Differential Effects of p21<sup>WAF1/CIP1</sup> Deficiency on MMTV-ras and MMTV-myc Mammary Tumor Properties

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ABSTRACT

p21<sup>WAF1/CIP1</sup> (p21) functions as a cyclin-dependent kinase (CDK) inhibitor and is a key mediator of p53-dependent growth arrest. However, its role in cell cycle regulation is complex, because it also appears to promote CDK activity in certain experimental contexts. Its potential role in tumor suppression was evaluated in MMTV-ras and MMTV-myc transgenic mice that were interbred to p21<sup>WAF1/CIP1</sup> knockout mice (p21<sup>−/−</sup>). p21 deficiency had differential effects on tumor incidence and age of onset, proliferation, and apoptosis in the presence of these two oncogenes. Tumors arising in MMTV-ras/p21<sup>−/−</sup> mice displayed higher S-phase fractions and correspondingly increased cyclin D1 and E/CDK activity than MMTV-ras tumors. In contrast, MMTV-myc/p21<sup>−/−</sup> tumors had lower S-phase fractions and levels of cyclin D1 and E/CDK activity than MMTV-myc tumors. In both tumor types, changes in cyclin D1 and E/CDK activity were paralleled by changes in the corresponding cyclin protein levels. Tumor cell apoptosis was also differentially influenced by p21 deficiency in the two models. MMTV-ras/p21<sup>−/−</sup> tumors exhibited a significant increase in spontaneous apoptosis as compared with MMTV-ras tumors, whereas p21 deficiency had minimal effect on apoptosis in MMTV-myc tumors. These results indicate that the effects of p21 expression on cellular proliferation are differentially affected by the expression of different oncogenes, and that p21 may play a role in promoting either growth arrest or proliferation, depending on the specific cellular context.

INTRODUCTION

Eukaryotic cell cycle progression is regulated by the sequential activation and inactivation of a series of protein kinase complexes, each consisting of a protein kinase subunit, referred to as a CDK, and an activating cyclin subunit (1). The activity of these complexes is cell cycle regulated by a number of interacting mechanisms, including oscillating synthesis and degradation of the cyclin component and phosphorylation/dephosphorylation of the kinase subunit (2). In addition, the cyclin/CDK complexes are regulated by the binding of a series of inhibitory subunits, referred to as CKIs (3). The CKIs fall into two structurally and functionally distinct families, the INK4 family (consisting of p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup>, and p19<sup>INK4d</sup>) and the CIP/KIP family (including p21<sup>WAF1/CIP1</sup>, p27<sup>KIP1</sup>, and p57<sup>KIP2</sup>). The INK4 family members specifically inhibit the CDK4 and CDK6 G1-phase kinase complexes (4–6). In contrast, the CIP/KIP family members are referred to as universal cyclin/CDK inhibitors, because they can interact with all of the cyclin/CDK complexes (7–9).

Since its initial discovery as a negative regulator of cell cycle progression (10–12), p21 has been implicated as a downstream effector of a number of different tumor suppressors, including p53 (10, 13), BRCA1 (14), WT1 (15), and transforming growth factor-β (16), although the extent to which p21 mediates the tumor suppression functions of any of these pathways is largely unknown. Its activity is best defined in the p53 pathway, where it functions as a key mediator of p53-dependent cell cycle arrest. p21 expression is rapidly elevated upon treatment of cells with agents that activate p53, such as ionizing or UV radiation or chemicals that induce DNA damage (13). Furthermore, p21-deficient cells are significantly impaired in their ability to undergo growth arrest in response to DNA-damaging agents (17, 18). In addition to its role in mediating growth arrest in response to DNA damage, p21 is also involved in the induction and maintenance of terminal differentiation (12, 19–22).

Because of the high rate of p53 mutations in tumors and the direct role for p21 in mediating p53-dependent growth arrest, one might predict that p21 would itself function as a tumor suppressor. Consistent with this prediction, p21 has been shown to suppress cell growth in culture and tumor formation in xenograft models (10, 22–24). In addition, p21 deficiency has been shown recently to accelerate tumor onset in MMTV-ras transgenic mice (25). However, its activity as a bona fide tumor suppressor remains in question because of the infrequency of p21 mutations in human tumors (26). Also, unlike p53-deficient mice, which exhibit a highly tumor-prone phenotype (27, 28), p21-deficient mice do not (17). Thus, although p21 plays an important role in mediating cell cycle arrest, it must perform additional functions that facilitate cell growth or survival, such that its loss is not selected for during tumorigenesis.

Studies on the specific effects of p21 binding to cyclin/CDK complexes have provided a possible explanation for the lack of p21 loss in tumors. Although the CIP/KIP CKIs were originally thought to inhibit all cyclin/CDK complexes, more recent studies have indicated that they have markedly different effects on different cyclin-containing complexes (reviewed in Ref. 29). Although they are in fact potent inhibitors of the cyclin E- and A-containing complexes, they actually promote cyclin D/CDK activity by multiple mechanisms, including facilitating cyclin D/CDK assembly and nuclear localization (30, 31). In fact, mouse embryo fibroblasts simultaneously deficient for both p21 and p27 showed no detectable cyclin D/CDK activity or nuclear localization of cyclin D protein (32). Cyclin D/CDK complexes also appear to function in part to sequester the CKIs, thereby preventing them from inhibiting cyclin E/CDK complexes (29).

Another possible explanation for the absence of p21 mutations in tumors is that p21-mediated growth arrest might afford protection from an alternate apoptotic response to DNA damage or cell cycle perturbations. Thus, there would be no selection for loss of p21 during tumorigenesis, because p21 expression would favor cell survival over apoptosis. p21 expression has been shown to confer protection from p53- or DNA damage-induced apoptosis in a number of cell types (33–36), and it has therefore been hypothesized that p21 may be a...
major determinant of the cellular decision to undergo growth arrest rather than apoptosis after p53 activation (33). However, this effect is clearly cell context specific, because p21 expression fails to abrogate p53-dependent apoptosis in other cell types (33).

To investigate the potential role of p21 in tumor suppression in vivo and to assess its effects on cell cycle progression and survival in the context of two different oncogenes, we have interbred mice deficient for p21 (18) with either MMTV-ras or MMTV-myc transgenic mice (37) to generate MMTV-ras or -myc mammary tumors that differ primarily in their p21 functional status (MMTV-ras/p21+/- versus MMTV-ras/p21-/- and MMTV-myc/p21+/- versus MMTV-myc/p21-/-). We found that p21 deficiency had opposite effects on tumor cell proliferation and apoptosis in the context of ras or myc, consistent with the hypothesis that p21 functions not simply as an inhibitor of cellular proliferation but rather as a modulator of cell growth, the function of which is highly dependent upon diverse regulatory signals.

MATERIALS AND METHODS

Animals. MMTV-ras and MMTV-myc transgenic mice (37) were originally obtained from Charles River Laboratories (Wilmington, MA), and a breeding license was obtained from E. I. DuPont de Nemours and Co. (Wilmington, DE) to permit the maintenance of these lines of mice and their interbreeding to other lines. Both lines were originally obtained in an inbred FVB genetic background but were subsequently maintained in our laboratory in an FVB × C57Bl/6 × BALB/c mixed genetic background. p21-/- mice (18) were generously provided by Tyler Jacks (in a 129/Sv × C57Bl/6 background) and through interbreeding to MMTV-ras and MMTV-myc mice were maintained in our laboratory in a similarly mixed genetic background. MMTV-ras/p21+/-, MMTV-ras/p21-/-, and MMTV-ras/p21+/- offspring were generated as littermates from common matings so that all animals in the study were of a comparably mixed genetic background, and the same was the case for the MMTV-myc × p21+/- matings. Offspring from matings between MMTV-ras or MMTV-myc mice and p21-/- (or p21+/-) mice were screened by PCR for their ras or myc and p21 status. DNA was extracted by standard methods from a small piece of tail tissue cut from each animal at the time of weaning (38). Primers used for the detection of the MMTV-ras transgene were 5’-CCCAAGGCTTAAGTAAGTTTTTGG-3’ (5’ sense primer) and 5’-GGG-CATAAGCACAGATAAAACACT-3’ (3’ antisense primer). Primers used for detecting the MMTV-myc transgene were 5’-GGTGAATGCTCTCCCTCATC-ATC-3’ (5’ sense primer) and 5’-GTGCCACCTGAGTCTGAAAGA-3’ (3’ antisense primer). For evaluating the p21 status of offspring, PCR reactions using three primers allowed for simultaneous detection of both the normal and mutant p21 allele in a single reaction. These primers consisted of a common 5’ sense primer (5’-AAGCCTTGTAGTGCTTTTGCC-3’) and two 3’ antisense primers, specific for p21 exon 2 (5’-TGAGCAAGATCATCCGTCG-3’) or the neo cassette (5’-GCTATAAGCATAGCAGTGGTC-3’). Reactions were run for 40 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min.

Tumor Onset and Growth Measurements. Twice weekly, animals were examined visually for the presence of tumors. Tumor incidence in the MMTV-ras/p21+/- versus MMTV-ras/p21-/- mice and MMTV-myc/p21+/- versus MMTV-myc/p21-/- mice was compared using the χ2 test. Differences in the age of tumor onset between mice of the same genotypic groups were compared using Cox proportional hazards analysis. Model fit was assessed by the log-likelihood method. Once a tumor was detected and had reached 200–400 mg in size, its growth was monitored for an additional 10–15 days, after which the animal was sacrificed. Tumor measurements were taken approximately every other day using hand calipers, and tumor volume was calculated according to the formula: tumor volume (mm3) = (W2 × L)/2, where W is width (mm) and L is length (mm). Tumor growth curves were generated by plotting tumor volume measurements against time. Using a curve-fitting algorithm, an exponential equation representing each curve was generated. Using these equations, the time to tumor doubling was calculated, and statistical differences between genotypes were tested using ANOVA analysis. Only female animals were included in this study, and to accelerate mammary tumor onset, females were housed with male breeders continuously from the time they reached sexual maturity until the time of their sacrifice. Although MMTV-ras transgenic females develop both mammary and salivary tumors (37, 39), MMTV-myc females develop only mammary tumors. Therefore, only mammary tumors from the various classes of mice were used to generate the data shown in Figs. 2–5.

Flow Cytometry. At the time of sacrifice, a 25–50-mg piece of tumor tissue was flash frozen in liquid nitrogen and stored at −80°C until analyzed by flow cytometry. Tumor samples were processed by standard methods using propidium iodide staining of tumor cell DNA (40). Each sample was analyzed with an EPICS ELITE flow cytometer (Coulter Cytometry, Miami, FL) using a 15 mW argon ion laser operated at 688 nm. Histograms were analyzed for cell cycle compartments using Multi Cycle-PLUS Version 3.0 (Phoenix Flow Systems, San Diego, CA). A minimum of 50,000 events were collected to maximize statistical validity of the compartmental analysis. Differences in S-phase fractions between groups were evaluated using the Student t test.

Cyclin D1 and Cyclin E-associated CDK Assays. Cyclin D1- and cyclin E-immunoprecipitation kinase assays were performed as described previously (41, 42). Tissues (normal or mouse mammary gland) were Dounce homogenized in lysis buffer [150 mM NaCl, 50 mM HEPES (pH 7.2), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, and 0.1 mM sodium orthovanadate (Sigma Chemical Co. Chemicals, St. Louis, MO)] at 4°C. Lysates were centrifuged at 10,000 × g for 5 min. Protein content was determined by the Bio-Rad protein assay, and 100 μg were used for each sample. The supernatants were precipitated for 12 h at 4°C with protein A-agarose beads precoated with saturating amounts of the cyclin D1 antibody, DCS-11 (NeoMarkers, Fremont, CA), or cyclin E antibody, M20 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitated proteins on beads were washed twice with 1 ml of lysis buffer and twice with kinase buffer [50 mM HEPES (pH 7.0), 10 mM MgCl2, 5 mM MnCl2, and 1 mM DTT]]. The beads were then resuspended in 40 μl of kinase buffer containing 10 μM ATP, 5 μCi of [γ-32P]ATP (6000 Ci/mmol; Amersham Corp., Arlington Heights, IL), and 2 μg of the protein substrate, either soluble glutathione S-transferase-pBR fusion protein for the cyclin D-dependent kinase assay or histone H1 for the cyclin E-dependent kinase assay. The samples were incubated for 20 min at 30°C with occasional mixing. They were then boiled in polyacrylamide gel sample buffer containing SDS and separated by electrophoresis. Phosphorylated proteins were quantified after exposure to autoradiographic film (Lab-scientific, Inc., Livingston, NJ) by densitometry using ImageQuant version 1.11 (Molecular Dynamics Computing Densitometer, Sunnyvale, CA).

Cyclin D1, Cyclin E, and p21 Western Blots. The abundance of cyclin D1, cyclin E, and p21 protein was determined by Western analysis as described previously (42, 43). Fifty μg of each tumor cell lysate were electrophoresed in an SDS–12% polyacrylamide gel and transferred electropheretically to a nitrocellulose membrane (Micron Separations, Inc., West- borough, MA). After transfer, the gel was stained with Coomassie blue as a control for blotting efficiency, and the membrane was stained with Ponceau S stain to verify equal protein loading (not shown). The membrane was incubated for 2 h at 25°C in T-PBS buffer supplemented with 5% (w/v) dry milk to block nonspecific binding sites. After a 6-h incubation with the primary monoclonal anti-cyclin D1 antibody DCS-6 (NeoMarkers) at a 1:1000 dilution in T-PBS buffer containing 0.05% (v/v) Tween 20, the membrane was washed with the same buffer. The membrane was then incubated with goat antimouse horseradish peroxidase secondary antibody (Santa Cruz Biotechnology) and washed again. The cyclin D1 protein was visualized by the Enhanced Chemiluminescence System (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The membrane was then stripped and reprobed with the rabbit polyclonal anti-cyclin E antibody M-20 (Santa Cruz Biotechnology) at a 1:500 dilution, followed by a goat antirabbit secondary antibody (Santa Cruz Biotechnology). For quantitation of p21, membranes were probed with the rabbit anti-p21 antibody M-19 (Santa Cruz Biotechnology) and the goat antirabbit horseradish peroxidase secondary antibody (Santa Cruz Biotechnology).

Apoptosis Analysis. At the time of sacrifice, tumor samples were fixed in 10% neutral buffered formalin and routinely processed for embedding in paraffin. Tumor tissue sections were in situ labeled for apoptotic cells using the TUNEL assay (44). Labeled slides were analyzed using light microscopy by counting positive cells within a 10 × 10-mm grid in the eyepiece in 10 × 450 fields. Necrotic regions of tumors, as evaluated by light microscopy of H&E-stained slides, were avoided. Apoptotic cells in nonnecrotic regions generally
appeared to be uniformly distributed through viable areas of tumor. The total number of tumor cells in each field ranged from 500 to 1000, depending on the histological pattern of the tumor. The percent of apoptosis was calculated by dividing the total number of positively labeled cells by the total cells in 10 \times 450 fields. Differences in apoptosis levels between groups were evaluated using the Student t test.

RESULTS

Effect of p21 Deficiency on Mammary Tumor Incidence, Age of Tumor Onset, and Tumor Growth Rates in MMTV-ras and MMTV-myc Mice. To determine the effect of loss of p21 on tumor properties in different oncogenic contexts, p21-deficient mice were bred to MMTV-ras and MMTV-myc transgenic mice to generate MMTV-ras/p21−/− and MMTV-myc/p21−/− mice. Female animals of each genotype were monitored twice weekly for the onset of tumors. We have demonstrated previously that deficiency of p53 in of each genotype were monitored twice weekly for the onset of tumor was first detected. Tumorigenesis was similarly accelerated when MMTV-ras mice were bred into a p21−/− background (P < 0.01; Fig. 1A), although in this case, there was a comparable acceleration of both mammary and salivary tumorigenesis. However, although there appeared to be an increase in overall tumor incidence in the MMTV-ras/p21−/− mice as compared with the MMTV-ras mice, it was not statistically significant (P = 0.2). In contrast, p21 deficiency had no effect on mammary tumor onset in MMTV-myc mice, when measured as the age at which 50% of the females had developed tumors (~7 months for both MMTV-myc and MMTV-myc/p21−/− mice). Surprisingly, however, there was a significant decrease in the overall tumor incidence in MMTV-myc/p21−/− mice, with nearly 35% remaining tumor-free at 18 months of age, as compared with 15% for MMTV-myc mice (P ≤ 0.05; Fig. 1B). Thus, although p21 appears to function as a tumor suppressor in the context of activated ras, it actually appears to promote tumorigenesis in the MMTV-myc mice. Because the MMTV-myc mice develop only mammary tumors, regardless of p21 status, the remaining studies include data for only the mammary tumors from each class of mice.

We have demonstrated previously that p53 deficiency in MMTV-ras mice resulted not only in accelerated tumor onset but higher tumor mean growth rates as well (39). However, the correlation between accelerated tumor onset and increased tumor mean growth rates does not always hold; for example, tumor onset is greatly accelerated in MMTV-ras × myc mice compared with either MMTV-ras or MMTV-myc mice, but the overall mean growth rates for tumors arising in all three classes of mice are nearly identical (45). It was therefore of interest to determine the effect of p21 deficiency on the rate of tumor growth. Once mammary tumors were detected in mice of each genotype, tumor growth was determined by caliper measurements over a period of approximately 10–15 days, and for each tumor, the time to tumor doubling was calculated as described in “Materials and Methods.” p21 deficiency was found to have no significant effect on the rate of mammary tumor growth in either the MMTV-ras or MMTV-myc mice (Fig. 2).

p21 Deficiency Has Opposite Effects on S-Phase Fractions in Mammary Tumors from MMTV-ras and MMTV-myc Mice. Although p21 knockout mice develop normally and do not exhibit an increased predisposition to tumorigenesis, cultured cells from these mice display defects in p53-dependent G1 checkpoint control (17, 18). Therefore, it was of interest to explore whether p21 deficiency had effects on the cell cycle profiles of mammary tumor cells from MMTV-ras/p21−/− and MMTV-myc/p21−/− mice, as compared with those from MMTV-ras and MMTV-myc mice. The relative fractions of cells in G0/G1, S, and G2/M phases of the cell cycle were determined using flow cytometric analysis of frozen tumor tissue that was collected at the time of sacrifice. Fig. 3 shows the average cell cycle distribution for mammary tumors arising in each class of mice. A significant increase (P = 0.002) was observed in the S-phase fractions in mammary tumors from MMTV-ras/p21−/− mice when compared with mammary tumors from MMTV-ras mice, much as we had observed previously in MMTV-ras/p53−/− mice (39). In contrast, a significant decrease (P = 0.03) in cells in S-phase was observed in mammary tumors from MMTV-myc/p21−/− mice as compared with MMTV-myc mice.

Cyclin D1 and Cyclin E-associated CDK Activity in Mammary Tumors from p21-deficient MMTV-ras and MMTV-myc Mice. The increased S-phase fractions observed in the tumors from MMTV-ras/p21−/− mice are consistent with p21 functioning primarily as a CDK inhibitor. However, the decrease in S-phase fractions in tumors from MMTV-myc/p21−/− mice suggests an alternative function for p21 in the context of myc overexpression. To further investigate the effects of p21 deficiency on CDK activity in these tumors, cyclin D1- and cyclin E-associated kinase assays were performed on protein extracts from mammary tumors from MMTV-ras, MMTV-ras/p21−/−, MMTV-myc, and MMTV-myc/p21−/− mice. Cyclin D1- and cyclin E-containing CDK complexes were immunoprecipitated using antibodies against cyclin D1 and E, respectively, and the immunoprecipitated complexes were evaluated for in vitro kinase activity using recombinant purified glutathione S-transferase-Rb as a substrate for the cyclin D1/CDK assay and histone H1 for the cyclin E/CDK assay. For both ras and myc tumors, the changes in cyclin D1- and E-associated kinase activity in p21-deficient tumors correlated with the changes in S-phase fractions; MMTV-ras/p21−/− tumors had significantly more cyclin D1- and cyclin E-associated CDK activity than MMTV-ras tumors, whereas MMTV-myc/p21−/− tumors had signif-

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Significantly decreased CDK activity compared with MMTV-myc tumors (Fig. 4, A and B). These data suggest that in the context of these two oncogenes, p21 may act either to inhibit or to stimulate cyclin/CDK activity.

To further evaluate the mechanism by which p21 deficiency influenced CDK activity in the context of either ras or myc, the levels of cyclin D1 and cyclin E protein in tumor lysates were assessed by Western blot analysis. For each of the tumor types evaluated, the levels of either cyclin D1 or cyclin E were found to parallel the corresponding CDK activity levels. In MMTV-ras tumors, p21 deficiency resulted in an increase in the levels of both cyclins, whereas in MMTV-myc tumors, the opposite was observed (Fig. 4, C and D). In addition, p21 protein levels were evaluated in both MMTV-ras and MMTV-myc mammary tumors and were found to be comparable in these two tumor types (data not shown). Biochemical studies have indicated that p21 alters cyclin/CDK activity through direct binding to the complexes (30). However, the results of this study suggest that an additional mechanism for CDK regulation accompanying p21 loss may involve direct or indirect modulation of cyclin protein levels.

Effect of p21 Deficiency on Apoptosis in Mammary Tumors from MMTV-ras and MMTV-myc Mice. The observed cyclin D1 and cyclin E-associated CDK activities, together with the cell cycle data, suggest that MMTV-ras/p21−/− tumors should display higher growth rates than MMTV-ras tumors, and that MMTV-myc/p21−/− tumors should display slower growth than MMTV-myc tumors. However, as shown in Fig. 2, comparisons of growth rates for tumors from p21-deficient mice and their wild-type p21 counterparts showed no significant differences in either MMTV-ras or MMTV-myc mice. Because tumor growth rates are influenced not only by the rate of cell cycle progression but also by the rate of cell death, the levels of apoptosis in mammary tumors from MMTV-ras, MMTV-ras/p21−/−, MMTV-myc, and MMTV-myc/p21−/− mice were determined using TUNEL analysis (Fig. 5). Mammary tumors from MMTV-ras mice displayed very low levels of spontaneous apoptosis (0.12%), whereas MMTV-ras/p21−/− tumors exhibited an almost 4-fold increase.
in the level of apoptosis ($P = 0.04$). Both MMTV-myc and MMTV-myc/p21−/− tumors had substantially higher levels of apoptosis than either class of ras tumor (3.8% in MMTV-myc tumors and 2.8% in MMTV-myc/p21−/− tumors), consistent with the known ability of c-myc overexpression to sensitize cells to apoptosis (46–48). However, the apoptosis values in the two classes of myc tumors were not statistically different, indicating that in the presence of c-myc overexpression, p21 status is not a significant determinant of apoptosis levels.

**DISCUSSION**

Although p21 was originally identified as a negative regulator of cell cycle progression (10–12), several lines of evidence suggest that
it may play complex roles in the regulation of both cell proliferation and survival. If p21 functioned solely as an inhibitor of cell cycle progression, one might expect there to be selective pressure for loss of p21 function during tumorigenesis, as there is for p53. However, the low frequency of p21 mutations in human tumors calls into question the notion that p21 is necessarily expected that p21 deficiency would result in phenotypic changes were paralleled by comparable increases in both cyclin D1- and cyclin E-associated kinase activity. These results indicate that loss of p21 function in the presence of activated ras results in loss of G1 to S-phase control and is consistent with a role for p21 in negatively regulating G1 cyclin/CDK activity.

The most striking result from this study was the contrasting effect that p21 deficiency had on cell proliferation in mammary tumors from MMTV-myc mice compared with those from MMTV-ras mice. MMTV-myc tumors have very high S-phase fractions (45), consistent with the known role of c-myc in overcoming G1-S checkpoints and promoting S-phase entry (51). In fact, c-myc has been shown to be able to override p21-mediated growth arrest (52). Thus, it was not necessarily expected that p21 deficiency would result in phenotypic changes in MMTV-myc tumors. However, MMTV-myc/p21−/− tumors displayed a significantly reduced number of cells in S-phase, accompanied by a decrease in both cyclin D1- and cyclin E-associated CDK activity, suggesting that in the context of c-myc overexpression, p21 promotes an increase in G1 cyclin/CDK activity, leading to the higher S-phase fractions seen in tumors from MMTV-myc mice.

The paradoxical possibility that p21 can function as either a negative or positive regulator of CDK activity has been suggested previously by both biochemical and cell culture studies (30, 31). Experiments investigating the biochemical mechanism of p21 action revealed a dual activity for p21 in regulating cyclin/CDK complexes, depending upon the binding ratio of p21 to these complexes (30). Furthermore, p21 has been found in association with active cyclin/CDK complexes in tumor cells in culture. In fact, it was shown that the major portion of cyclin D1-associated kinase activity from a human mammary epithelial cell line was associated with p21 (31). Taken together, these data suggest that p21 regulates the activity of cyclin/CDK complexes through direct interaction, but that the mode of regulation, either positive or negative, depends on the intracellular concentration of p21 relative to that of the complexes it regulates.

Intriguing results from a recent study in which MMTV-wnt-1 transgenic mice were interbred to p21 knockout mice support the notion that modest changes in p21 levels can markedly alter cyclin/CDK activity and tumor growth, because tumors arising in MMTV-wnt-1/p21−/− had elevated mean growth rates and cyclin/CDK activity compared with either MMTV-wnt-1 or MMTV-wnt-1/p21−/− tumors (53).

We found that loss of p21 resulted in an increase in the activity of G1 cyclin/CDKs in MMTV-ras tumors, which have low intrinsic cyclin D1- and E-associated CDK activity, but was associated with a decrease in cyclin/CDK activity in MMTV-myc tumors, which have higher D1- and E/CDK activity. On the basis of the assumption that p21 was regulating the activity of these complexes through direct interaction, we expected that the absolute cyclin...
levels would not be affected by p21 status. To examine this, we determined the levels of cyclins D1 and E in tumor cell lysates from MMTV-ras and MMTV-myc mice by Western blot analysis. Surprisingly, in both myc and ras tumors, changes in cyclin D1 and E protein levels were found to parallel the corresponding CDK activity levels as a function of p21 status. These results suggest that in addition to regulating the activity of cyclin/CDK complexes through direct interaction, p21 can also either directly or indirectly lead to changes in cyclin protein levels. A recent study examining the effects of loss of both p21 and p27 in mouse embryo fibroblasts demonstrated not only a loss of cyclin D/CDK activity but also greatly reduced cyclin D protein levels (32). Although it is not clear whether the effect of p21 on cyclin D1 levels is direct or indirect, there is evidence for p21-induced transcription of cyclin D1 (54), possibly mediated through pRb activation.

In addition to its dual role in both positively and negatively regulating cell proliferation, another possible explanation for the infrequency of p21 mutations in human tumors is that loss of p21 may sensitize cells to the induction of apoptosis. Several studies have shown that expression of p21 can provide a survival advantage by protecting cells from undergoing apoptosis (33–36). For example, p21-deficient cell lines display an increased sensitivity to apoptosis induced by DNA-damaging agents or irradiation (34). In an in vivo study, it was shown that p21 deficiency resulted in increased sensitivity to ionizing radiation and delayed onset of lymphomagenesis in atm−/−/p21−/− double-knock-out mice (36). This implies that in the context of atm deficiency, loss of p21 contributed to tumor suppression of lymphomas, potentially explaining why its loss would not be selected for during tumorigenesis. In the current study, a significant increase in apoptosis was observed in mammary tumors from MMTV-ras/p21−/− mice compared with MMTV-ras mice. Thus, in this context, p21 may confer a survival advantage upon mammary tumor cells. It is interesting to note that although loss of p21 in MMTV-ras mice did result in increased tumor cell proliferation, it also resulted in an increase in tumor cell death, thereby negating any growth advantage gained by p21 loss. In contrast, in MMTV-myc mice, which display much higher apoptosis levels as well as increased S-phase fractions, p21 deficiency caused a decrease both in S-phase fractions and apoptosis (although the latter change was not statistically significant).

The fact that, with the exception of p16INK4a, none of the known CKIs are frequently found to be mutated in human tumors suggests that they may have multiple roles in the regulation of cell proliferation and survival, such that their loss is not selected for during tumorigenesis. It has been suggested that CKIs serve as entry points for regulation by numerous oncogenes and tumor suppressor genes and function to coordinate diverse regulatory signals (50). p21 in particular appears to perform a number of alternative functions in the regulation of the cell cycle and apoptosis, because it can either promote or inhibit CDK activity and may or may not confer protection from apoptosis, depending upon the cellular context. It is becoming increasingly clear that the signals that govern cellular processes, such as entry and exit from the cell cycle, differentiation, and programmed cell death, function in complex regulatory networks rather than simple linear pathways, and that these networks may be wired differently in different cell or tumor types. In this study, we demonstrate opposite effects of p21 deficiency on tumor incidence and cell cycle parameters in two tumor models that differ only in the oncogene driving tumorigenesis. Our results thus highlight the importance of considering cellular context in interpreting the function of regulatory proteins like p21.

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**Note Added in Proof**

Although p21-deficient mice were originally reported to lack a tumor-prone phenotype (17), Martin-Caballero et al. have recently reported that when a large cohort of p21-deficient mice was followed for greater than 2 years of age, they were found to spontaneously develop a variety of tumor types, with a mean age of onset of 16 months.

**REFERENCES**


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