis, we used a panel of antibodies that specifically recognize individual pRB phosphopeptides that are preferentially phosphorylated by either cyclin D·cdk4 or cyclin E·cdk2 *in vitro* (35). These antibodies were used to screen immunoblots of pRB immunoprecipitates from either wild-type or *p21−/−* irradiated cells (Fig. 4). Cyclin D·cdk4 is known to specifically phosphorylate Ser-780 of human pRB with a 20- to 60-fold higher efficiency than either cyclin E·cdk2 or cyclin A·cdk2 (35). Antibodies specific for phospho-Ser-780 recognized both the partially and the hyperphosphorylated form of pRB, indicating that cdk4 contributes to the phosphorylation of both of these species (Fig. 4). In contrast, an antibody directed against a cdk2-specific pRB site, α-phospho-Ser-811 of human pRB (ref. 12 and Y.T., unpublished data), recognized the hyperphosphorylated form of pRB in *p21−/−* irradiated cells but failed to detect the partially phosphorylated pRB species in either wild-type or *p21−/−* irradiated cells (Fig. 4). Similar results were obtained with a second cdk2-specific phosphorylation site antibody, α-phospho-Thr-350 (Fig. 4). Taken together, these data indicate that cdk4 is responsible for the partial phosphorylation of pRB *in vivo*, but active cdk2 is required to phosphorylate the additional sites specifically modified in hyperphosphorylated pRB. Most importantly, our data suggest that the p53 → p21 pathway arrests cells in response to low doses of γ-irradiation by specifically inhibiting cdk2 and thereby preventing the transition of pRB from the partially phosphorylated to the hyperphosphorylated form. Given the requirement of pRB for the integrity of the G_1 arrest, we conclude that partially phosphorylated pRB is essential for this growth arrest.

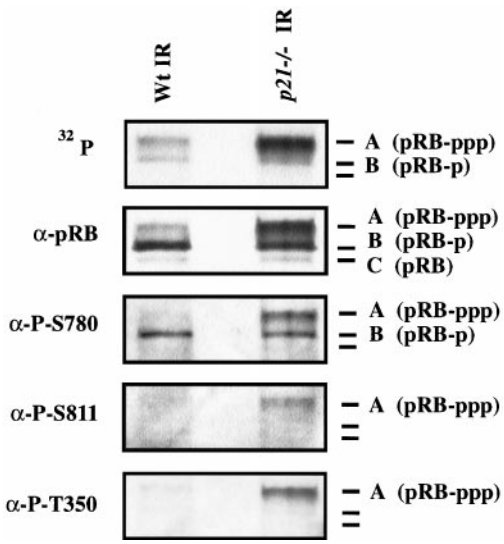

FIG. 4. Phosphorylation status of cdk2-specific and cdk4-specific pRB phosphorylation sites. Immunoblot analysis of immunporecipitated pRB with α-P-S780 (cdk4 site), α-P-S811 (cdk2 site), and α-P-T350 (cdk2 site).

DISCUSSION

The ability of cells to arrest in G_1 in response to DNA damage depends on the accumulation of the tumor suppressor, p53 (20). This p53-dependent G_1 arrest largely is mediated through the induction of the cdk inhibitor p21 (26, 27). By analyzing primary cell lines lacking specific cell cycle regulators, we have investigated the mechanism by which p21 brings about a G_1 arrest after treatment with low-dose γ-irradiation. Our data indicate that the induction of p21 results in the specific inhibition of cdk2, but not cdk4, activity, and that the consequent G_1 arrest depends on the presence of functional pRB. This dependence is consistent with a recent report by Harrington *et al.* (39) that demonstrates a requirement for *Rb* in G_1 arrest induced by a variety of DNA damaging agents.

As pRB is known to regulate the expression and activity of a number of proteins that play central roles in cell cycle control, pRB has the potential to affect the G_1 arrest pathway through multiple mechanisms. However, our biochemical data suggest that the genetic dependence of this pathway upon *Rb* arises primarily from the ability of p21 to block cdk2-mediated phosphorylation of pRB. The absence of pRB does not affect the ability of p21 to down-regulate cdk2 in response to γ-irradiation (see Fig. 2*A*). This down-regulation results in a dramatic p21-dependent alteration in the phosphorylation status of pRB, which reflects the loss of phosphorylation at cdk2-specific, but not cdk4-specific, sites. These observations strongly suggest that pRB acts as a downstream target of the activity of p21. We therefore favor a model in which p21 acts to impose G_1 arrest by specifically inhibiting cdk2 and thereby preventing inactivation of the growth suppressive properties of the pRB tumor suppressor (Fig. 5). In addition, it is apparent

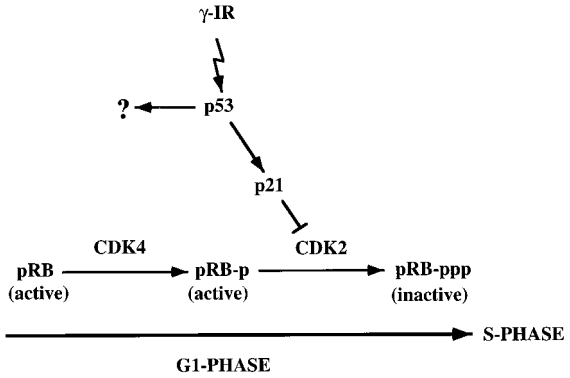

FIG. 5. Model of DNA damage-induced G_1 arrest.

that loss of *Rb* results in a small increase in cdk2-associated activity (see Fig. 2). This deregulation of cdk2 activity may further compromise the ability of the p21-dependent G_1 arrest pathway to down-modulate cdk2 activity in response to DNA damage. As both a target and a regulator of this pathway, pRB plays a critical role in determining whether or not a cell will initiate DNA replication in the presence of damaged DNA.

The proposed role of p21 in this pathway raises important questions regarding the mechanism through which p21 brings about cell cycle arrest. Ample evidence suggests that p21 protein is capable of inhibiting both cdk2- and cdk4-associated activity (23–25). However, our analysis of cdk2 and cdk4 activities in the context of DNA damage-induced G_1 arrest is consistent with the specific p21-dependent inhibition of cdk2, but not cdk4. Indeed, previous work has shown that much of the p21 protein induced by UV irradiation associates with cdk2 and not with cdk4 and that elevated levels of p21 alone are insufficient to inhibit cdk4 (38). This hypothesis does not rule out the possibility that p21 induction is necessary for DNA damage-induced inhibition of cdk4, or that elevation of p21 levels past a threshold may become sufficient to block cdk4 activity. However, our data do reveal a level of p21-target specificity that has significant consequences for the mechanism of DNA damage-induced G_1 arrest and imply that cdk2-specific inhibition is sufficient to produce a p21-dependent G_1 arrest. Clearly, additional study is required to determine the extent to which such specificity underlies the biological properties of p21.

Our observations also provide considerable insight into the role of pRB in normal cell cycle control (see Fig. 5). Recent studies suggest that pRB is phosphorylated in a two-step process during the normal cell cycle (16, 17, 35). Cyclin D·cdk4/6 specifically phosphorylates pRB at a subset of its phosphorylation sites. However, complete phosphorylation of pRB requires cyclin E·cdk2 to specifically target the remaining phosphorylation sites. In both of these studies, inhibition of cdk2 resulted in a G_1 block that correlates with inhibition of the second step of pRB phosphorylation (16, 17). Our current data are entirely consistent with the notion that cyclin D·cdk4/6 and cyclin E·cdk2 mediate the sequential phosphorylation of pRB through the phosphorylation of distinct subsets of sites within this protein. These findings further indicate that the p53 → p21 checkpoint pathway is able to impose a G_1 block by specifically inhibiting cdk2 activity and thereby only the second step of pRB phosphorylation. These findings do not rule out the possibility that there are other cdk2 substrates whose phosphorylation is critical for S-phase entry. Indeed, Serrano *et al.* (40) have shown that overexpression of p21 can inhibit cell cycle entry in an pRB-negative tumor cell line. However, our data strongly suggest that partially phosphorylated pRB retains the ability to prevent cell cycle progression. By extension of this logic, cyclin D·cdk4-dependent phosphorylation is insufficient to inactivate the growth suppressive properties of pRB. Although these conclusions were derived from the analysis of the DNA damage response, it seems highly likely that the same mechanisms will control the timing of S-phase entry in the normal cell cycle.

This model raises clear questions about the role of cyclin D·cdk4 in the regulation of pRB. It is possible that this kinase affects a pRB function that is unrelated to the control of cell cycle entry. However, there is extensive data to suggest that cyclin D·cdk4 plays a critical role in overriding the growth suppressive properties of pRB (8, 10, 14, 15). Alternatively, the inactivation of pRB by cyclin E·cdk2 may depend entirely on the prior phosphorylation of pRB by cyclin D·cdk4. This mechanism would provide two distinct points at which extracellular signals and/or checkpoint pathways could influence the state of pRB phosphorylation and therefore the cell division process. Significantly, very high doses of irradiation can result in the inhibition of both cdk2 and cdk4 activity (37, 41). Together with our data, this observation suggests that the DNA damage checkpoint can block pRB phosphorylation by specifically inhibiting either one (cyclin E·cdk2) or both (cyclin D·cdk4 and cyclin E·cdk2) of the pRB kinases depending on the severity of the DNA damage. The mechanism of inactivation of cyclin D·cdk4 in this response has yet to be established. However, it is now clear that the modulation of the site-specific phosphorylation state of pRB is a critical control point in both normal cell cycle regulation and the DNA damage checkpoint.

Our observation that pRB is a key component of the p53-dependent G_1 arrest also may help to explain how chemotherapeutic agents target tumor versus normal cells. Many antineoplastic treatments cause DNA damage that results in the activation of p53. Significantly, these events have a differential effect on normal and tumor cells; the tumor cells are more likely to undergo p53-dependent apoptosis whereas their normal cellular counterparts preferentially activate the p53-dependent G_1 arrest pathway (42). Given the high frequency of pRB inactivation in human tumors (reviewed in ref. 1), our data suggest that the propensity of tumor cells to undergo p53-dependent apoptosis could arise from their inability to enforce a pRB-dependent cell cycle arrest. This hypothesis is directly supported by the observation that certain antineoplastic drugs cause *Rb*+/+ and *Rb*+/− fibroblasts to arrest but induce *Rb*−/− fibroblasts to apoptose (43). Taken together, these findings suggest that the clinical efficacy of chemotherapeutic agents will be influenced by both the *p53* and *pRB* status of the target tumor.

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