p16\textsuperscript{INK4A} Participates in a G\textsubscript{1} Arrest Checkpoint in Response to DNA Damage

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Members of the INK4 protein family specifically inhibit cyclin-dependent kinase 4 (cdk4) and cdk6-mediated phosphorylation of the retinoblastoma susceptibility gene product (Rb). p16\textsuperscript{INK4A}, a prototypic INK4 protein, has been identified as a tumor suppressor in many human cancers. Inactivation of p16\textsuperscript{INK4A} in tumors expressing wild-type Rb is thought to be required in order for many malignant cell types to enter S phase efficiently or to escape senescence. Here, we demonstrate another mechanism of tumor suppression by implicating p16\textsuperscript{INK4A} in a G\textsubscript{1} arrest checkpoint in response to DNA damage. Calu-1 non-small cell lung cancer cells, which retain Rb and lack p53, do not arrest in G\textsubscript{1} following DNA damage. However, engineered expression of p16\textsuperscript{INK4A} at levels compatible with cell proliferation restores a G\textsubscript{1} arrest checkpoint in response to treatment with \gamma-irradiation, topoisomerase I and II inhibitors, and cisplatin. A similar checkpoint can be demonstrated in p53\textsuperscript{-/-} fibroblasts that express p16\textsuperscript{INK4A}. DNA damage-induced G\textsubscript{1} arrest, which requires the expression of pocket proteins such as Rb, can be abrogated by overexpression of cdk4, kinase-inactive cdk4 variants capable of sequestering p16\textsuperscript{INK4A}, or a cdk4 variant incapable of binding p16\textsuperscript{INK4A}. After exposure to DNA-damaging agents, there was no change either in overall levels of p16\textsuperscript{INK4A} found in complex with cdk4 and 6. Nonetheless, p16\textsuperscript{INK4A} expression is required for the reduction in cdk4- and cdk6-mediated Rb kinase activity observed in response to DNA damage. During tumor progression, loss of p16\textsuperscript{INK4A} expression may be necessary for cells with wild-type Rb to bypass this G\textsubscript{1} arrest checkpoint and attain a fully transformed phenotype.

Another possibility is that p16\textsuperscript{INK4A} plays a role in the maintenance of genome integrity (34). Frequently, following DNA damage normal cells arrest their proliferation at cell cycle checkpoints, the most prominent of which occur at the G\textsubscript{1}-S and G\textsubscript{2}-M boundaries. Arrest allows time for repair prior to continued cell cycle progression. One G\textsubscript{1} arrest checkpoint is controlled by p53 (5, 18). In response to DNA damage, p53 levels increase by a posttranscriptional mechanism, resulting in the transcriptional activation of p21\textsuperscript{WAF1}, a universal inhibitor of cyclin-dependent kinases, which can mediate G\textsubscript{1} arrest (6, 11, 42). Inactivation of p53 is the most common genetic event in human cancer, suggesting that loss of a DNA damage-induced G\textsubscript{1} checkpoint is an essential step in tumor progression. This allows damaged DNA to be replicated, which leads to the accumulation of additional mutations and the eventual emergence of a malignant clone.

DNA damage also induces alterations in cyclin D1-cdk4 activity. For example, UV irradiation can lead to decreases in cyclin D1 levels and to inhibitory phosphorylation of cdk4 (24, 27, 37). Such perturbations may contribute to G\textsubscript{1} arrest following DNA damage. In the present study, we have investigated whether p16\textsuperscript{INK4A} may also be involved in the response to DNA damage. We have used non-small cell lung cancer (NSCLC) cells, which lack p53 and do not arrest in G\textsubscript{1} following DNA damage. When these cells are engineered to express p16\textsuperscript{INK4A} at levels compatible with proliferation, the ability to arrest in G\textsubscript{1} in response to DNA damage is restored. Furthermore, we demonstrate that p53\textsuperscript{-/-} fibroblasts maintain a similar G\textsubscript{1} arrest checkpoint in response to DNA damage, which correlates with the level of p16\textsuperscript{INK4A} they express. Although neither overall p16\textsuperscript{INK4A} levels nor the amount complexed to cdk4 and cdk6 changes following DNA damage, the presence

p16\textsuperscript{INK4A} is a specific inhibitor of cyclin dependent kinase 4 (cdk4) and cdk6, which participate in the cyclin D-dependent phosphorylation of the retinoblastoma susceptibility gene product, Rb (31). Hyperphosphorylation of Rb inactivates its growth-suppressive properties, allowing cells to enter S phase. Several lines of evidence indicate that p16\textsuperscript{INK4A} is a tumor suppressor. First, its gene maps to 9p21, a chromosomal locus deranged in many human cancers (15). Second, p16\textsuperscript{INK4A} is commonly deleted, mutated, or hypermethylated and transcriptionally silenced in tumors that retain wild-type Rb, and ectopic expression of p16\textsuperscript{INK4A} in these cells at high levels results in G\textsubscript{1} arrest (17, 19, 22, 30, 33, 35). Furthermore, p16\textsuperscript{INK4A} deficient mice are susceptible to several types of malignancies (32), and germ line mutations of p16\textsuperscript{INK4A} in humans are associated with familial syndromes involving malignant melanoma and pancreatic cancer (8, 14, 16, 40).

The precise mechanism by which p16\textsuperscript{INK4A} exerts its tumor-suppressive effects is less clear. One straightforward suggestion is that inactivation of p16\textsuperscript{INK4A} is required for malignant cells to enter S phase efficiently. However, many normal cells express p16\textsuperscript{INK4A} throughout G\textsubscript{1} and are able to proliferate, suggesting that other mechanisms of tumor suppression must be operating. An alternative mechanism involves the recently identified link between p16\textsuperscript{INK4A} expression and cellular senescence (1, 10, 28, 32). As fibroblasts or epithelial cells age, p16\textsuperscript{INK4A} levels increase dramatically, and it has been proposed that loss of p16\textsuperscript{INK4A} expression is required for cells to escape senescence during their progression to malignancy.

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of p16INK4A causes a decrease in cdk4- and cdk6-mediated Rb kinase activity and results in G1 arrest, even in the absence of p53.

MATERIALS AND METHODS

Cell lines. Calcium phosphate precipitation (4) was used to transfect BING packaging cells (provided by Warren Pear, Massachusetts Institute of Technology, Cambridge, Mass.) with pBPSTR1 (25) or pBPSTR1 into which a cDNA encoding human p16INK4A had been cloned. A To the left is shown an immunoblot of p16INK4A and Rb in a mass population of Calu-1 cells infected with pBPSTR1 and in three pBPSTR1-p16INK4A clones (clones 6, 14, and 18) in the presence (+) and absence (−) of tetracycline. To the right is shown an immunoblot analysis of p16INK4A expression in three pBPSTR1-p16INK4A clones in the absence of tetracycline compared to extracts from WI38 diploid human fibroblasts and NHBE cells. Blots were stripped and reprobed for proliferating-cell nuclear antigen as a loading control. B p16INK4A-expressing clones were radioiodinated with [35S]methionine, and extracts were subjected to immune precipitation with anti-p16INK4A. Immune precipitates were dissociated (33, 38), divided into three aliquots, and precipitated separately with anti-p16INK4A, anti-cdk4, and anti-cdk6. SDS-PAGE and autoradiographic analysis of these second precipitates demonstrate that cdk4 and cdk6 were present in the initial p16INK4A immune precipitates.

Immune precipitations. Cells were metabolically radiolabeled with [35S]cysteine and [35S]methionine, and lysed in Nonidet P-40 (NP-40)-containing lysis buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 1.0% NP-40, and 1 mM phenylmethylsulfonyl fluoride). Lysate from a 10-cm-diameter plate was subjected to immune precipitation using an anti-p16INK4A monoclonal antibody (ZJ11) raised against a glutathione S-transferase (GST)–p16INK4A fusion protein (a gift from James DeCaprio, Dana-Farber Cancer Institute) or with rabbit antisera raised against peptides derived from the C-terminal domains of cdk4 (Clontech, Palo Alto, Calif.) or cdk6 (Santa Cruz Biotechnology, Santa Cruz, Calif.). Double immune precipitations for p16INK4A, cdk4, and cdk6 were performed as described previously (33, 38). For immune depletion experiments, lysates were subjected to five rounds of immune precipitation using ZJ11 prior to analysis.

Immune blotting. Cells were lysed in cold NP-40-containing lysis buffer, and 100 to 150 µg of cellular protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was electrophoretically transferred to Immobilon-P membranes (Millipore, Danvers, Mass.) in 10 mM 3-(cyclohexylamino)-1-propane sulfonic acid (pH 11) and 15% methanol. Nonspecific binding sites were blocked by incubating the membrane in Tris-buffered saline (TBS)–10% nonfat dried milk. Primary antibody incubations were carried out in TBS–1% milk with the following: anti-human p16INK4A, anti-murine p16INK4A, and anti-cdk4 (Santa Cruz Biotechnology), anti-Rb (clone G3-245; PharMingen, San Diego, Calif.), anti-p21WAF1 (Oncogene Research Products, Cambridge, Mass.), anti-IA (BAbCO, Richmond, Calif.), anti-E7 (a gift from James DeCaprio, Dana-Farber Cancer Institute), and anti-cyclin D1 (Neomarkers, Fremont, Calif., or Upstate Biotechnology, Inc., Lake Placid, N.Y.). After being washed membranes were incubated in horseradish peroxidase-conjugated secondary antibodies and developed with enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

DNA damage treatments. Prior to treatment, Calu-1 cells were plated at 104 cells/ml and fibroblasts from p53−/− mice were plated at 5 × 105 cells/ml. In both cases, this provided cultures that were 50 to 70% confluent at the time of treatment. Twenty-four hours after plating, Calu-1 cells were treated with the following concentrations of chemotherapeutic agents for 48 h: 0.035 mM adriamycin (ADR), 2.5 µM etoposide, 5 µM camptothecin, and 2.6 µg/ml cisplatin. In some experiments, cells were cultured for an additional 36 h in the same concentration of drug along with nocodazole (0.4 µg/ml). For γ-irradiation of Calu-1 cells, 10 Gy of irradiation was delivered by a 137Cs source at 118 cGy/min. This dose was adequate to induce a G2 arrest in parental Calu-1 cells. Twenty-four hours after plating, fibroblasts from p53−/− mice were treated for 24 h with ADR (0.033 µg/ml), etoposide (2 µM), and cisplatin (1 µg/ml). In some experiments, cells were cultured in the same concentration of drug along with nocodazole (0.4 µg/ml) for an additional 16 h. For γ-irradiation, cells were treated with 20 Gy of irradiation. This dose was required in order to induce a G2 arrest in late-passage p53−/− embryo fibroblasts.

Fluorescence-activated cell sorting (FACS) analysis. Cells were collected by trypsinization, washed, and resuspended in 1 ml phosphate-buffered saline. An additional 1 ml of 80% ethanol was added, and cells were fixed overnight at 4°C. Fixed cells were centrifuged and resuspended in 0.5 ml of 500-µg/ml RNase A and incubated for 45 min at 37°C. Cells were centrifuged and resuspended in 0.5 ml of 60 µM propidium iodide in 38 mM sodium citrate and incubated at room temperature for a minimum of 30 min. Cells were then analyzed for DNA content by flow cytometry (Becton Dickinson, Hialeah, Fla.).

FIG. 1. Characterization of Calu-1 cells engineered to express p16INK4A. Calu-1 cells were infected with the retrovirus pBPSTR1 or the same retrovirus into which a cDNA encoding human p16INK4A had been cloned. A To the left is shown an immunoblot of p16INK4A and Rb in a mass population of Calu-1 cells infected with pBPSTR1 and in three pBPSTR1-p16INK4A clones (clones 6, 14, and 18) in the presence (+) and absence (−) of tetracycline. To the right is shown an immunoblot analysis of p16INK4A expression in three pBPSTR1-p16INK4A clones in the absence of tetracycline compared to extracts from WI38 diploid human fibroblasts and NHBE cells. Blots were stripped and reprobed for proliferating-cell nuclear antigen as a loading control. B (p16INK4A-expressing clones were radioiodinated with [35S]methionine, and extracts were subjected to immune precipitation with anti-p16INK4A. Immune precipitates were dissociated (33, 38), divided into three aliquots, and precipitated separately with anti-p16INK4A, anti-cdk4, and anti-cdk6. SDS-PAGE and autoradiographic analysis of these second precipitates demonstrate that cdk4 and cdk6 were present in the initial p16INK4A immune precipitates.

FIG. 2. Growth curves. Calu-1 cells were plated at 104 cells/10-cm-diameter dish and counted daily. Solid symbols, presence of tetracycline; open symbols, absence of tetracycline; ■ and ▲, mass culture of Calu-1 cells infected with pBPSTR1; ● and ○, clone 6; □ and ○, clone 14; A and △, clone 18.
Rb kinase assays. Cells were lysed for 1 h at 4°C in 50 mM HEPES (pH 7.2)–150 mM NaCl–1 mM EDTA–2.5 mM EGTA–1 mM dithiothreitol–0.1% Tween 20 supplemented with 10% glycerol–1 mM NaF–0.5 mM sodium orthovanadate–aprotinin (1 μg/ml)–leupeptin (1 μg/ml)–10 mM β-glycerophosphate–phenylmethylsulfonyl fluoride (100 μg/ml). Lysates were clarified by centrifugation at 10,000×g for 10 min, and the supernatants were precleared with rabbit immunoglobulin G prior to immune precipitation using 300 ng each of rabbit antisera against cdk4 and cdk6 (described above). Immune precipitates were collected by using protein A-Sepharose beads equilibrated with lysis buffer containing 4% bovine serum albumin. Beads were washed four times with lysis buffer and then twice with kinase buffer containing 50 mM HEPES (pH 7.2), 10 mM MgCl₂, 5 mM MnCl₂, and 1 mM dithiothreitol. After the final wash, 25 μl
TABLE 1. G1 arrest in p16INK4A-expressing Calu-1 cells in response to γ-irradiation

| h after irradiation | % G1 DNA content* | pBPSTR1 | Clone 6 |
|---------------------|-------------------|---------|---------|
| 0                   | 52.4              | 52.4    |         |
| 4                   | 36.2              | 47.6    |         |
| 8                   | 22.0              | 39.6    |         |
| 12                  | 5.1               | 35.3    |         |
| 16                  | 4.6               | 34.1    |         |
| 18                  | 3.1               | 30.9    |         |

* A mass population of Calu-1 cells infected with a control retrovirus (pBSTR1) or a clone (clone 6) of Calu-1 cells infected with a retrovirus expressing p16INK4A were treated with 10 Gy of irradiation. At the indicated times, cells were collected and DNA content was determined by FACS analysis. Similar results were obtained with two additional p16INK4A-expressing clones. The same proportions of G1 DNA were obtained in the presence and absence of nocodazole at 18 h after treatment.

of kinase reaction mix was added, consisting of kinase buffer with 20 µM ATP, 10 µCi of [γ-32P]ATP, and 1 µg of GST-Rb ( amino acids 792 to 928). Samples were incubated at 37°C for 30 min with occasional mixing, boiled in SDS-PAGE sample buffer, and fractionated by electrophoresis through 10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose, and phosphorylated GST-Rb was visualized by autoradiography. The nitrocellulose filter was then subjected to Western blotting using cdk4- and cdk6-specific antibodies, to ensure that equivalent amounts had been immune precipitated for each kinase assay.

To prepare the GST-Rb substrate, a culture of Escherichia coli transformed with pGEX-Rb (792-928) was induced with IPTG (isopropyl-β-d-thiogalactopyranoside) and lysed as previously described (21). Fusion protein was captured on glutathione-Sepharose 4B and released by incubation with reduced glutathione. The concentration of the soluble fusion protein was estimated by Coomassie blue staining of electrophoresed protein in comparison to protein standards of known concentration.

RESULTS

Engineered expression of p16INK4A in Calu-1 cells. Calu-1 cells are NSCLC cells which have wild-type Rb, deleted p53, and transcriptionally silent and hypermethylated p16INK4A loci (3, 33). Ectopic expression of p16INK4A at high levels in these and other cell types that retain wild-type Rb produces G1 arrest (17, 19, 22, 30, 33, 35). In the present experiments, we generated Calu-1 cells expressing levels of p16INK4A compatible with proliferation by infection with a tetracycline-suppressible retroviral vector, pBPSTR1 (25), encoding human p16INK4A (31). Figure 1A shows that several independently isolated clones expressed p16INK4A in the presence of tetracycline and that expression could be enhanced three- to fourfold by tetracycline withdrawal. Levels of p16INK4A expression in the absence of tetracycline were comparable to those seen in WI38 diploid human fibroblasts and somewhat higher than those seen in normal human bronchial epithelial (NHBE) cells (Fig. 1A). Levels of p16INK4A expressed by engineered Calu-1 cells in the presence of tetracycline were similar to those in NHBE cells. Although ectopically expressed p16INK4A bound to cdk4 and cdk6 (Fig. 1B), the levels of p16INK4A expressed in these cells did not affect growth rates (Fig. 2). Immune depletion of p16INK4A from extracts of these cells revealed populations of free cdk4 and cdk6 (see Fig. 10), and depletion of these cdk4 and cdk6 were present in excess over p16INK4A in these cells, resulting in sufficient Rb phosphorylation to allow S-phase entry.

Response of p16INK4A-expressing Calu-1 cells to DNA-damaging agents. When control Calu-1 cells that do not express p16INK4A were treated with ADR or etoposide for 48 h, nearly all cells arrested in G2 (Fig. 3A). On average, 3% of these cells had a G1 DNA content after ADR treatment, and 10% had a G1 DNA content after etoposide treatment (Fig. 3B). In contrast, cells expressing p16INK4A responded to ADR or etoposide treatment by growth arrest with a significantly larger proportion of cells in G1 (Fig. 3A). After ADR treatment, on average, 12 and 27% of cells had a G1 DNA content in the presence and absence of tetracycline, respectively (Fig. 3B). After etoposide treatment, the G1 DNA proportions were 21 and 32% in the presence and absence of tetracycline, respectively. Other chemotherapeutic agents, such as camptothecin and cisplatin (Fig. 4), as well as γ-irradiation (Fig. 4 and Table 1) also induced G1 arrest in Calu-1 cells only when they expressed p16INK4A.

The response to DNA-damaging agents was a true cell cycle arrest since the proportion of cells in S phase decreased from 30% in untreated cells to 5 to 10% after treatment, and the G1 arrest was stable during continued culture in the presence of nocodazole (Fig. 5A). In addition, the G1 arrest was reversible since arrested cells reentered S phase after cessation of DNA-damaging treatments (Fig. 5B). Thus the G1 arrest had the characteristics of a checkpoint response.

Response of p53−/− fibroblasts to DNA damaging agents. To ensure that the results observed with Calu-1 cells were not restricted to an engineered cell line, we performed similar experiments on fibroblasts derived from p53−/− mice. Figure

FIG. 5. Stability of G1 arrest in the presence and absence of nocodazole. (A) Calu-1 cells infected with pBPSTR1 alone show a shift from a normal DNA profile for exponentially growing cells to a G2 arrest after 36 h of treatment with nocodazole. p16INK4A-expressing clones (clone 14) show a G1 arrest response after ADR or etoposide treatment (as in A) which does not change after an additional 36 h of nocodazole treatment. Similar results were observed with the other p16INK4A-expressing clones. (B) Calu-1 cells expressing p16INK4A (clone 14) in the absence of tetracycline were induced to arrest in G1 with ADR or γ-irradiation, resulting in less than 10% of these cells having an S-phase DNA content. FACS analysis performed 18 h after withdrawal of the DNA-damaging treatment shows that cells have reentered the cycle, with more than 20% having an S-phase DNA content.
6A shows that early-passage (passage 5) fibroblasts expressed p16\(^{INK4A}\) and that levels of expression decreased with continued passage. Because of the absence of p53 in these cells, the expectation was that a G\(_1\) arrest checkpoint in response to DNA damage would be absent. Surprisingly, however, Fig. 6B shows that these cells have a persistent G\(_1\) arrest response. The proportion of cells arresting in G\(_1\) in response to \(\gamma\)-irradiation, ADR, etoposide, or cisplatin correlated with the amount of p16\(^{INK4A}\) expressed, so that DNA damage induced a significant proportion of early- but not late-passage cells to arrest in G\(_1\).

The G\(_1\) arrest in p16\(^{INK4A}\)-expressing early-passage fibroblasts was stable during continued culture in the presence of nocodazole (Fig. 6B). Intermediate-passage cells expressed intermediate levels of p16\(^{INK4A}\) and the proportion of cells arresting in G\(_1\) in response to DNA-damaging agents correlated with these levels of expression (data not shown). In addition, similar to Calu-1 cells, engineered expression of p16\(^{INK4A}\) in late-passage cells at levels compatible with cell proliferation restored a G\(_1\) arrest response to DNA damage (Fig. 7).

**Effect of cdk4 variants and HPV E7 expression on the G\(_1\) arrest response.** To confirm that the G\(_1\) arrest in Calu-1 cells depended on p16\(^{INK4A}\), we expressed a cdk4 variant in which lysine-35 is replaced by methionine (20) (Fig. 8A). This variant has no kinase activity but binds p16\(^{INK4A}\) efficiently (30), and when overexpressed, it sequesters p16\(^{INK4A}\), thereby preventing its interaction with endogenous cdk4. Figure 9 shows that expression of cdk4\(^{K35M}\) reversed the G\(_1\) arrest response in p16\(^{INK4A}\)-expressing Calu-1 cells. A similar result was obtained by expressing cdk4\(^{D185N}\), another kinase-defective variant that may also sequester p16\(^{INK4A}\) (39) (Fig. 8A and 9).

These results suggest that kinase-defective cdk4 variants prevent p16\(^{INK4A}\) from inhibiting endogenous cdk4, thereby permitting Rb phosphorylation and reversal of the G\(_1\) arrest response to DNA damage. Consistent with this model, overexpression of wild-type cdk4 overcame the G\(_1\) arrest response in p16\(^{INK4A}\)-expressing cells (Fig. 8A and 9). This was further confirmed by the ability of another cdk4 variant, cdk4\(^{R24C}\) to reverse the G\(_1\) arrest response (Fig. 8A and 9). This variant has
full kinase activity but does not bind and is therefore not inhibited by p16\(^{INK4A}\) (41). Finally, the G\(_1\) arrest response was abrogated by expressing the HPV E7 protein, which inactivates pocket proteins (Fig. 8B and 9). Thus, the p16\(^{INK4A}\)-mediated G\(_1\) arrest in response to ADR is dependent on p16\(^{INK4A}\) itself and pocket proteins such as Rb and further depends on inhibition of cdk4.

Effect of DNA-damaging agents on cell cycle protein expression and cdk activity. G\(_1\) arrest following DNA damage involves p53-dependent and -independent events. In cells with wild-type p53, DNA damage results in increased levels of p53 (5, 18), which induce increased expression of the non-INK4 cdk inhibitor, p21\(^{WAF1}\) (6), leading to G\(_1\) arrest (6, 11, 42). Figure 10A shows that levels of p21\(^{WAF1}\) did not rise following ADR or etoposide treatment, indicating that the G\(_1\) arrest response was not due to p53-independent recruitment of the p21\(^{WAF1}\) pathway. Similarly, there was no change in levels of ectopic p16\(^{INK4A}\) in Calu-1 cells (Fig. 10A), in levels of endogenous p16\(^{INK4A}\) in mouse embryo fibroblasts (Fig. 10B), or in levels of cdk4 in both cell types (Fig. 10A and 11B) following DNA damage. In addition, DNA damage did not increase the amount of p16\(^{INK4A}\) complexed to cdk4 and cdk6 (Fig. 10C).

In contrast, ADR treatment and \(\gamma\)-irradiation routinely produced lower levels of cyclin D1 in engineered Calu-1 cells and in mouse embryo fibroblasts whether or not they expressed p16\(^{INK4A}\) (Fig. 11). In the absence of p16\(^{INK4A}\), this decrease was not associated with a measurable reduction in cdk4- or cdk6-mediated Rb kinase activity. However, in the presence of p16\(^{INK4A}\), kinase activity was reduced (Fig. 12).

**DISCUSSION**

Our data implicate p16\(^{INK4A}\) in a G\(_1\) arrest checkpoint in response to DNA damage. We demonstrated this first in Calu-1 NSCLC cells, which express wild-type Rb but do not express p53 or p16\(^{INK4A}\) and undergo cell cycle arrest in G\(_1\) in response to DNA damage. Although high levels of ectopic p16\(^{INK4A}\) expression in these cells produce G\(_1\) arrest (33), the levels engineered in the present experiments were compatible with cell proliferation, presumably because cdk4 and cdk6 were in stoichiometric excess compared to p16\(^{INK4A}\) as demonstrated by depletion experiments. While these levels of p16\(^{INK4A}\) expression did not inhibit cell proliferation, they did result in G\(_1\) arrest in response to a wide variety of DNA-damaging agents that exert their effects in different ways. In addition, the proportion of cells arrested in G\(_1\) correlated with the amount of p16\(^{INK4A}\) expressed by the cell population.

The G\(_1\) arrest response in these cells depended on expression of p16\(^{INK4A}\) since it was abrogated by kinase-defective cdk4 variants that sequester p16\(^{INK4A}\). These variants prevent p16\(^{INK4A}\) from inhibiting endogenous cdk4, thereby allowing cells to proceed through G\(_1\) into S phase. Overexpression of wild-type cdk4 achieved a similar result, again most likely due to p16\(^{INK4A}\) sequestration. Furthermore, G\(_1\) arrest was reversed by a cdk4 variant incapable of binding p16\(^{INK4A}\) and by the HPV E7 protein, indicating that the G\(_1\) arrest response was dependent on the ability of p16\(^{INK4A}\) to inhibit cdk4 activity in a cell with active pocket proteins, such as Rb.

The G\(_1\) arrest response induced by DNA damage was accompanied by decreases in cyclin D1 levels. Although Calu-1 cells engineered to express low levels of p16\(^{INK4A}\) proliferated like their parental cells, it is possible that this amount of p16\(^{INK4A}\) artificially sensitized them to decreases in cyclin D1 and that p16\(^{INK4A}\) is not normally involved in this DNA damage checkpoint. This is unlikely to be the case for several reasons. First, the amount of p16\(^{INK4A}\) expressed by engineered Calu-1 cells was similar to that expressed by WI38 diploid fibroblasts and NHBE cells, indicating that the levels of
expression approximated physiological levels seen in nontransformed cells in culture.

In addition, our results were not confined to cell lines in which p16INK4A was ectopically expressed but were also observed in primary embryo fibroblasts from p53^2/2 mice. We found that early-passage cells expressed high levels of p16INK4A and underwent G1 arrest in response to several DNA-damaging agents. This was a surprising result and indicates that DNA damage-induced G1 arrest does not solely depend on the presence of p53 or on events immediately downstream from p53 such as induction of p21^WAF1. Late-passage cells expressed much lower levels of p16INK4A and no longer demonstrated a G1 arrest response after DNA damage.

Other reports on studies using human diploid fibroblasts or mouse embryo fibroblasts from wild-type mice have described progressive increases in p16INK4A levels with continued passage (1, 10, 32, 43). The behavior of the p53^-/- mouse embryo fibroblasts was different in our experiments, although we did not analyze p16INK4A levels at every passage, so increases in p16INK4A prior to a subsequent decrease may have been missed. Nonetheless, as p16INK4A expression diminished, the proportion of cells arresting in G1 in response to DNA damage progressively decreased. Admittedly, a variety of other genetic (or epigenetic) alterations which might have influenced the DNA damage-induced G1 arrest response could have occurred in these cells during passage. However, there was a clear correlation between the level of endogenous p16INK4A in these p53^-/- fibroblasts and their degree of G1 arrest in response to DNA-damaging agents.

The precise mechanism by which p16INK4A functions in a G1 arrest checkpoint remains to be elucidated. We have demonstrated that DNA damage does not induce an increase in p16INK4A levels. Instead, we and others have observed significant decreases in levels of cyclin D1 expression following DNA damage (24, 27). While this response also occurred in transformed cells that do not express p16INK4A, it did not result in a diminution of total cdk4 and cdk6 kinase activities as determined by an in vitro kinase assay. In contrast, when transformed cells were engineered to express p16INK4A, DNA damage-mediated decreases in cyclin D1 levels were associated with a significant decrease in cdk4- and cdk6-mediated Rb kinase activity. Thus, although DNA damage does not produce a change in the levels of p16INK4A in cells that express it, its presence is required for a decrease in Rb kinase activity and resultant G1 arrest following DNA damage.

One simple explanation of these findings would be that lower levels of cyclin D1 after DNA damage permitted increased amounts of p16INK4A to bind to cdk4 and cdk6. However, our data indicate that this is not the case. The depletion experiments (Fig. 10) demonstrated that cdk4 are in excess and that there are roughly equivalent amounts of p16INK4A-free cdk4 and cdk6 both before and after exposure to DNA damage. Although we cannot exclude the possibility that subtle quantitative changes in the amount of p16INK4A complexed to target kinases could result in a biological effect, it is clear that dramatic accumulation of p16INK4A in cdk4 or cdk6 complexes does not occur following DNA damage.

Rather, it is likely that p16INK4A plays a passive but essential role in the context of other perturbations that occur in the cyclin D-ccdk4-Rb axis after DNA damage. For example, tyrosine phosphorylation of cdk4 is required in order for cells to arrest in G1 after UV light treatment (37). Therefore, it is possible that a proportion of cdk4 molecules in the cells we examined becomes tyrosine phosphorylated in response to DNA damage. In the absence of p16INK4A, there must be sufficient amounts of active cyclin D-ccdk4 complexes to allow phosphorylation of Rb and bypass of the checkpoint. However, in the presence of p16INK4A, the available cdk4 following DNA damage that is not inhibited by either tyrosine phosphorylation or p16INK4A association may become limiting, leaving insufficient active cyclin D-ccdk4 to allow cell cycle progression. In this scenario, steady-state levels of p16INK4A or the amount of
FIG. 10. Effect of DNA-damaging agents on expression of p16INK4A, p21\(^{WAF1}\), and cdk4 and distribution of p16INK4A. (A) Calu-1 cells infected with pBPSTR1 or clone 6 cells expressing p16\(^{INK4A}\) were grown in the presence or absence of tetracycline (\(+\)tet or \(-\)tet, respectively) and were left untreated (NT) or were treated with ADR (A) or etoposide (E) for 48 h as described in the legend to Fig. 3. Cell lysates were analyzed by immunoblotting for p16\(^{INK4A}\) or were treated with ADR (A) or etoposide (E) for 48 h as described in the legend to Fig. 6, and cell lysates were examined by immunoblot for cyclin D1 expression. In addition to recognizing the primary cyclin D1 protein, the anti-cyclin D1 antibody used in this analysis also recognizes a less abundant protein of higher apparent molecular weight in murine cells which has been shown by protease mapping to be cyclin D1 as well (C). Similar results were observed with a second anti-cyclin D1 antibody. The blots were stripped and reprobed for cdk4 to demonstrate equal loading.

P16\(^{INK4A}\) complexed to cdk4 need not change following DNA damage, but the presence of P16\(^{INK4A}\) is nonetheless essential for G1 arrest to occur.

Another p53-independent contribution to G1 arrest following DNA damage may come from the redistribution of p27\(^{kip1}\). Unlike INK4 family members, which appear to displace D-type cyclins from cdkks, p27\(^{kip1}\) can exist in complex with the cyclin-cdk holoenzyme (26). In growing cells, most p27\(^{kip1}\) is associated with cyclin D1-cdk4 (27). UV irradiation has been reported to reduce the levels of both cyclin D1 and p27\(^{kip1}\), but since the reduction in cyclin D1 is greater, p27\(^{kip1}\) redistributes to cyclin A-cdk2, causing a reduction in cdk2 kinase activity and G1 arrest (27). p27\(^{kip1}\) is readily detectable in the cells we examined, and its levels do not change after DNA damage (data not shown). We have not yet investigated the association of p27\(^{kip1}\) with cyclin-cdk complexes in these cells, but it is tempting to speculate that a more significant redistribution of p27\(^{kip1}\) to cdk2 complexes might occur in the presence of p16\(^{INK4A}\) after DNA damage. If so, this mechanism would again be consistent with a necessary but passive role for p16\(^{INK4A}\) in the G1 arrest response.

The ability of P16\(^{INK4A}\) to participate in G1 arrest following DNA damage depends on its expression during G1. In this respect, p16\(^{INK4A}\) differs from other closely related members of the INK4 family, all of which are potent inhibitors of cdk4 and cdk6. For example, in many cell types, p15\(^{INK4B}\) expression depends on transforming growth factor \(\beta\) treatment (9, 29), while the expression of p16\(^{INK4C}\) and p16\(^{INK4D}\) is cell cycle regulated and restricted to S phase (13). p16\(^{INK4A}\), expression during G1 in a wider variety of normal cell types than other INK4 family members (34, 36) may explain why it is a more frequent target for inactivation. One would predict that any INK4 family member that is expressed during G1 in a given cell type might be a tumor suppressor in that cell. Consistent with

FIG. 11. Effect of DNA-damaging agents on expression of cyclin D1. (A) Calu-1 cells infected with pBPSTR1 or with pBPSTR1-p16\(^{INK4A}\) (clone 6) were left untreated (NT) or were treated with ADR (A) or \(\gamma\)-irradiation (\(\gamma\)) as described in the legend to Fig. 3, and cell lysates were analyzed by immunoblot for cyclin D1 expression. The blots were stripped and reprobed for cdk4 to demonstrate equal loading. (B) Early- and late-passage fibroblasts from p53\(^{-/-}\) mice were untreated or treated with \(\gamma\)-irradiation, ADR, or etoposide (E) as described in the legend to Fig. 6, and cell lysates were examined by immunoblot for cyclin D1 expression.

FIG. 12. Dependence of reduced Rb kinase activity on p16\(^{INK4A}\) expression after DNA damage. Calu-1 cells infected with pBPSTR1 (C) or pBPSTR1-p16\(^{INK4A}\) (clone 14) were untreated (NT) or treated with ADR (A) as described in the legend to Fig. 3. Lysates were subjected to immune precipitation using anti-cdk4 and anti-cdk6 antibodies, and the precipitates were used to phosphorulate GST-Rb in vitro. Kinase assays were analyzed by SDS-PAGE followed by electrophoretic transfer to nitrocellulose. Phosphorylated GST-Rb was detected by exposing the nitrocellulose filter to X-ray film. The presence of equal amounts of cdk4 and cdk6 in the precipitates was then determined by staining the nitrocellulose filter with anti-cdk4 and anti-cdk6 antibodies.
this idea, p15INK4B is expressed during G1 in T lymphocytes and is a tumor suppressor in T-cell leukemias (12, 36).

During tumor progression, loss of the G1 arrest checkpoint in response to DNA damage is essential for the eventual emergence of a malignant clone. Our data indicate that unless p16INK4A activity is lost, the cell types we examined can still arrest in G1 after DNA damage, even in the absence of p53. This may explain why most tumors with wild-type Rb inactivate both p16INK4A and p53 and why the frequency of p16INK4A inactivation in human cancers rivals that of p53. However, while inactivation of p16INK4A in tumors with wild-type Rb is nearly universal, some malignant cells do express wild-type p53. Our data predict that these cells may not have a fully competent G1 arrest checkpoint in response to DNA damage, despite the presence of p53, and that p16INK4A expression may augment this response. We are currently testing this hypothesis in tumor cells that express wild-type p53 and in fibroblasts from p16−/− mice.

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