p16 proteins from melanoma-prone families are deficient in binding to Cdk4

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The tumor suppressor candidate p16$\text{INK4a}$ is a cyclin-dependent kinase inhibitor that inhibits cell proliferation. The p16 coding gene is often mutated in glioblastomas, pancreatic adenocarcinomas and melanoma-prone pedigrees, but, until recently, the significance of these allelic variants has remained unclear. Here, we used interaction mating and coprecipitation to measure interaction of seven p16 allelic variants detected in melanoma-prone pedigrees with Cyclin-dependent kinases (Cdkks). We found that most variants were deficient in interaction with Cdk4 and Cdk6. One defective variant was found in both cancer prone families and in the control population and therefore previously defined as a common polymorphism. Another variant, which is weakly linked to familial cancer, is only slightly affected in interaction with Cdk6. These results are consistent with the idea that p16 allelic variants that decrease Cdk interaction predispose individuals who carry them to an increased risk of cancer. Moreover, they suggest that determination of affinity between p16 mutants and partner proteins may help identify functionally-significant allelic variants not detected by classical human genetic techniques.

Keywords: p16$\text{INK4a}$; Cdk4; Cdk6; Cki, melanoma; two-hybrids

Introduction

Cell cycle transitions are promoted by sequential activation of cyclin-dependent kinases, which consist of a catalytic subunit called Cdk (5), and activating subunits called Cyclins. Activity of these proteins is orchestrated by transcription of specific Cyclins, specific phosphorylation and dephosphorylation events, and association with Cdk-inhibitory proteins (Ckis) (reviewed in Hunter and Pines, 1994; King et al., 1994; Sherr, 1994; Morgan, 1995).

One Cki, p16$\text{INK4a}$ (p16), specifically binds to and inhibits the activity of Cyclin D/Cdk4 complexes, and thus regulates cellular proliferation (Serrano et al., 1993; Serrano et al., 1995). This small protein is composed of four ankyrin repeats, protein motifs which have been shown to direct protein-protein interactions between the $\alpha$ and $\beta$-subunits of the $\alpha$-binding protein (Thompson et al., 1991), between Cactus and Dorsal (Kidd, 1992), and between cdc10p and acl1p (Reymond and Simanis, 1993). p16 maps to chromosome 9p21, the site of the multiple tumor suppressor (MTS1) locus, which is rearranged, deleted, or mutated in many tumor derived cell lines (reviewed in Kamb, 1995), suggesting that p16 might be the MTS1 product. Consistent with this idea, p16 genes isolated from primary tumors often show sequence differences compared to wild-type. Moreover, p16 is often mutated in members of melanoma-prone families. However, the functional significance of these variations has not yet been assessed (reviewed in Kamb, 1995). Recently, p16 has also been shown to inactivate complexes containing cdk6, but the significance of this inhibition is so far not known (Hannon and Beach, 1994).

Here, we examined interactions between Cdkks and p16 variants isolated from melanoma-prone families. Six of these variants are thought to be bona fide mutants linked to the development of melanoma, and two of them 'common polymorphisms', also found in the general population (Hussussian et al., 1994). We assayed binding by both interaction-mating and in vitro binding assays. No allelic variant showed a broadened specificity of interaction. All but two allelic variants were also deficient in binding Cdk6. All but one allelic variant, including one encoded by a common polymorphism, showed decreased affinity for Cdk4 and did not compete with cyclin D1 for Cdk4 binding.

Our results suggest that decreased affinity of p16 for Cdk4 and Cdk6, and its resulting inability to compete with Cyclin D for binding, contributes to tumorigenesis. Moreover, for cases in which mutants of p16 are only impaired in their binding to Cdk4, they suggest that loss of inhibition of Cdk4 is the mechanism by which these alleles lead to tumorigenesis. They also suggest that the p16 common polymorphism that shows diminished binding to Cdk4 may also contribute to human cancer, even though it is also found in the population at large. Because the interaction mating technique used here simplifies the task of assaying binding by different alleles, our results suggest that screening for p16-Cdk4 interactions in yeast provides a functional test to reveal other alleles of p16 that may contribute to cancer, and may provide a useful supplement to more established human-genetic techniques.

Results

We studied a set of seven p16 mutants isolated from melanoma-prone families. We used interaction mating, an extension of the interaction trap two-hybrid system (Finley and Brent, 1994), to characterize the interaction of these proteins with a panel of cyclin dependent kinases.

Here, we name the mutations according to their distance from the ATG that begins the coding sequence. Because the first published sequence of p16 assigned an incorrect ATG, our numbering system...
differs from that used by Hussussian et al. (1994) and Ranade et al. (1995). Four of the p16 allelic variants: p16-N71S, -R87P, -G101W and -V126D (formerly, -N63S, -R79P, -G93W, and V118D) are missense mutations transmitted through the germ line of individuals from affected families. Two of them, p16-I49T and -A148T (formerly, I41T and A140T), are found in affected families, but are also found in members of the control population, and are thus termed 'common polymorphisms' (Hussussian et al., 1994). The last variant, p16-P81L (formerly, P73L), is a missense mutation that apparently arose somatically in a melanoma-prone pedigree. For the interaction matings, the reporter gene was the medium-high sensitivity pSH18-34 (8 LexA operators, Hanes and Brent, unpublished, Finley and Brent, 1994), which in haploid cells detects interactions between lambda repressor C termini with Kd < 10⁻⁶ M (Estojak, Brent, and Golemis, submitted), but whose sensitivity is somewhat reduced in diploids (Finley and Brent, 1994; Finley, personal communication).

The interaction mating experiments (Figure 1A and 1B) show that, as previously reported, wild-type p16 specifically interacts with Hs Cdk4 and Hs Cdk6, but...
p16 mutants defective in Cdk interaction
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Figure 1. p16 melanoma-prone family mutants show decreased affinity for Cdk4 and Cdk6. (A) Interaction-mating assays between strains carrying Cdk4s (baits) and melanoma-prone families p16 mutants (preys). Bait strains containing plasmids that expressed LexA fusions to Homo sapiens Cdk2, Cdk3, Cdk4, Cdk6, and Ca and Saccharomyces cerevisiae CDC28 were mated to EGY48 derivatives that contained B42 fusions to p16 or p16 algal variants. Plates were ura-1 his-1 trp X-gal and contain either glucose or galactose/raffinose. (B) Interaction-mating assays between strains carrying melanoma-prone families p16 mutants (baits) and Cdk4 (preys). Bait strains containing plasmids that expressed LexA fusions to p16 or p16 algal variants were mated to EGY48 derivatives that contained B42 fusions to Homo sapiens Cdk2, Cdk3, Cdk4, Cdk6, Saccharomyces cerevisiae CDC28 and Drosophila melanogaster Cdc2c. Plates are as in panel A. (C) β-galactosidase activity induced by interactions between Cdk4 and p16 WT or p16 mutants. Activities are normalized to the β-galactosidase activity induced by the Cdk4-p16 wild type interaction. (D) β-galactosidase activity induced by interactions between Cdk4 and p16 WT or p16 mutants. Activities are normalized to the β-galactosidase activity induced by the Cdk4-p16 WT interaction.

not with Cdk4 bait occupancy by the p16-P81L, -R87P, -G101W and -V126D proteins was decreased to 70 to 95% compared to wild type p16 (Figure 1C). Surprisingly, p16-I49T, previously defined as a common polymorphism, based on its occurrence in individuals not genetically related to melanoma gene carriers, showed a similar decrease in occupancy, showing that its affinity for Cdk4 is also impaired. By contrast, the other allele variant defined as a common polymorphism, p16-A148T, showed no impairment in its interaction with Cdk4. One of the germline mutations p16-N71S, retained substantial affinity for Cdk4 (20% decrease in Cdk4 occupancy).

Most variant proteins also showed decreased interaction with Cdk6. For example, LexA-Cdk6 bait occupancy by p16-N71S, -P81L, -R87P, and -V126D, was drastically reduced compared to wild type, while binding by the p16-I49T and -A148T polymorphisms was almost unimpaired. However, one protein, p16-G101W, which showed decreased interaction with Cdk4, interacted normally with Cdk6 (Figure 1D).

p16 interacts with Cdk4 in the absence of Cyclin D (Xiong et al., 1993; Serrano et al., 1993) and competes with Cyclin D for binding to Cdk4 in vitro (Parry et al., 1995). The decreased affinity of the melanoma-prone family p16 allelic variants for Cdk6 suggested that they should be impaired in their ability to compete with cyclin D for binding to Cdk4. To test this idea, we used an in vitro association assay. We added increasing amounts (721 fM to 721 nM final concentration) of GST-p16 or GST-p16 variants to mixtures of Cyclin D1 and Cdk4 made by translation in vitro, and quantitated the amount of Cdk4 that remained associated with Cyclin D1. As shown in Figure 2A...
and 2B, immunoprecipitations with an anti-cyclin D1 antibody show that the level of Cyclin D1-associated Cdk4 was decreased proportionally to the amount of GST-p16 or GST-p16-A148T 'common polymorphism' added to the mixture. By comparison, it required 50 times as much of the p16-N71S mutant, and >100 times as much of the p16-P81L and -G101W mutants, and of the p16-149T common polymorphism, to compete half-maximally for Cyclin D1. No concentration of the p16-R87P and -V126D mutants competed cyclin D1.

Because the interaction mating experiments had shown that p16-G101W was specifically impaired in interaction with Cdk4, and not with Cdk6, we tested its ability to associate with Cdk6 in vitro. We added increasing amounts of GST-p16, GST-p16-R87P, or GST-p16-G101W to Cyclin D1 and Cdk6 translated in vitro, and quantitated the ability of the fusion proteins to block cyclin D binding as above. By contrast with wild type GST-p16, GST-p16-R87P was unable to compete with Cyclin D1 for Cdk6 binding (Figure 3A and 3B). Competition was also achieved by increasing amounts of GST-p16-G101W, confirming that this allelic variant is not impaired in Cdk6 binding.

Discussion

We measured binding of allelic variants of p16 to Cyclin Dependent Kinases. Both interaction mating experiments suggested that all p16 allelic variants are unable to bind Cdk6/Cyclin D1, whereas p16-WT and p16-R87P bound Cdk6/Cyclin D1 with similar affinity. As the former two genes are in such allelic families, the above data are not yet consistent with the hypothesis that p16 alleles have different affinity for Cdk6/Cyclin D1.

In a next step we suggest that the allelic variants p16-R87P and p16-G101W may compete with each other for Cdk6/Cyclin D1. These data may functionally explain that p16 alleles may behave like gene products of one gene.

Our results (Rana et al., 1996) suggest that p16 alleles may be involved in binding Cdk6/Cyclin D1, whereas p16-WT and p16-R87P bound with similar affinity. As the binding of p16-R87P to Cdk6/Cyclin D1 is diminished, the idea that p16-R87P is affected by a 1D, and the other allele retains its binding ability, is not supported. This suggests that p16-R87P and Cdk6/Cyclin D1 may bind with reduced affinity and could be a consequence of the disease.

On the basis of the existing data, we propose that the V126D allele is the only allele of all p16 alleles that is not impaired in its interaction with Cdk6. This is consistent with data showing that V126D is a dominant negative allele with respect to its interaction with Cdk6. The data presented here also support the hypothesis that p16 alleles are able to compete with each other for Cdk6/Cyclin D1 binding, and that this competition may contribute to the disease process.
experiments and in vitro competition experiments show that affinity for Cdk4 is strongly diminished for four allelic variants (p16-P81L, -R87P, -G101W and -V126D) found specifically in melanoma-prone families, and for one allelic variant (p16-I49T), found in such families but also in the control population, and thought to be a common polymorphism. One of these allelic variants, p16-G101W, is not diminished in its affinity for Cdk6. Another allelic variant, p16-N71S, shows only a slight decrease in its affinity for Cdk4.

In summary, both in vitro and in vivo experiments suggest that the melanoma-prone family p16 mutants are functionally defective in their ability to inhibit Cdk4/Cdk6 kinase activity. Their impaired affinity for Cdk4 and Cdk6 results in a decreased capability to compete with Cyclin D for binding to these kinases. These results support the notion that loss of p16 function may contribute to tumorigenesis and suggest that p16 is an excellent candidate for the MTS1 locus gene product.

Our results differ in two regards from a recent study (Ronad et al., 1995). First, Ronad et al. deemed the allelic variant p16-I49T a common polymorphism, whereas we showed that it has an impaired ability to bind Cdk4 and compete with cyclin D1. However, it is worth noting that our interaction results (Figure 1C) and the kinase assays of Ronad et al. in fact gave very similar results: we show that p16-I49T retains 43% of the binding activity of p16 (Figure 1C) while Ronad et al. show that p16-I49T retains about ~50% of the kinase inhibitory activity of p16-6T, consistent with a diminution in its binding function. Consistent with this idea, our communoprecipitation experiments (see Figure 2B) also show that the allelic variant p16-I49T is affected in binding. Second, our experiments (Figure 1D, and Figure 3) clearly show that p16-G101W retains binding activity to Cdk6, while Ronad et al. show that purified p16-G101W apparently does not inhibit Cdk6/cyclin D1 kinase activity in vitro. If the p16-G101W used by Ronad et al. was active, this result suggests that this allelic variant can bind Cdk6, and compete for cyclin D1 binding without inhibiting its kinase activity, a possibility we are investigating.

On the other hand, our findings correlate well with existing human genetic data. Consistent with our interaction data, possession of p16-R87P, -G101W or -V126D alleles is highly correlated with melanoma in familial pedigrees, whereas possession of p16-N71S is only weakly correlated (Hussussian et al., 1994). Moreover, because our results show that the p16-G101W protein is specifically affected in binding to Cdk4, but not Cdk6, our results strongly suggest impaired interaction with Cdk4 is the mechanism by which this mutant contributes to cancer. However, there is one case in which our results differ from those predicted by the human-genetic studies. Our results show that the p16-I49T allele, which occurs in both melanoma-prone pedigrees and in control populations (Hussussian et al., 1994), is deficient in interaction with Cdk4. This result suggests that p16-I49T may well dispose individuals who carry it to increased cancer risk. We suggest that the interaction-mating assay used in this study will facilitate demonstration of loss-of-interaction for allelic variants of p16 and of other proteins. It is thus possible that interaction mating assays may augment existing human genetic strategies by providing alternative means to detect weakly penetrant mutations, and those that are not confined to genetically-distinct family cohorts.

Materials and methods

Plasmids

LexA- and B42- containing plasmids are derivatives of pEG202 and pJG4-5 plasmids respectively (Gyuris et al., 1993; Zervos et al., 1993). All were constructed by standard techniques (Ausubel et al., 1987–1995). All sequences generated by PCR were confirmed by direct DNA sequencing.

LexA derivatives (baits)

pLexA-p16: Ecor1I/Hind3I endl ended fragments that contained the entire coding regions for p16 or p16 allelic variants were introduced into the Ecor1I/Hind3I site of pEG202AAAT, a modified version of the plasmid pEG202, which adds one nucleotide upstream of the Ecor1 restriction site and thus directs the synthesis of fused moieties in a different frame (E. Golemis, unpublished; Gyuris et al., 1993). pLexA-Cdks: Ecor1I/Hind3I, Ecor1I/Hind3I or Hind3I/SalI fragments DNAs that contained the coding regions for Cdkks were cloned in the Ecor1I/SalI site of pEG202 (Gyuris et al., 1993; Finley and Brent, 1994 and this work).

B42 derivatives (preys)

pB42-p16: EcoRI/XhoI ended coding regions for p16 or p16 allelic variants, as above, were cloned into pBG4-5AT, a modified version of the plasmid pJG4-5, which expresses fused moieties in a different frame due to the fact that it carries an additional A upstream of the EcoRI restriction site (E. Golemis, unpublished; Gyuris et al., 1993). pBG2-Cdks: coding regions for Cdkks were cloned into EcoRI/XhoI pJG4-5, as above (Gyuris et al., 1993 and this work).

Yeast manipulations

Standard microbiological techniques and media were used throughout (Guthrie and Fink, 1991; Ausubel et al., 1987–1995). Yeast minimal dropout media are designated by the nutrients component(s) left out. They contain, in addition to the dropout mix, either 2% glucose or 2% galactose/1% raffinose as a carbon source. X-Gal minimal drop out plates contained X-Gal and phosphate buffer at pH 7.0. (Ausubel et al., 1987–1995). Yeast transformation was carried out as described by Gietz et al. (1992). Interaction mating assays were performed using the yeast strains RPY206 (Mato his3Δ100 leu2-2 lys2Δ201 met15-801 trp1Δ1, hinG) and EGY48 (Mata his3 leu2-3 lys2Δ201 met15-801 trp1Δ1, hisG) that contained the pSH18-34 reporter plasmid as described in Finley and Brent (1994).

Expression and purification of p16 proteins

Coding regions for p16 or p16 allelic variants were cloned in frame into pGEX4T (Pharmacia). E. Coli BL21 carrying the plasmids were grown to an OD600 of 0.5 at 37°C, IPTG was added to the media to a final concentration of 0.33 mM and the culture was grown 10 h at 20°C. Cells were harvested by centrifugation, frozen and thawed twice by immersing the tubes in dry ice and a 37°C waterbath, and resuspended in 1/50 of the starting culture volume of PBS pH7.2, 5 mM EDTA, 1 mM DTT, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin A, 1 mM PMSF and 2 mg/ml lysozyme (Sigma). After 30 min on ice,
Triton X-100 was added to a final concentration of 1% and the cells were sonicated until the viscosity was reduced. The supernatant was collected by centrifugation and the protein was bound to 100 μl of Glutathione-sepharose resin (Pharmacia) for 60 min. The resin was washed three times with washing buffer (WB: PBS, 1% Triton X-100, 5 mM EDTA, 1 mM DTT, 2 μg/ml aprotopin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM PMSF), three times with WB containing 250 mM KCl and equilibrated by two washes with 50 mM Tris-HCl pH 8.0, 25% glycerol. GST fusion proteins were eluted in 50 mM Tris-HCl pH 8.0, 10 mM glutathione, 25% glycerol, aliquoted and stored at -80°C. Protein quantitation was done by the Bradford Coomassie dye-binding method according to the manufacturer's instructions (BioRad).

**In vitro binding assays**

Fragments that carried the coding sequence for human Cdk4, Cdk6 and Cyclin D1 were subcloned into pGEM derived plasmids (Promega) to allow transcription with the T7 RNA polymerase. Proteins were synthesized in vitro using the T7-coupled transcription/translation system and L-[35S]methionine according to the manufacturer's instructions (Promega). Samples were incubated for 30 min at 30°C. Equal amounts of in vitro translated Cyclin D1 and Cdk4 were mixed with GST-p16 fusion, diluted in 3 μl of 20 mM Tris-HCl pH 8.0, 150 mM NaCl, and incubated for 30 min at 30°C. Volume was then adjusted to 500 μl with ice cold 20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.25% Nonidet P40 (Sigma). After centrifugation the supernatant was immunoprecipitated with 5 μg of a rabbit anti-human cyclin D1 antibody (UBI). The immune complexes were collected with protein A-Sepharose as described, run on 10 or 12% SDS acrylamide gels (Protogel, National Diagnostics), stained and visualized and quantitated using a PhosphoImager (Molecular Dynamics).

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**References**


