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Temperature-Sensitive Mutants of p16^{CDKN2} Associated with Familial Melanoma

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Altered expression or function of the p16^{CDKN2} tumor suppressor gene on chromosome 9p21 occurs in a wide range of human tumors, and mutations in the gene have been shown to segregate with familial predisposition to malignant melanoma. We have used a variety of assays to examine the functional properties of tumor-associated alleles, including eight premature termination mutants, eight missense mutants, and three isoforms of p16 initiated at different amino-terminal methionine codons. The amino- and carboxy-terminal domains of the protein, outside the ankyrin-like repeats, appeared to be dispensable, but the majority of the premature termination mutations led to loss of function. Of the missense mutations tested, four displayed clear loss of function whereas two behaved like the wild type under all conditions tested. The remaining two mutations, a G-to-W mutation at position 101 (G101W) and V126D, both of which are associated with familial melanoma, were found to be temperature sensitive for binding to Cdk4 and Cdk6 in vitro, for inhibiting cyclin D1-Cdk4 in a reconstituted pRb-kinase assay, and for increasing the proportion of G_1-phase cells following transfection. These findings clarify previous disparities and argue strongly that p16^{CDKN2} is a bona fide tumor suppressor associated with familial melanoma.

The *CDKN2* gene (also referred to as *MTS1*, *MLM*, and *INK4a*) on human chromosome 9p21 encodes a 156-amino-acid product, p16, that binds to and inhibits the activity of the cyclin D-dependent protein kinases (Cdks) Cdk4 and Cdk6 (22, 37, 48). As these kinases are thought to regulate progression through the G_1 phase of the cell cycle by contributing to the phosphorylation and functional inactivation of the retinoblastoma gene product, pRb, *CDKN2* has the potential to be a tumor suppressor gene (for reviews, see references 50 and 51). Loss of p16 would lead to increased Cdk4 and/or Cdk6 activity and would promote the phosphorylation of pRb. A large body of data now supports this contention and indicates that alteration of some component of the p16-cyclin D1-Cdk4-pRb pathway is a common feature of human tumors (reviewed in reference 14).

In addition to the mutations and deletions observed in the *RB1* gene itself, both the cyclin D1 and the Cdk4 genes map to regions of DNA that are consistently amplified in certain tumor types (14). For the cyclin D1 gene, most evidence relates to the amplification of the chromosome 11q13 region in breast and squamous cell carcinomas (7), whereas *CDK4* lies in a region of chromosome 12q13 that is amplified in sarcomas and gliomas (25). In addition, cyclin D1 expression can be activated by chromosomal translocations (14), and Cdk4 can sustain mutations that reduce its sensitivity to inhibition by p16^{CDKN2} (60, 64). However, the more recent literature is dominated by reports that the p16 gene is incapacitated in several different ways and in a wide variety of tumors. The mechanisms include transcriptional silencing due to methylation of the CpG island in exon 1α (10, 19, 33, 40), extensive deletions that remove either one copy or both copies of the gene (21, 22, 37; for a review, see reference 14), smaller intragenic deletions and insertions that affect the expression of a functional protein (3, 11, 17, 34, 39, 58, 62), and point mutations that substitute amino acids, cause frameshifts or premature termination of the protein, or affect the splicing of the mRNA (3, 17, 20, 22, 34, 38, 42, 55, 58, 63).

Despite this weight of evidence, the credentials of p16 as a tumor suppressor have been the subject of some controversy (1, 32). Initial interest in the locus stemmed from observations that, in some kindreds, inherited predisposition to malignant melanoma segregated with markers on 9p21 (4, 9, 36). However, not all families that show linkage to 9p21 have detectable mutations in p16 (20, 23, 58). Similarly, numerous studies have shown loss of heterozygosity on the short arm of chromosome 9 in a variety of sporadic tumors, but the frequency of p16 mutations on the remaining allele turns out to be much lower than expected, implying that there could be another tumor suppressor within the region of minimal loss (2, 38, 56). A possible candidate would be the *CDKN2b*/*MTS2*/*INK4b* gene, which is tandemly linked to p16 and encodes a functionally similar protein, designated p15 (12, 15, 21, 22). This now seems unlikely, as most of the data indicate that the mutations affect primarily p16 and that deletion of p15 occurs usually because of a concomitant deletion of p16 (18, 21, 35, 53). An alternative explanation would be the silencing of the remaining p16 allele by methylation or by mutations outside the coding region, but such possibilities have not been fully explored.

A further point of controversy is that the frequency of p16 alterations appears to be higher in cell lines than in primary tumors, leading to suggestions that they reflect a tissue culture artifact (2, 38, 52, 63). The counterargument has been that it is technically more demanding to obtain unambiguous data from heterogeneous-tumor biopsies than from clonal cell lines. Although there may well be an additional selection for p16 loss during the immortalization of cultured cells (16, 29, 47), there is no doubt that p16 alterations occur at significant frequencies in primary tumors (14).

Several groups have tried to confirm the role of p16 as a tumor suppressor by demonstrating that tumor-specific mutations in p16 affect its function (27, 30, 45, 46, 59, 61). While most reports conclude that tumor-associated mutations cause loss of function, there are significant discrepancies in the re-

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ported effects and indications that some mutants behave differently in different assays. The assays include various ways of measuring direct binding of p16 to Cdk4 and Cdk6 in vitro, inhibition of cyclin D-Cdk activity in baculovirus-infected insect cells, protein-protein interaction in *Saccharomyces cerevisiae*, and cell cycle arrest following introduction of p16 into cultured mammalian cells. Here, we describe the most extensive series of tumor-associated mutants analyzed thus far and provide an explanation for some of the disparities in previous reports. Whereas most of the nonsense mutations result in loss of p16 function, the missense mutations produce a range of effects. In particular, two of the missense alleles associated with familial melanoma confer a temperature-sensitive phenotype, so that assays performed at different temperatures can yield conflicting results. Such conditional behavior can be revealed by a simple Cdk binding assay.

MATERIALS AND METHODS

Antisera. Several different antisera against human p16 were used in this work. A rabbit polyclonal antiserum against the carboxy-terminal peptide of p16 (DPAR6) and a mouse monoclonal antibody that recognizes the same domain (DCS-50) have been previously described (30, 41). An additional polyclonal antiserum (DPAR12) was prepared by using bacterially expressed histidine-tagged p16 as the immunogen. This antiserum efficiently precipitated all the p16 mutants described here, including the carboxy-terminal truncations.

Purification of bacterially expressed p16. The p16 coding sequences were cloned into the pRSET-A vector (Invitrogen) for expression in bacteria. The recombinant proteins were expressed in *Escherichia coli* BL21(DE3)pLysS and recovered from insoluble inclusion bodies by solubilization in 6 M urea and affinity chromatography on chelating Sepharose (Pharmacia), using the buffers and conditions recommended by the supplier. The purified proteins were dialyzed to remove urea and concentrated by ultrafiltration. The protein concentration was determined with a Pierce bicinchoninic acid protein assay kit, and the purity of each preparation was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% polyacrylamide gel and staining with PAGE Blue-83. Colored ^{14}C-labeled protein markers (Amersham) were included as molecular mass standards. Histidine-tagged wild-type p16 migrated with an apparent molecular mass of 20 to 21 kDa.

Construction of mutant forms of p16. Two methods were used to generate mutant forms of p16 cDNA. Carboxy-terminal truncations were constructed by using a fixed 5′ oligonucleotide and different 3′ oligonucleotides to amplify the intervening DNA by PCR. The 5′ primer was based on the sequences encoding the first eight amino acids and carried a *Bam*HI site, whereas the 3′ primers each had the requisite termination codon followed by an *Eco*RI site to facilitate cloning into either Bluescript or pRSET-A vector. For the missense mutants, the p16 coding sequences were transferred into M13mp18 to generate a single-stranded template. Mutagenesis was then performed, using mismatched oligonucleotides to prime synthesis of the complementary strand. Subsequent selection for phage containing the mutated DNA strand relied on incorporation of α-S-dATP and selective nicking and degradation of the wild-type strand according to the protocols described by the manufacturer (Amersham). Filling-in and ligation generated double-stranded M13 DNA carrying the required base change on both strands. All the mutations were verified by DNA sequencing.

In vitro binding assays. In vitro binding assays were performed as described elsewhere (13, 41). Briefly, [^{35}S]methionine-labeled Cdk2, Cdk4, and Cdk6 were synthesized by coupled transcription and translation of plasmid DNAs using the TNT expression system (Promega). Similar conditions were used to synthesize either labeled or unlabeled p16 expressed in the pRSET-A vector, which introduces an amino-terminal histidine tag. Samples (2 μl) of the reaction products were mixed and incubated for 30 min at 30°C or at different temperatures as specified below. In some experiments, various amounts of bacterially expressed p16 were added instead of the in vitro-translated protein. After incubation, the mixtures were diluted to 1 ml with ice-cold buffer containing 500 mM NaCl, 1% (vol/vol) Nonidet P-40, and 3% (wt/vol) bovine serum albumin and centrifuged to remove debris, and the supernatants were precipitated with 5 μl of polyclonal antiserum against p16. The immune complexes collected with protein A-Sepharose were analyzed by SDS-PAGE in 12% polyacrylamide gels, and the labeled proteins were visualized by autoradiography.

Kinase inhibition assays. Sf9 insect cells were coinfected with the appropriate recombinant baculoviruses and whole-cell extracts were prepared 48 h postinfection as described by Kato et al. (24). Extracts (5 μl) were preincubated with an equal volume of histidine-tagged p16 (serial 10-fold dilutions) for 15 min at 30°C or at different temperatures as specified. Phosphorylation reactions (final volume, 40 μl) were performed in buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and initiated by adding 100 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 500 ng of glutathione *S*-transferase (GST)-Rb bound to glutathione-Sepharose beads. After 15 min, the reactions were terminated by the addition of 1 ml of ice-cold buffer comprising 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40. The glutathione-Sepharose beads were recovered by centrifugation and boiled in dissociation buffer, and proteins were analyzed by SDS-PAGE in a 10% polyacrylamide gel.

Cell cycle arrest assays. Analyses of cell cycle effects were based on the method described by van den Heuvel and Harlow (57). The U2-OS human osteosarcoma cell line was maintained in Dulbecco modified Eagle's medium containing 10% fetal calf serum. Subconfluent monolayers in 50-mm-diameter dishes were transfected with 10 μg of plasmid DNA encoding p16, or the vector alone, together with 5 μg of a plasmid expressing human CD8 antigen. The transfection protocol using lipofectamine was as described by the manufacturer (Gibco-BRL). The cells were harvested 48 h after removal of the transfected DNA precipitates, stained for CD8 antigen and DNA content, and analyzed by multiparameter flow cytometry. The data are expressed as histograms of 1,000 CD8-positive cells in which cell number (*y* axis) is plotted against DNA content. The results are the averages of two independent transfections, with error bars showing the deviation from the mean.

RESULTS

Effect of variant amino termini on p16 function. In the initial description of p16 as a protein that interacts with Cdk4, the published cDNA sequence encoded a protein of 148 amino acids (48). It was later realized that the open reading frame extended to a more upstream methionine codon and that the resultant translation product would comprise 156 amino acids (references 15 and 39 and unpublished results). This has led to confusion in the literature because of the use of different numbering systems for specific residues; here, we number residues according to the corrected 156-amino-acid sequence. In addition, recent studies have indicated that in some melanoma-prone kindreds, there has been a 24-bp insertion at the amino terminus of the p16 coding domain, duplicating the first eight amino acids (58). Thus, versions of the p16 gene with three different start sites for translation have been reported (Fig. 1a).

To examine whether these differences affect the function of the resultant proteins, we constructed the three variant amino termini by PCR-based amplification and examined the ability of the proteins to bind to Cdk4 and Cdk6 in vitro. The 164- and 156-amino-acid versions of the cDNA were generated in the same reaction, since the 5′ oligonucleotide primer that includes the ATG can hybridize at two different positions in the wild-type p16 sequence (Fig. 1a); a different primer was used to generate the 148-amino-acid form. Each p16 variant was synthesized in rabbit reticulocyte lysates by coupled transcription and translation and mixed with similar lysates programmed by cDNA for Cdk2, Cdk4, or Cdk6. The mixtures were then immunoprecipitated with an antiserum against the carboxy-terminal peptide of p16, and the precipitated proteins were analyzed by SDS-PAGE (Fig. 1b). As previously reported, p16 binds to Cdk4 and Cdk6 in this assay but not to Cdk2 (41). There did not appear to be any differences in the binding properties of the three amino-terminal variants (Fig. 1b) or in their ability to inhibit Cdk4 or Cdk6 kinase activity in vitro (not shown). These data suggest that amino-terminal sequences upstream of the methionine at residue 9 (M-9) are not essential for Cdk binding.

Effect of carboxy-terminal deletions on p16 function. Apart from inherited alleles, the most frequent tumor-associated mutations in the p16 gene, on a per-codon basis, appear to be those leading to premature termination of the protein. For example, R80ter and W110ter mutations have been recorded for multiple tumors, and R58ter has been identified as a predisposing mutation in melanoma kindreds (20). Curiously, among all the possible single base changes that could generate translational stop codons, only a subset have so far been observed in tumors. To determine whether these seven prema-

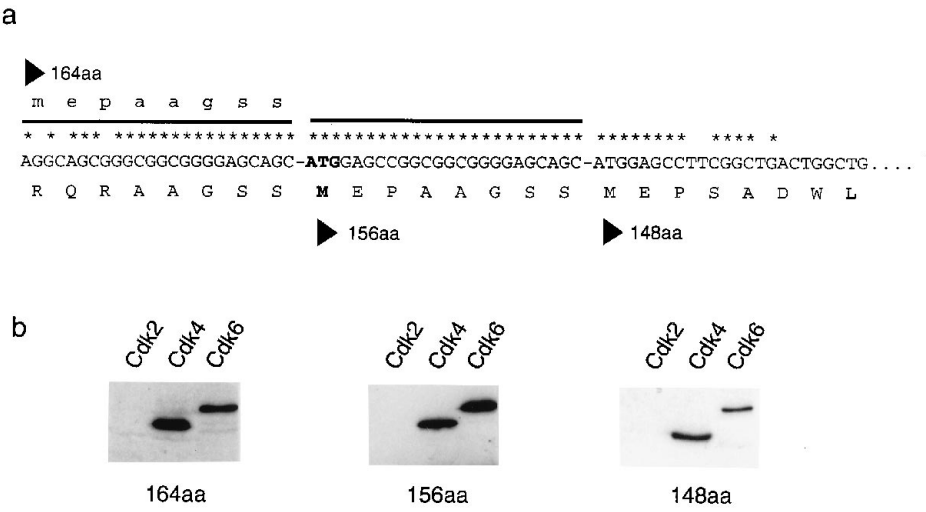

FIG. 1. Cdk binding activity of amino-terminal variants of p16. (a) Nucleotide sequence surrounding the presumed translation start site of p16 (boldface) with the corresponding amino acid sequence in single-letter code. Initiation at this position would specify a protein of 156 amino acids. A second ATG occurs 24 nucleotides downstream, and initiation at this position would specify a protein of 148 amino acids. The bases following this second ATG are very similar to those following the major ATG (identity in 13 of 16 nucleotides, as indicated by the asterisks). Similarly, the 24 bases upstream of the translation start site constitute an almost perfect direct repeat (21 of 24 bases match). Thus, an oligonucleotide based on the first 24 nucleotides of the coding domain (line) can hybridize at two alternative positions, resulting in different PCR products. Priming at the upstream site results in a perfect repeat of the first 24 bp and would yield a product of 164 amino acids. A direct repeat of this type has been linked to familial melanoma (58). (b) The three variants of p16, comprising 164, 156, or 148 amino acids, were translated in vitro and tested for their ability to associate with Cdk2, Cdk4, and Cdk6 in a standard in vitro binding assay. The mixed products were immunoprecipitated with an antiserum against p16 and analyzed by SDS-PAGE in a 15% polyacrylamide gel. The labeled products were detected by autoradiography. aa, amino acids.

ture-termination mutants all cause loss of function, the relevant cDNAs were constructed by PCR, with different oligonucleotides as 3′ primers, and the resultant proteins were tested for binding to Cdk4 and Cdk6. One additional mutation corresponding to termination at the end of exon 2 was included in these analyses. Although this is not a true termination mutant, several tumors have been shown to have mutations affecting splicing of exons 2 and 3, resulting in a product that terminates aberrantly at a stop codon in the intron. For the present purposes, we have considered this termination at D-153.

The eight mutants, effectively representing a carboxy-terminal deletion series, were subcloned into the pRSET-A vector to enhance their translation efficiency and to add an amino-terminal histidine tag. As illustrated in Fig. 2a, the respective proteins were expressed at roughly equivalent levels and could all be immunoprecipitated with a polyclonal antiserum against the full-length histidine-tagged p16 protein expressed in bacteria. The ^{35}S-labeled p16 variants were then mixed with labeled Cdk2, Cdk4, or Cdk6, immunoprecipitated, and analyzed by SDS-PAGE (Fig. 2a). The only variant that retained the ability to bind to Cdk4 and Cdk6 in this assay was the D153ter mutant that extended to the end of exon 2. All the other truncations resulted in loss of binding, including W110ter and E120ter, which retain three complete ankyrin repeats (Fig. 2b).

Effect of missense mutations on p16 function. Over 70 point mutations that result in changes in the amino acid sequence of p16 have been reported, and this number is likely to increase as more tumors are analyzed. However, some of these differences may simply reflect polymorphisms, as confirmed for the A-to-T mutation at position 148 (A148T) which occurs in about 3 to 5% of the population (20, 23). Others are clearly associated with familial melanoma, since they segregate with the disease in several distinct kindreds (20, 23, 58), but most appear to arise sporadically in human cancers. We chose to examine a selection of these missense mutants, including three that are associated with familial melanoma, R87P, G101W, and V126D. Single base changes were introduced into the wild-type cDNA by standard in vitro mutagenesis strategies, and each mutant DNA was then transferred into the pRSET-A vector to introduce an amino-terminal histidine tag. To provide a more quantitative comparison of their properties, each protein was expressed in bacteria and partially purified by chromatography on chelating Sepharose. Analysis by SDS-PAGE confirmed that the different proteins were of equivalent purity and could be precipitated by a polyclonal antiserum against the carboxy-terminal domain of p16.

Cdk binding assays were performed as described above but with 50 ng of each mutant protein. In titration experiments, this amount was shown to be within the linear range for the quantitative coprecipitation of Cdk4 and wild-type p16. Under standard binding conditions, at 30°C, several of the mutant forms of p16 were unable to bind to Cdk4 and Cdk6, including R87P, H98P, and A100P, although there was some residual degree of binding with A100P (Fig. 3a). Mutant P114L was included as a nonbinding control in these assays (30). In contrast, the other missense mutants behaved essentially like wild-type p16. Although this was not surprising in the case of the A148T polymorphism, the two inherited mutations, G101W and V126D, were expected to cause some loss of function. Some groups have reported that these mutants are impaired in their ability to bind to and inhibit Cdk4 (45, 59), while others noted some ambiguity when the G101W mutant was tested in different assays (27, 61).

Temperature-sensitive binding of p16 mutants to Cdk4 and Cdk6. We reasoned that the ambiguous behavior of some p16 mutants might be indicative of a conditional effect, such as temperature sensitivity. The Cdk binding assays were therefore repeated at 42°C to try to accentuate such effects. Significantly different results were obtained with the G101W and V126D mutants, which appeared unable to bind detectably to Cdk4 or Cdk6 at the higher temperature (Fig. 3b). This contrasts with wild-type p16 and the A148T variant, whose binding was unaffected by temperature. Two other missense mutants, G98S and A100V, retained apparently wild-type properties at both

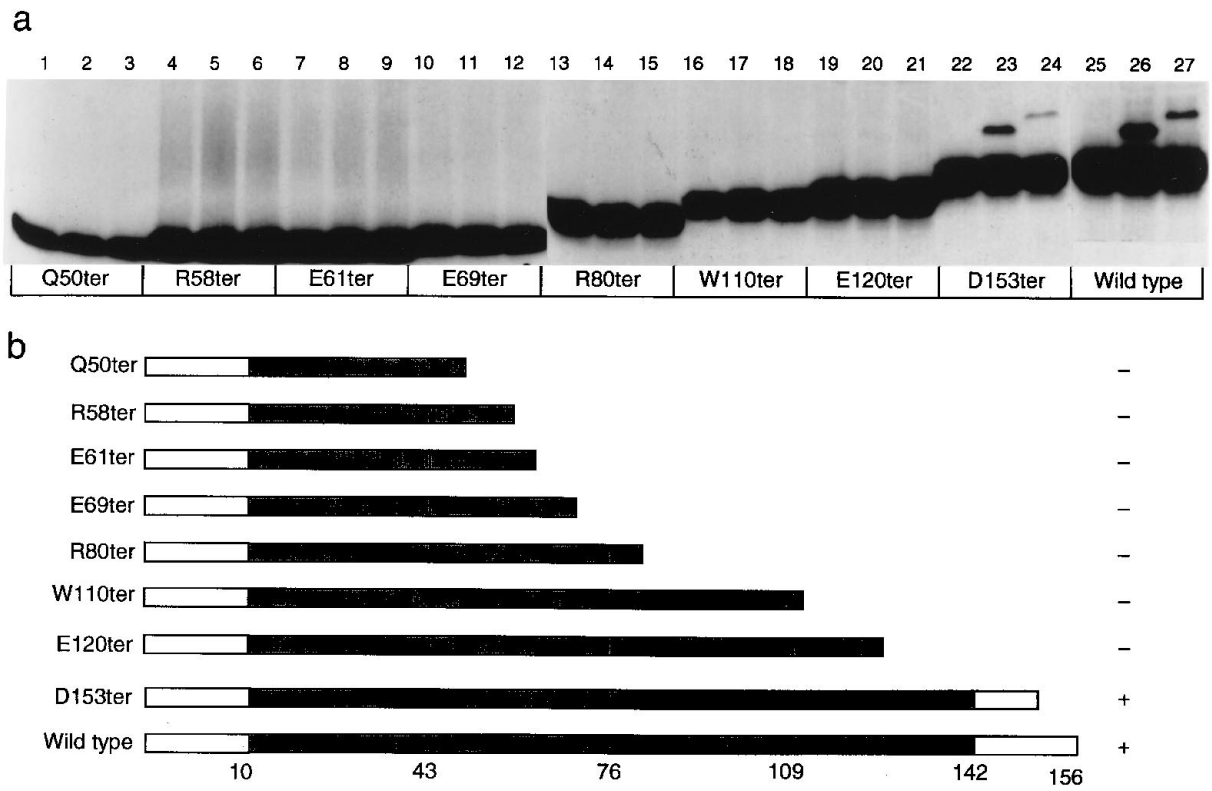

FIG. 2. Cdk binding activity of C-terminally truncated forms of p16. (a) Carboxy-terminal deletions of p16 were generated by PCR using 3′ oligonucleotides that introduced termination codons at specific positions as indicated. These corresponded to tumor-specific mutations, apart from D153ter, which represented termination at the end of exon 2. Each variant was translated in vitro and tested for its ability to bind to Cdk2 (lanes 1, 4, 7, 10, 13, 16, 19, 20, and 25), Cdk4 (lanes 2, 5, 8, 11, 14, 17, 20, 23, and 26), and Cdk6 (lanes 3, 6, 9, 12, 15, 18, 21, 24, and 27). The strongly labeled bands represent the progressively shorter forms of p16 translated from the pRSET vector. Binding to Cdk4 and Cdk6 was observed only with the full-length and D153ter variants, as summarized in panel b. (b) Diagram relating the locations of the termination codons to the four ankyrin-like repeats (shaded boxes), with the corresponding amino acids in the p16 sequence numbered below. The ability (+) or inability (−) of the proteins to bind to Cdk4 and Cdk6 is indicated on the right.

30 and 42°C, suggesting that they are either polymorphisms or sequencing artifacts or that they are affected in some other property of p16. The true loss-of-function mutants, R87P, H98P, and P114L, did not bind to Cdk4 or Cdk6 at either temperature, and any residual activity of the A100P mutant was not apparent at 42°C.

These conditional properties suggested that the p16 variants were likely to show quantitative differences in the Cdk4 and Cdk6 binding assays rather than all-or-nothing effects. The three melanoma-linked mutants were therefore analyzed in more detail and over a range of temperatures. First, the amounts of labeled Cdk4 that coprecipitated with increasing amounts of p16 were quantitated by phosphorimaging. Over the linear range of these assays, for example with 50 ng of purified p16, there was a clear difference in the binding capacities of the mutants assayed at 30°C. As illustrated in Fig. 4, the amount of Cdk4 binding to the G101W mutant was approximately 75% of the amount binding to wild-type p16. With the V126D mutant, this value was around 45%, while the R87P mutant showed virtually no binding at 30°C. When the assays were performed at different temperatures, it was clear that the binding capacities of the G101W and V126D mutants decreased essentially linearly between 30 and 42°C. At 42°C, the binding was reduced to <10% of the wild-type value, but this residual figure reflects a slight increase in the background of the assay at higher temperatures. Interestingly, the activities of the two mutants have a similar temperature dependence; at 37°C, this equates to only 27 and 16% of wild-type activity for G101W and V126D, respectively.

Inhibition of cyclin D-Cdk4 kinase activity by p16 mutants. Although the G101W and V126D mutants were clearly temperature sensitive in their ability to form binary complexes with Cdk4 and Cdk6, we considered it essential to determine whether this also applied to the inhibition of kinase activity of cyclin D-Cdk4 complexes. As previously described, lysates of Sf9 insect cells coinfected with recombinant baculoviruses expressing cyclin D2 and Cdk4 support the efficient phosphorylation of bacterially expressed GST-Rb fusion proteins (24, 41). This activity can be quantitatively inhibited by wild-type p16 but not by a mutant such as P114L that fails to bind Cdk4 (27, 30). To assess their temperature sensitivity, serial dilutions of mutant p16 proteins were preincubated with the Sf9 cell lysates for 15 min at either 30 or 42°C. The appropriate buffer, substrate, and [γ-^{32}P]ATP were then added, and the reactions were allowed to proceed at the different temperatures for a further 15 min. The products were then analyzed by SDS-PAGE (Fig. 5). The inhibitory properties of the G101W and V126D mutants were clearly temperature dependent, whereas those of wild-type p16 and the R87P mutant were not. One additional mutant, A100V, was included in these analyses to confirm that its wild-type properties in the Cdk binding assay could be substantiated in a different assay system.

Inhibition of S-phase entry by p16 mutants. We also tested whether the p16 mutants were conditionally impaired in their

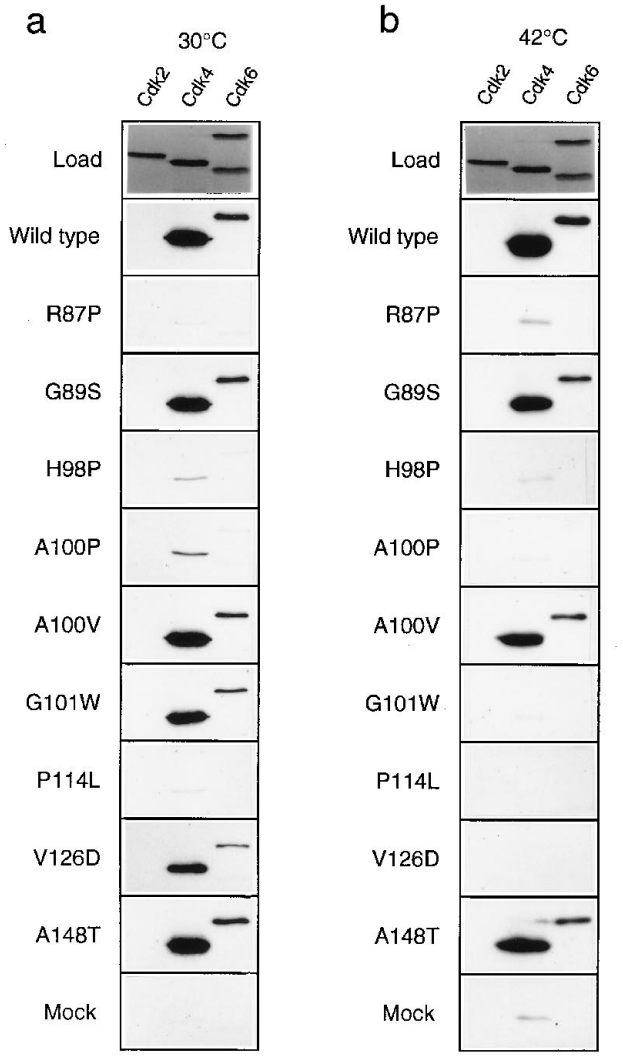

FIG. 3. Cdk binding of missense mutants of p16 at 30°C (a) and at 42°C (b). Missense mutations were introduced into the p16 coding sequences, and the corresponding proteins, carrying a histidine tag, were expressed in bacteria and purified on chelating Sepharose. Equivalent amounts (~50 ng) of each protein were assayed for binding to labeled Cdk2, Cdk4, and Cdk6 synthesized by in vitro translation. Labeled proteins were analyzed by SDS-PAGE in a 15% polyacrylamide gel and visualized by autoradiography. Load, direct analysis of the in vitro translation products prior to binding; Mock, binding reaction with proteins recovered from a mock purification from pRSET-A-transfected bacteria.

ability to induce a cell cycle arrest in transfected cells. To perform these assays, each mutant cDNA was transferred into a eukaryotic expression vector, pcDNA3, so that transcription was directed from a cytomegalovirus promoter. The various plasmids were then introduced into the U2-OS osteosarcoma cell line by electroporation, along with a plasmid encoding the cell surface protein CD8. The cells were maintained at either 34 or 40°C; more-extreme temperatures were found to adversely affect their growth or viability, but the in vitro assays (Fig. 5) suggested that some degree of temperature sensitivity should be apparent at 40°C. Two days after transfection, the cells were stained with propidium iodide and a fluorescein isothiocyanate-conjugated antibody against CD8 and analyzed by flow cytometry. From the combined data, it was possible to calculate the percentage of CD8-positive cells in different phases of the cell cycle. Figure 6 compares the percentages of CD8-positive cells with G_1-phase DNA content at the two temperatures. When cells were transfected with the vector alone, approximately 45 to 50% of them were judged to have a G_1-phase DNA content, whereas this proportion increased to around 70% with a plasmid encoding wild-type p16 (Fig. 6). Although there was a slight temperature effect, with more cells in G_1 phase at 40 than at 34°C, we do not consider this to be significant. Allowing for some variations in the levels of expression achieved with different plasmids, the A100V mutant again appeared to behave like the wild type at both temperatures. However, a very different picture was obtained with the inherited mutations. With G101W, the degree of G_1 arrest at 34°C was close to that with wild-type p16, but the proportion of cells in G_1 was markedly reduced at 40°C. Similarly, the V126D mutant gave an intermediate effect at 34°C but had almost no effect at 40°C. Finally, the R87P mutant had only a marginal effect on the proportion of cells in G_1 at either temperature. Within the limits of this type of assay, these data confirm that the behavior of the temperature-sensitive mutants in vivo is broadly in line with their properties in the in vitro assays.

DISCUSSION

The only known function of $p16^{CDKN2}$ is to bind to Cdk4 and Cdk6 and thereby inhibit their activity as Cdks (15, 41, 48). In contrast to the p21 family of Cdk inhibitors, this binding is direct and results in the formation of binary complexes between p16 and Cdk without the participation of a cyclin (13). With these considerations in mind, we reasoned that of the various assays available with which to test p16 function, the most reliable was likely to be one that depended on semiquantitative measurements of direct binding to Cdks, as shown in Fig. 1 to 4. Experience with the kinase inhibition assay based on expression of the cyclin-Cdk complexes from baculovirus vectors suggests that inhibition of kinase activity by p16 may not be strictly linear (Fig. 5) (27, 41, 48, 61). This could reflect the inability of p16 to disrupt preformed cyclin-Cdk complexes under the conditions employed for the kinase assays, and the true stoichiometry of these inhibition assays has not been properly evaluated.

There are also inherent problems with the G_1 arrest assays, since it is difficult to make them properly quantitative. Ideally, this requires some control for the amount of protein expressed in the transiently transfected and sorted cells, and there is likely to be a considerable degree of cell-to-cell variability. Moreover, even with a cell line such as U2-OS, which has a characteristically small G_1-phase fraction and a large S-phase fraction, the effects amount to $<50\%$ increases in the G_1 fraction compared with that of controls (Fig. 6). Finally, assays based on protein-protein interactions in *S. cerevisiae* (not attempted here) are inappropriate because they have to be performed at temperatures at which the conditional properties of mutants such as G101W and V126D would not be apparent.

We nevertheless considered it essential to validate the in vitro binding data by comparing the p16 mutants in kinase inhibition (Fig. 5) and cell cycle arrest (Fig. 6) assays. In our experience, the results from different assays are consistent. Mutant forms of p16 that fail to bind to Cdk4 and Cdk6, such as R87P, H98P, A100P, and P114L, and the various truncation mutants all failed to inhibit the Rb-kinase activity of cyclin D2-Cdk4 and failed to induce a G_1 arrest in U2-OS cells, at least under the conditions used (Fig. 5 and 6 and additional data not shown). More importantly, the mutants that were temperature sensitive for binding to Cdk4 and Cdk6 also displayed temperature sensitivity in the kinase inhibition and cell cycle arrest assays.

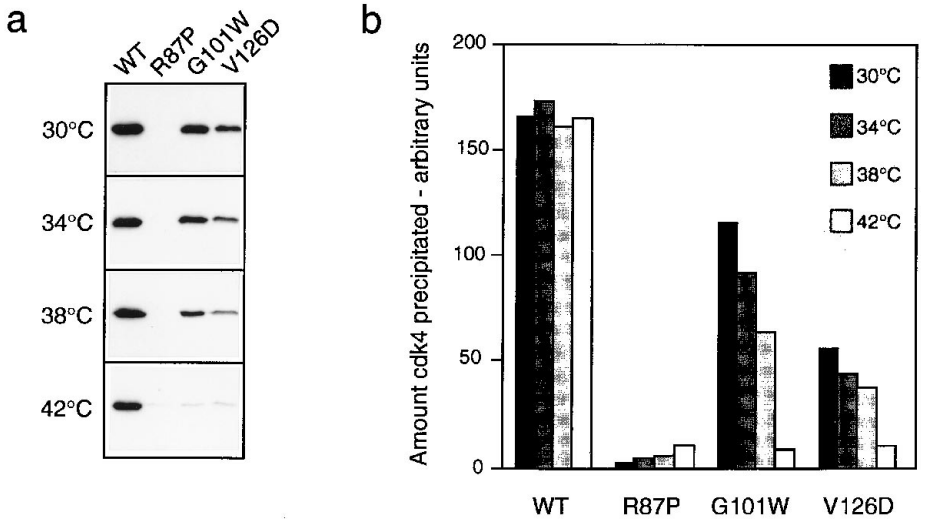

FIG. 4. Temperature sensitivity of p16 mutants. In vitro binding assays were performed using in vitro-translated Cdk4, labeled with [^{35}S]methionine, and 50 ng of partially purified histidine-tagged p16. The assays were performed at the indicated temperatures, and the immunoprecipitated proteins were analyzed by SDS-PAGE in a 15% polyacrylamide gel. The labeled products were visualized by autoradiography (a), and the amounts of labeled Cdk4 coprecipitating with each variant of p16 were quantitated by phosphorimaging (b). WT, wild type.

The two mutations that confer temperature-sensitive properties on p16 map in the third and fourth of the four ankyrin-like repeats that make up the bulk of the protein (48). This suggests that the overall structure or folding of p16 must be critical for binding to Cdks, and the fact that other loss-of-function mutations are distributed throughout the molecule agrees with this conclusion. Moreover, virtually all of the prematurely terminated forms of p16 had lost binding activity (Fig. 2). The exact requirements for C-terminal sequences have not been defined, but the related p15^{CDKN2b} protein, which terminates at the equivalent of T-137 in p16, is fully functional (12, 15, 21, 43, 53; additional data not shown). Conversely, Yang et al. reported that deletion to R-131 or removal of the fourth ankyrin repeat abolishes Cdk binding (61). Taken together, the data implicate the sequences between E-120 and T-137 as essential for the functional integrity of p16. However, it seems unlikely that this region is directly involved in the interaction with Cdks. Recently, Fåhraeus et al. have defined a 20-amino-acid peptide from the third ankyrin repeat of p16 that is able to bind to and inhibit the activity of Cdk4 (6). Presumably, this will represent a minimal interaction domain that is contextually influenced by the remainder of the protein.

The variant amino termini had little influence on the properties of the resultant protein (Fig. 1). Indeed, some previous studies of p16 mutants have been based on the sequence be-

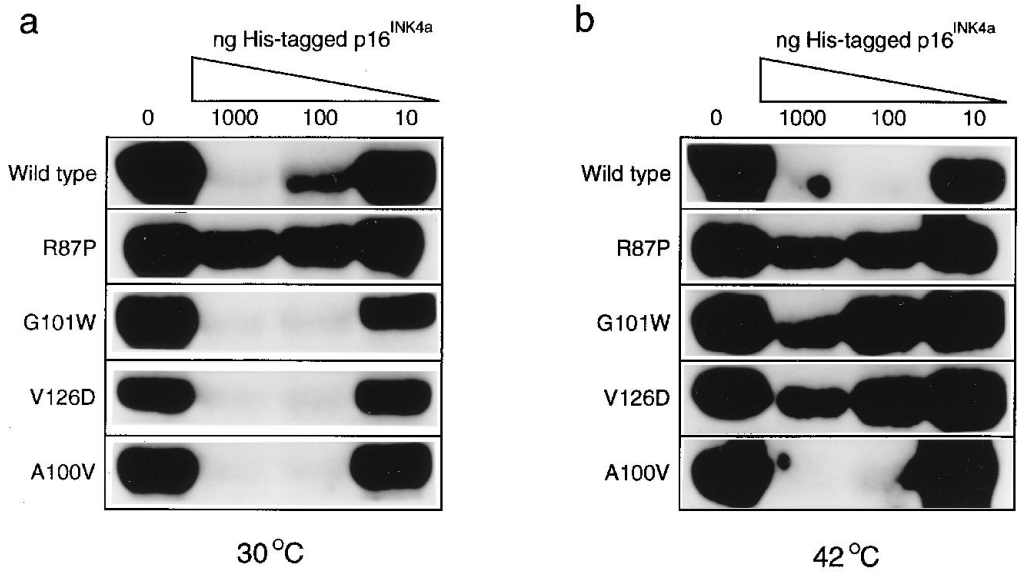

FIG. 5. Inhibition of cyclin D2-Cdk4 kinase activity by mutant forms of p16. Cyclin D2-Cdk4 complexes were generated by coinfecting Sf9 insect cells with recombinant baculoviruses, and the cell lysates were incubated with increasing amounts of bacterially expressed p16 (as indicated above the panels) at either 30 or 42°C. The ability of these complexes to phosphorylate GST-Rb fusion protein at 30°C (a) or 42°C (b) was assayed as previously described (24, 41).

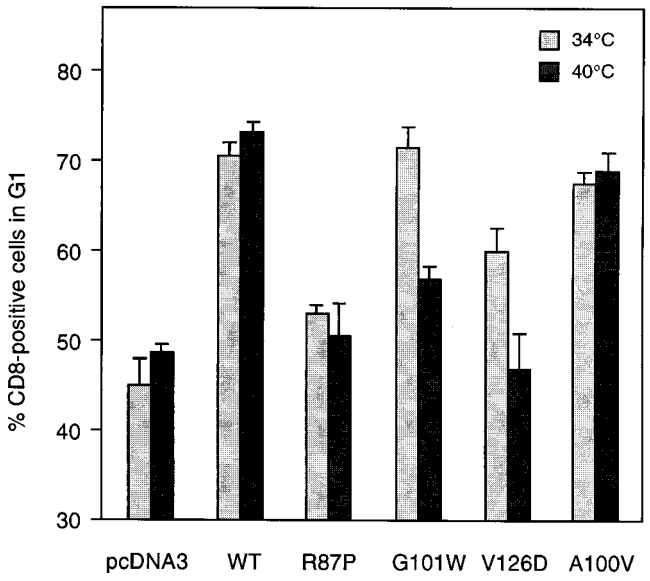

FIG. 6. Inhibition of S-phase entry by mutant forms of p16. U2-OS human osteosarcoma cells were transfected with equivalent amounts of either vector alone (pcDNA3) or plasmids encoding wild-type (WT) or variant forms of p16, as indicated. A plasmid encoding CD8 was included as an indicator of successfully transfected cells. After 48 h, the cells were stained with a fluorescein isothiocyanate-conjugated antibody against CD8 and with propidium iodide to measure their DNA content. The cells were then analyzed by multiparameter flow cytometry, and the data were expressed as the proportion of CD8-positive cells with a G_1 DNA content. The results are averages of two independent transfections, with error bars showing the deviations from the means.

ginning at M-9 (45, 59). Since the first residue that clearly conforms to the ankyrin repeat consensus is L-16, it would be interesting to determine whether further truncations have any effect on the properties of the protein; in this study, we confined the analyses to naturally occurring variants. One of these variants, with the insertion of 24 bp, has been shown to segregate with familial melanoma (58), yet the protein appears to be functional, at least in the assays used. Since our constructs were engineered for efficient expression, further work will be required to determine whether the additional sequences affect the efficiency of either transcription or translation or some as-yet-undefined property of the protein.

Another four of the mutations analyzed segregate with familial melanoma, R58ter, R87P, G101W, and V126D (20, 23). All of these confer complete or conditional loss of function, confirming the status of p16 as a tumor suppressor. Although similar conclusions have been drawn by others (27, 45, 46, 59, 61), there are some serious discrepancies between the different reports. This is particularly applicable to G101W, which in some studies was classified simply as a loss-of-function mutation (45, 59) whereas in others it was regarded as partially impaired or gave conflicting results in different assays (27, 46, 61). These disparities can be largely explained by the temperature-sensitive behavior of G101W. Moreover, our data suggest that the direct Cdk binding assay provides the simplest test of conditional behavior, since it can be readily performed over a range of temperatures.

One of the reasons for analyzing tumor-specific forms of p16 is to distinguish between true loss-of-function mutations and rare polymorphisms. In the present study, we identified two changes that had no discernible effect on the function of p16 (G89S and A100V), and several others have been noted previously (27, 45, 46, 61). Some, such as A148T, are now recognized as polymorphisms, but the growing number of apparently neutral alterations implies that the gene is extremely polymorphic. Alternatively, such base changes might occur in tumors because of mutations elsewhere in the genome that affect the fidelity of DNA replication. Another possibility is that the neutral mutations in p16 might affect the product of the alternatively spliced transcript derived from the *CDKN2* locus (5, 31, 44, 54). Transcripts incorporating exon 1α encode p16, whereas those incorporating exon 1β have the capacity to encode a completely unrelated protein, $p19^{ARF}$, in a different reading frame (44). Of the neutral variants analyzed here, the G89S change in p16 would equate with a G-to-E change in $p19^{ARF}$, whereas the A100V mutation would not affect the coding capacity of the alternative reading frame.

In summary, the in vitro binding assay can reveal mutations in p16 that cause complete, conditional, or no loss of function, and it will be interesting to extend these analyses to the increasing number of mutations reported in the literature. We are particularly intrigued about the existence of other temperature-sensitive alleles and the physiological processes that might reveal their conditional behavior in vivo. Why should germ line G101W and V126D mutations confer a predisposition to melanoma? The preliminary evidence from $CDKN2a^{-/-}$ mice suggests some connection between p16 function and melanocyte differentiation, but the nullizygous mice sustain fibrosarcomas and lymphomas rather than melanomas (49). Moreover, the spectrum of sporadic mutations found in human melanomas supports a role for UV light (28, 42), consistent with a link between melanoma and chronic sun exposure. Since skin temperatures are normally several degrees lower than that of the body core, the temperature-sensitive forms of p16 are likely to retain some of their activity (Fig. 4), although this will depend on the location on the body and whether it is clothed or unclothed. For example, a temperature-sensitive form of tyrosinase has been shown to underlie the variable peripheral pigmentation seen in type 1 oculocutaneous albinism, so that warm areas show lighter pigmentation and cool areas are darker (8, 26). It might therefore be interesting to determine whether melanomas show different distributions in kindreds carrying temperature-sensitive mutations versus those with temperature-insensitive lesions.

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