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p21$^{WAF1/CIP1}$ Mutants Deficient in Inhibiting Cyclin-dependent Kinases (CDKs) Can Promote Assembly of Active Cyclin D/CDK4(6) Complexes in Human Tumor Cells[1]

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Abstract

The cyclin-dependent kinase (CDK) inhibitor p21$^{WAF1/CIP1}$ is a multidomain, multifunctional protein and a candidate tumor suppressor. Here, we show that, among rationally designed and tumor-associated mutants of human p21 ectopically expressed in U-2-OS cells, those that are selectively deficient in binding to either cyclin or CDK are partially impaired in inhibiting endogenous CDK activities but efficiently promote assembly of active cyclin D/CDK4(6) complexes. These results provide mechanistic insights into the p21-cyclin/CDK interplay *in vivo* and suggest a functional subclassification of tumor-specific aberrations of p21. Intriguingly, the subclass exemplified by the melanoma-derived N50S mutant may promote tumorigenesis, by both attenuating CDK-inhibitory function and concomitantly activating the proto-oncogenic cyclin D-dependent kinases.

Introduction

Inhibitors of CDKs[3] negatively regulate progression through the cell division cycle during development and maintenance of tissue homeostasis and represent candidate tumor suppressors, the deficiency or loss of which may contribute to oncogenesis. The prototypic CDK inhibitor p21$^{WAF1/CIP1}$ has been implicated in fundamental biological processes, including G_1 arrest, as part of a p53-controlled checkpoint pathway in response to DNA damage, modulation of DNA synthesis, differentiation, apoptosis, and cellular senescence (1–4). These diverse functions appear to be mediated by protein-protein interactions of the multimodular p21 protein, the most prominent among them being associations with cyclins and CDKs via distinct sequences within the NH_2-terminal domain of p21 and interactions with proliferating cell nuclear antigen, a nuclear localization signal, and a second cyclin-binding motif within the COOH-terminal domain (3–11). The biological effects of p21 depend on protein abundance, as exemplified by the inhibition of cyclin-CDK activities at high levels (12, 13), in contrast to promoting assembly of the active cyclin D-CDK complexes by lower levels of p21 (13), respectively. Despite the accumulating knowledge about the control of p21 expression by both p53-dependent and -independent pathways (1–4) and the molecular mechanisms underlying the diverse functions, the significance of p21 as a plausible target of tumorigenic aberrations remains largely unclear. For instance, although the expression of p21 is severely downmodulated in p53-deficient tumors, mutations or deletions of the *p21* gene are rare in human cancer (14–18), and mice that are homozygously deleted for p21 develop normally and do not show a tumor-prone phenotype (19). In addition, the recently proposed promotion of cyclin D-CDK assembly by p21 (13) suggested a positive rather than negative effect on cell cycle progression, a function that is not easily reconciled with the widely appreciated candidacy of p21 as a tumor suppressor. To address the complex issue of the positive (assembly-promoting) *versus* inhibitory effects of p21 on cyclin-CDK activity in relation to tumorigenesis, we have examined the structure-function relationship of p21 in a series of engineered as well as naturally occurring tumor-associated point missense mutants of *p21* upon ectopic expression in human cells.

Materials and Methods

Plasmids and Site-directed Mutagenesis. p21 mutants were generated in the pCMV-HAp21 vector coding for HA-tagged wild-type human p21 (provided by K. Helin, European Institute of Oncology, Milan, Italy), using the Quick Change method (Stratagene) according to the manufacturer's instructions, and confirmed by DNA sequencing. The following mutations (and combinations thereof) were introduced, based on published information about the functionally relevant residues L21H with P24A for ΔC (9), D52A for ΔK (8), and R156D with L157D for ΔC2 (11) and the tumor-derived mutations N50S (from a melanoma; Ref. 18), F63L (from a Burkitt's lymphoma; Ref. 16), and R94W (from a breast carcinoma; Ref. 15).

Cell Culture and Transient Transfection. The human osteosarcoma cell line U-2-OS was maintained in DMEM with 5% FCS. For transient transfections, cells were seeded into 6-cm dishes and transfected by the calcium phosphate method according to standard protocols for 12 h, washed, and cultured for further 24 h before lysing the cells in an immunoprecipitation buffer (20) for biochemical analyses.

Antibodies, Immunochemical Analyses, and Kinase Assays. For immunoprecipitation, cellular proteins were extracted on ice for 45 min, lysates were cleared by centrifugation, the supernatants were examined for total protein content using the Bradford method, and the protein (1 mg) was used for immunoprecipitations with antibodies against cyclin D1 and D2 (mAb 5D-4), cyclin E (mAb E-172), cyclin A (rabbit serum), and p21 (mAb DCS-61). Immunocomplexes were washed and split for analyses by immunoblotting or kinase assays.

For kinase assay, beads were equilibrated in kinase assay buffer (20), and the kinase reaction was performed in 30 μl of buffer containing 5 μCi of [γ-^{32}P]ATP and 1 μg of GST-pRb (c-terminus) or histone H1 for 20 min at 30°C. The reaction was stopped by addition of Laemmli sample buffer, and samples were separated using denaturing gel electrophoresis (20) and analyzed by PhosphorImager (Molecular Dynamics). Gel electrophoresis of either the total cell lysates or immunoprecipitated protein complexes, followed by Western blotting, were performed as described previously (20).

Results and Discussion

p21$^{WAF1/Cip1}$ Mutants Impaired in Association with Either Cyclin or CDK Promote the Assembly of Active Cyclin D/CDK4(6) Complexes. To complement the previous reports on the structure-function relationship of p21, obtained largely by means of pure *in vitro* approaches or deletion mutants (9, 11, 13), we set up a transient transfection assay, allowing for comparison of the relative impact of wild-type p21 and its point mutation-containing derivatives within the known cyclin- and/or CDK-binding domains on composition and kinase activity

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[3] The abbreviations used are: CDK, cyclin-dependent kinase; HA, hemagglutinin; mAb, monoclonal antibody; GST, glutathione *S*-transferase; pRb, retinoblastoma protein.

of the cyclin/CDK complexes in human U-2-OS cells. Consistent with the published *in vitro* data (10, 12), transient expression of wild-type p21 quantitatively inhibited the endogenous cyclin E-associated kinase activity toward both the GST-pRb and histone H1 substrates (Fig. 1*A*). Mutants of *p21* that were deficient in association with either cyclin (designated ΔC; Ref. 9) or CDK (designated ΔK; Ref. 8) were less efficient relative to the wild-type control in inhibiting the cyclin E/CDK2 kinase, yet they retained significant residual inhibitory activity when they were expressed to high levels (Fig. 1*A*, *left*). The observed partial deficiency in kinase inhibition was not caused by inefficient binding because their respective abilities to enter cyclin E/CDK2 complexes were virtually unaffected (Fig. 1*A*, *right*). In contrast, simultaneous disruption of the cyclin- and the CDK-binding domains of p21 resulted in a p21 mutant (designated ΔCK) that completely lost its ability to associate with cyclin E/CDK2 (Fig. 1*A*, *right*) and to inhibit cyclin E-associated kinase activity (Fig. 1*A*, *left*).

A moderate kinase activity associated with cyclins D1 and D2 was detectable when they were assayed on the pRb substrate in lysates from control, pCMV-transfected cells, and this activity was moderately elevated upon transient expression of the wild-type p21 (Fig. 1*A*, *left*). Kinase activity toward histone H1 remained at its background level, consistent with the preference of the cyclin D-dependent kinases for the pRb substrate. Unexpectedly, expression of either the ΔC or the ΔK p21 mutant resulted in a considerable, 10–20-fold increase of the kinase activity coimmunoprecipitated with the endogenous D-type cyclins (Fig. 1*A*, *left*). Despite the significant amount of endogenous p21 already associated with D-type cyclins (as evidenced in vector-transfected control cells), both the ΔC and ΔK mutants entered the endogenous cyclin D complexes as effectively as the wild-type p21 (Fig. 1*A*, *right*). The combined ΔCK mutant, on the other hand, almost entirely lacked any influence on the cyclin D-associated kinase activity (Fig. 1*A*, *left*) and did not associate with cyclin D/CDK complexes (Fig. 1*A*, *right*).

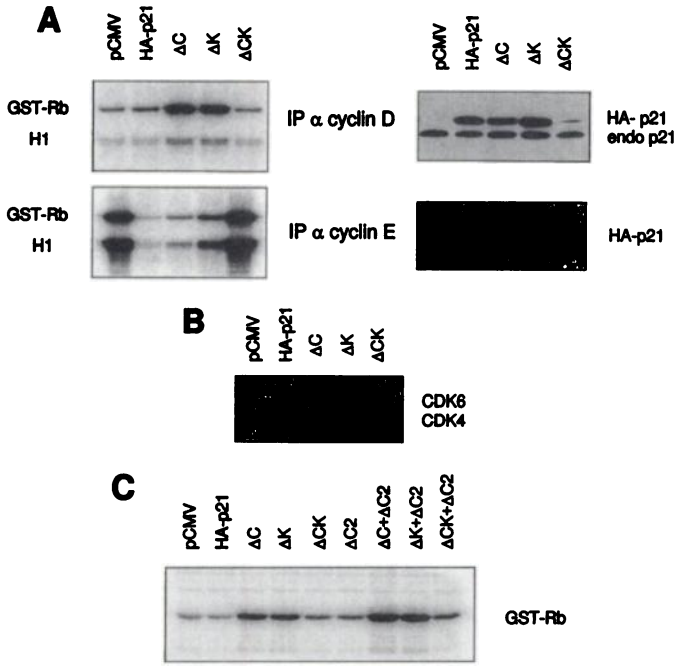

Fig. 1. Enhanced assembly and kinase activity of cyclin D-CDK4(6) complexes mediated by p21 mutants deficient in binding to either cyclin or CDK. *A*, p21 mutated in either cyclin- or CDK-binding site supports cyclin D-associated kinase activity. U-2-OS cells were transiently transfected with empty vector (pCMV) or expression plasmids for HA-tagged wild-type p21 (HA-p21), p21 derivatives defective in the cyclin-binding domain (ΔC), the CDK-binding domain (ΔK), or both domains (ΔCK). Twenty-four h later, cell lysates were prepared and immunoprecipitated with antibodies to cyclins E and D1/D2 as indicated. After washing, the immunocomplex-containing beads were divided and analyzed for the associated kinase activity toward GST-pRb and histone H1 (*left*) and for the amount of the associated *p21* protein (*right*). *B*, assembly of D-type cyclins with CDK4 and CDK6 is stimulated by both cyclin- and kinase-deficient mutants of *p21*. Cyclin D-containing immunocomplexes were prepared essentially as described for *A* and analyzed by immunoblotting for the presence of the associated cognate kinases, CDK4 and CDK6. *C*, integrity of the COOH-terminal second cyclin-binding motif has no influence on the cyclin D-dependent kinase activity stimulated by the ΔC and ΔK mutants of *p21*. U-2-OS cells were transfected as described in *A* (*Lanes 1–5*, from *left* to *right*) as well as with the expression plasmids for the selected p21 versions containing an additional mutation within the COOH-terminal RRL motif (*Lanes 6–9*) and analyzed for the cyclin D-associated kinase activity.

In an attempt to explain the stimulatory effect of the ΔC and ΔK mutants on cyclin D-dependent kinase activity, we investigated the amount of CDK4 and CDK6 associated with D-type cyclins in the transfected cells. In agreement with the proposed function of p21 as an assembly factor for D-type cyclins and CDK4(6) (13), overexpression of wild-type p21 significantly increased the amounts of CDK4 and, especially, CDK6, in the cyclin D1/D2 immunoprecipitates, whereas the ΔCK p21 mutant had no effect (Fig. 1*B*). Importantly, expression of either ΔC or ΔK mutants also led to a dramatic increase of cyclin D/CDK4(6) complexes, similar to the effect of wild-type p21 (Fig. 1*B*). Thus, both the ectopic wild-type p21 and its derivatives that were deficient in either cyclin- or CDK-binding sites are capable of promoting and/or stabilizing the cyclin D/CDK4(6) complex formation, but only the mutants (ΔC and ΔK) can concomitantly support, rather than inhibit, the associated kinase activity.

Apart from the major cyclin-binding site in the NH_2-terminal domain of p21, there is another RRL-containing motif close to the COOH terminus that has been proposed to function as a potential second cyclin-binding site (5, 11). To test whether the integrity of this COOH-terminal motif has any influence on the cyclin D/CDK4(6) assembly and/or activity, we constructed additional mutants by disrupting the second cyclin-binding site in wild-type p21 (11) and by combining the ΔC2 with ΔC, ΔK, or both. When transiently expressed in U-2-OS cells, the ΔC2 mutation did not cause any detectable effects, in that both ΔC and ΔK, with or without the additional ΔC2 mutation, enhanced the cyclin D-associated kinase activity (Fig. 1*C*) and the cyclin D/CDK4(6) assembly (data not shown) to the same extent.

The Cyclin D-associated Kinase Activity Supported by the ΔC and ΔK Mutants of p21 Is Generated Specifically by CDK4 and CDK6. The preferential phosphorylation of GST-pRb indicated that the cyclin D-associated kinase activity supported by the ΔC and ΔK mutants of p21 is mediated by the cyclin D-cognate kinases CDK4 and CDK6. However, because the D-type cyclins also assembled with CDK2 and CDK1 upon expression of p21 in these experiments (data not shown), we wanted to examine the CDK specificity in kinase assays, using two different approaches.

First, we reproduced the kinase assay essentially as described for Fig. 1*A* (*left*), with the exception that we let the kinase reaction proceed in the presence of increasing concentrations of Roscovitine, a specific inhibitor of CDK2 and CDK1. As a positive control, we measured the impact of Roscovitine on cyclin A-associated kinase known to be composed of both cyclin A/CDK2 and cyclin A/CDK1 complexes. Indeed, the kinase activity immunoprecipitated with anti-cyclin A antibody from U-2-OS cells was severely compromised by addition of Roscovitine in a dose-dependent manner. On the other hand, the cyclin D-associated kinase activity stimulated by ΔC or ΔK mutants of p21 appeared unchanged even if challenged with the Roscovitine concentration that reduced the cyclin A-associated kinase activity by >95%, compared to the mock-treated samples (Fig. 2*A*).

Second, we took advantage of the ability of our newly generated anti-p21 antibody, mAb DCS-61, to preferentially recognize p21 that was associated with cyclin D/CDK4(6) complexes, rather than the CDK2- or CDK1-containing cyclin/CDK complexes. Because the DCS-61 epitope resides between amino acid residues 61 and 80, overlapping with the previously mapped CDK-binding domain of p21,

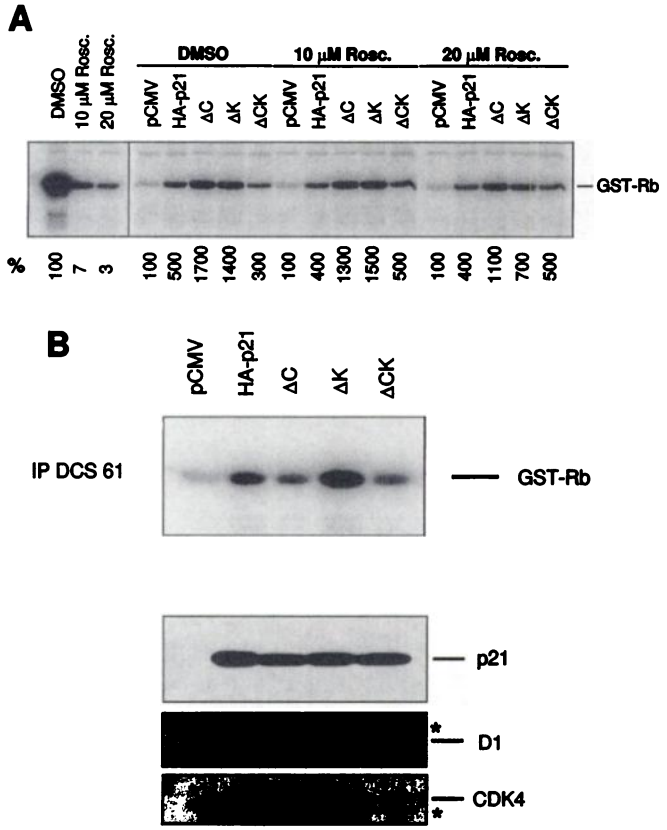

Fig. 2. The cyclin D-associated kinase activity stimulated by the p21 cyclin/CDK-binding mutants is specific for CDK4 and CDK6. *A*, the enhancement of kinase activity associated with cyclin D and dependent on the ΔK and ΔC mutants of p21 is resistant to Roscovitine. U-2-OS cells were transfected with the indicated expression plasmids and immunoprecipitated by the mAb 5D4 against cyclin D1 and D2. The immobilized immunocomplexes were divided into three parts and equilibrated in the kinase assay buffer containing either DMSO (0.1%) or 10 and 20 μM Roscovitine. Subsequently, the kinase reactions (in the presence of DMSO or the indicated concentrations of Roscovitine) were performed using GST-pRb as a substrate. As a control sensitive to Roscovitine, endogenous kinase activity immunoprecipitated by antibody to cyclin A is shown in the three lanes on the left, measured under identical conditions. The values below the autoradiogram indicate the relative changes in phosphate incorporation into the GST-pRb substrate. *B*, mAb DCS-61 directed to an epitope within the CDK-binding domain of p21 and preferentially recognizing cyclin D/CDK4(6) complexes, stimulates kinase activity associated with wild-type p21. Cells were transfected as indicated and immunoprecipitated with the mAb DCS-61. Immunocomplexes were split and analyzed for the associated kinase activity toward GST-pRb (*top*) and for the associated cyclins/CDKs (*bottom*). *, position of immunoglobulin fragments recognized by the secondary antibody.

we reasoned that, when attached to p21, such an antibody may compete with CDK association to p21 and thus mimic the effect of the ΔK mutant. To test this prediction, we transiently overexpressed wild-type p21 and the cyclin/CDK-binding mutants in U-2-OS cells; lysed the cells in the presence of the DCS-61 mAb, which was then immunoprecipitated along with the associated p21-cyclin D/CDK4(6); and measured the associated kinase activity using GST-pRb as a substrate. In Fig. 2*B* (*top*), we show a typical outcome of such experiment. Although the activity associated with endogenous p21 from control (pCMV)-transfected cells was barely measurable, DCS-61 was able to support significant activity in cells overexpressing wild-type p21. Because this level of p21 overexpression otherwise inhibits endogenous cyclin/CDKs, we argued that the activity associated with DCS-61 resulted from efficient disruption of the CDK-binding domain on p21 and, indeed, mimicked the assembly and stimulatory effect of the ΔK mutant on cyclin D/CDK4(6). Consistent with such an interpretation, DCS-61 brought down significant kinase activity from cells transfected with ΔK but not with the ΔC mutant of p21, the latter scenario mimicking the combined ΔCK mutant because all cyclin D1 and CDK4 were lost from the immunoprecipitates (Fig. 2*B*, *bottom*). Collectively, these results provided independent evidence for the ability of p21, deficient in CDK association, to assemble and activate the cyclin D/CDK4(6) complex.

Melanoma-associated Mutant of p21 Supports Cyclin D/CDK4(6) Kinase Activity. The data presented thus far were obtained with the mutants that were "rationally designed," based on published *in vitro* experiments (8, 9). To investigate whether some of the naturally occurring p21 mutations identified in different types of human malignancies (15–18) may have similar impact on cyclin/CDK assembly and/or activity, we individually introduced three distinct tumor-associated point mutations, localized within or adjacent to the CDK-binding domain, into our HA-tagged p21 expression plasmid and analyzed their impact on cyclin D-associated kinase activity upon overexpression in U-2-OS cells. As a positive control, we also expressed the ΔK mutant that promotes the assembly of active cyclin D/CDK4(6) complexes (see Fig. 1, *A* and *B*). All of the p21 versions were equally expressed (data not shown) and associated to a similar extent with cyclin D-containing complexes (Fig. 3*A*, *bottom*). One of the mutants, the N50S substitution isolated from a malignant melanoma cell line (18), significantly stimulated cyclin D-associated kinase activity toward GST-pRb to an extent comparable to the ΔK mutant (Fig. 3*A*, *top*). Although the other two tumor-derived mutants, previously reported to be partially deficient in their CDK-inhibitory abilities (15, 16), promoted the assembly/stabilization of the cyclin D/CDK complexes to a similar extent as the N50S mutant, they were still inhibitory in our experiments when expressed at high levels (Fig. 3 and data not shown). To further substantiate the cyclin D-stimulatory potential of the N50S mutant that localizes to the p21 region essential for CDK binding (7, 8), we performed a titration experiment by gradually increasing the amount of the expression plasmids coding for either wild-type p21 or the N50S mutant. Fig. 3*B* demonstrates that the lowest concentration of the wild-type p21 had some stimulatory effect on the cyclin D-associated kinase activity, which was gradually reduced upon increasing the amount of the expression plasmid, and almost totally inhibited at the highest concentration. On the contrary, N50S-mediated stimulation of the cyclin D/CDK4(6) activity steadily increased and remained high, even at the highest plasmid concentration.

Implications for the Role(s) of p21 in Regulation of Cyclin/CDKs and Oncogenesis. As emphasized in a recent review article by Hengst and Reed (3), there are currently a number of controversial issues concerning the biological and biochemical properties of p21, one of them being the nature of molecular interactions and stoichiometry of p21 within the complexes with cyclins and CDKs. At least one reason for such discrepancies may be the fact that the majority of the studies performed to date were conducted with recombinant proteins under *in vitro* conditions (7–12) and, thus, should be extrapolated to *in vivo* situation with caution. Our approach has been to mimic the *in vivo* situation as closely as possible, and therefore, we based this study on the most subtle yet effective (8, 9, 11) point missense mutations, to minimize any potential nonspecific effects on p21 structure, and used those under *in vivo* conditions, relying on analyses of effects on endogenous cyclin/CDKs. This approach has also allowed us to compare the rationally designed mutants with the point mutants naturally occurring in human malignancies, a major point addressed in the present experiments. In the study that inspired this work by proposing the role for p21 as an assembly factor of cyclin D/CDKs, LaBaer *et al.* (13) also used the U-2-OS cell culture model, yet the authors used larger deletion mutants in the cyclin- and CDK-binding domains of p21, examined ectopically expressed rather than endogenous cyclin D and CDK4, and did not analyze any naturally occurring tumor-derived mutants. The data obtained in our present study suggest that, in human cells, p21 can assemble/stabilize the cyclin D/CDK complexes via recruitment through at least one strong (wild-type) binding site, interacting with either cyclin

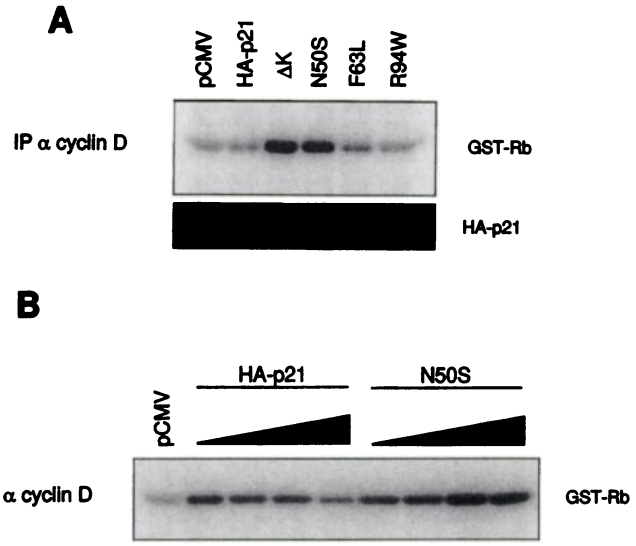

Fig. 3. Melanoma-associated p21 mutant, N50S, stimulates the cyclin D-associated kinase activity. *A*, U-2-OS cells were transfected with an empty vector (pCMV) or the expression plasmids for HA-tagged wild-type p21 (HA-p21), p21 mutant deficient in CDK binding (ΔK), and three p21 mutants isolated from different types of human malignancies: N50S (familial melanoma), F63L (Burkitt lymphoma), and R94W (breast carcinoma). Cyclin D1/D2 immunocomplexes from the transfected cells were subsequently analyzed for the kinase activity toward GST-pRb (*top*) and for the amount of the associated p21 (*bottom*). *B*, U-2-OS cells were transfected with increasing amounts (0.1, 0.3, 1, and 3 μg of expression plasmid per 60-mm dish) of either wild-type p21 (HA-p21) or the melanoma-associated p21 mutant (N50S) and analyzed for the cyclin D-associated kinase activity using GST-pRb as a substrate.

or CDK, and that the integrity of the suggested second, COOH-terminal cyclin-binding site of p21 (5, 11) appears irrelevant for the observed effects. We, furthermore, show that the activity of the cyclin D/CDK4(6) complexes assembled by the p21 alleles severely impaired in either kinase or cyclin interaction appear noninhibitable, even at high p21-to-cyclin/CDK ratios. Thus, our results are compatible with the notion that strong interaction with both the cyclin and CDK is necessary for inhibition but not for assembly of the cyclin D/CDK complexes *in vivo*. Mechanistically, the observed 10–20-fold increase of endogenous cyclin D/CDK4(6) kinase activity promoted by p21 mutants severely defective in (8, 9) but probably not entirely deprived of the binding to either cyclin or CDK likely reflects a combination of efficient assembly/stabilization and attenuated inhibition, even in the presence of an abundance of p21.

Testing the prediction that, due to its cell cycle-inhibitory function downstream of p53, p21 could be a potential tumor suppressor has thus far produced ambiguous results. Unlike *p53*, the *p21* gene does not localize to a tumor suppressor locus, and the p21-nullizygous mice do not develop cancer with increased frequency (19). On the other hand, a large proportion of human malignancies harbor defects of p53 and, consequently, express very low levels of p21, and intragenic mutations of p21 were reported to occur with low frequency in several types of tumors; in contrast, no gene deletions have been found to date (3, 4, 14–18). Thus, it appears that the vast majority of human tumors may express either low levels of wild-type p21 or mutated p21 proteins with apparently attenuated CDK-inhibitory activity. In contrast to classical tumor suppressors, however, there is no selection pressure to eliminate the p21 function during multistep oncogenesis entirely. One possible explanation for these intriguing findings, also indirectly supported by our present results with the tumor-derived mutants of p21, is that it might be beneficial for tumor cells to preserve the p21 function at low levels, known to promote the assembly and nuclear targeting of the proto-oncogenic cyclin D-dependent kinases (Ref. 13 and this study), while avoiding the high p21 levels that are necessary to inhibit the CDKs. All three tumor-associated mutants examined here were able to promote the assembly of cyclin D/CDK complexes, and although the breast cancer-derived (15) and the lymphoma-derived (16) mutants inhibited the assembled complexes when they were overexpressed, the melanoma-associated N50S mutant supported the activity of cyclin D/CDK4(6), even at high levels (see Fig. 3). Our data, therefore, point to a functional diversity among the tumor-associated aberrant alleles of *p21*, and it is tempting to speculate that the subclass exemplified here by the N50S mutant might result in deregulated cyclin D-dependent kinase activity, for instance, in p53 wild-type tumors exposed to DNA damage. Further experimentation is clearly needed to resolve the role of p21 in oncogenesis, but a tantalizing possibility arises that this multifunctional protein could join the emerging group of cellular regulators such as the E2F-1 transcription factor, the impact of which may be proto-oncogenic or tumor suppressive, dependent on the level of expression, type of mutation, and, perhaps, on specific cellular environment.

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