[CANCER RESEARCH 54, 6353–6358, December 15, 1994]

Advances in Brief

Deletion of *p16* and *p15* Genes in Brain Tumors[1]

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Abstract

We have used molecular genetic methods to examine the status of cell cycle-inhibitory genes in human brain tumors. We found that *p16* and a neighboring gene, *p15*, were often homozygously deleted in glioblastoma multiformes but not in medulloblastomas or ependymomas. The deletions occurred in both primary tumors and their derived xenografts, but no intragenic mutations in either of the two genes were found. The *p15* gene was expressed in a more widespread pattern in normal tissues than *p16*, but the products of both genes had similar capacities to bind to cyclin D-dependent kinases 4 and 6. These data suggest that the target of deletion in glioblastoma multiforme includes both *p15* and *p16* genes. The reason that homozygous deletions, rather than intragenic mutations, are so common in these tumors may be that deletion is a more efficient mechanism for simultaneous inactivation of both genes.

Introduction

The Cdks[3] are essential enzymes with activities that are tightly regulated by protein phosphorylation and association with other proteins, particularly the cyclins (1, 2). Sequential activation of the cyclin-Cdk complexes is thought to be responsible for orderly transitions through the cell cycle. The abnormal activation of Cdk activity, through a variety of mechanisms, is expected to underlie part of the uncontrolled growth that characterizes cancer.

Two classes of genes whose products can inhibit Cdks have recently been identified. The first class consists of *p21* [also called *CIP1, WAF1, SDI1, CAP20* (3–8)] and *p27* [also called *KIP1,* (9–11)] which have a high degree of sequence similarity and can inhibit a variety of Cdk subtypes. The expression of p21 is regulated by p53, providing a link between tumor suppressor genes and cell cycle regulation (5). The second class is represented by *p16* (also called *MTS1*), which encodes a protein with ankyrin-like repeats that can inhibit Cdk4 and Cdk6 but not other Cdks (12, 13). Cdk4 and Cdk6 are closely related in primary sequence and are both activated by D type cyclins during G_1 (14, 15).

Previous studies have demonstrated that many tumors, particularly melanomas, leukemias, and gliomas, contain hemizygous or homozygous deletions of chromosome 9p21 (16–18). Additionally, a gene responsible for melanoma predisposition has been mapped to this same chromosomal region by linkage analysis (19). The *p16* gene resides within the deleted 9p21 region within these tumors (20, 21). Subtle mutations of the *p16* gene have been reported in a variety of tumor cell lines and in some primary tumors (20–23). Moreover, germline alterations of the *p16* gene have been found in a subset of patients with familial melanoma (21, 24, 25). There has been considerable controversy over the role of *p16* in neoplasia, in part because of conflicting results that have been reported (20–29).

In this study, we have searched for genetic alterations of *p16* in human brain tumors. During the course of this work, we were led to characterize a neighboring gene, *p15,* that proved to have biochemical properties similar to those of *p16.* Our results suggest that both of these genes may play a role in glioblastoma tumorigenesis.

Materials and Methods

Tumor and Nucleic Acid Sources. Brain and colorectal tumor xenografts were established in nude mice as described previously (30). DNA from primary and xenografted tumors were prepared by standard methods (31). RNA from normal human adult tissues and GM xenografts were purified by a guanidine isothiocyanate based method (Promega) following the manufacturer's instructions. Placenta and HeLa cell polyadenylated mRNA were purchased from Stratagene (San Diego, CA).

PCR Amplification and Sequencing of Tumor DNA. Primers used to amplify *p16* exons are listed in Table 1. All PCR reactions were carried out in 50-μl reactions using 100 ng genomic DNA. The PCR solutions have been described (32), and the reaction used a 95°C, 2-min initial denaturation step followed by 30 to 35 cycles at 95°C for 30 s, 58°C for 1 min, and 70°C for 1 min. The PCR products were separated by electrophoresis on 1.5% agarose or 10% nondenaturing polyacrylamide gels. Mouse spleen DNA was used to ensure that the PCR primers were specific for human *p16.* Sequencing of the PCR products was performed with SequiTherm Polymerase (Epicentre) as described by the manufacturer, using the primers listed in Table 1. Amplification of *p15* was carried out similarly, using the primers described in Table 1. A 500-base pair genomic Cdk4 fragment (33) was amplified in parallel PCR experiments to control for the integrity of DNA samples.

***p15* cDNA Cloning.** The 5′ end of the *p15* cDNA was obtained through the rapid amplification of cDNA ends procedure, using placenta cDNA as template (34, 35). The rapid amplification of cDNA ends anchor primer (Clontech) was combined with either primer P15-2R or primer P15-S4 (Table 1) for the first and second rounds of PCR amplification, respectively. A PCR product of about 800 base pairs generated after the second PCR amplification was cloned directly into the pCRII vector (Invitrogen). The sequences of the clones were determined using T7 and SP6 primers as well as additional primers from the *p15* gene as its sequence was generated. The sequence was confirmed by direct sequencing of a P1 clone (P2420) containing the entire *p15* and *p16* genes using *p15*-specific primers. The P2420 clone was isolated by PCR screening of a human P1 genomic library (Genome Systems, Inc.)

Functional Analyses of *p15*. RT-PCR products containing the complete coding sequence of the *p16* and *p15* genes were generated from HeLa and placenta mRNA, respectively, using primers containing signals for *in vitro* transcription by T7 polymerase and *in vitro* translation by reticulocyte lysates (Table 1, primers TNT-P16 and P16-S13 for *p16* and primers TNT-P15 and P15-S2R for *p15*). The p16 and p15 proteins for *in vitro* binding assays were synthesized using these RT-PCR products and an *in vitro* transcription-translation kit (Promega) as described previously for other genes (36, 37).

Received 10/11/94; accepted 11/3/94.

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[1] This work was supported by the Preuss Foundation; the Clayton Fund; the National Foundation for Cancer Research; Baylor SPORE in Prostate Cancer; NIH Grants CA-58204 (J. W. H.), AG-11085 (S. J. E., J. W. H.), CA-43733 and CA-51504 (S. H. B.), CA-11898 and NS-20023 (D. D. B.), CA-57208 (S. M.), CA-51183 (J. W.), CA-41183 (B. V.), and CA-09243 (J. J.). B. V. is an American Cancer Society Research Professor.

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; MTS, multiple tumor suppressor gene; CIP1, Cdk-interacting protein; WAF1, wild-type *p53*-activated fragment; SDI1, senescent cell-derived inhibitor; CAP1, Cdk-associated protein; KIP1, Cdk-inhibitory protein; RT, reverse transcriptase; GM, glioblastoma multiforme; ORF, open reading frame.

Table 1 *Primer sequences for p16 and p15 analyses*

Name	Sequences	Primary use
P16-1F	5′-GAAAGGAGAGGAGGGGCT-3′	Amplify *p16*, exon 1[a]
P16-1R	5′-GCGCTACCTGATTCCAATTC-3′	Amplify *p16*, exon 1[a]
P16-2F	5′-GGAAATTGGAAACTGGAAGC-3′	Amplify *p16*, exon 2[a]
P16-2R	5′-TCTGAGCTTTGGAAGCTCT-3′	Amplify *p16*, exon 2[a]
P16-3F	5′-TTTTCTTTCTGCCCTCTGCA-3′	Amplify *p16*, exon 3
P16-3R	5′-TGAAGTCGACAGCTTCCG-3′	Amplify *p16*, exon 3
P16-S1	5′-GCCTTCGGCTGACTGGC-3′	Sequence *p16*, exon 1
P16-S2	5′-GCGCCACCGCCTCCAGC-3	Sequence *p16*, exon 1
P16-S4	5′-TCAGCCAGGTCCACGGGC-3′	Sequence *p16*, exon 2
P16-S5	5′-TTCCTGGACACGCTGGTG-3′	Sequence *p16*, exon 2[b]
P16-S13	5′-GGCCCTGTAGGACCTTCG-3′	Sequence *p16*, exon 3
P15-1F	5′-CCAGAAGCAATCCAGGCGCG-3′	Amplify *p15*, exon 1
P15-1R	5′-AATGCACACCTCGCCAACG-3′	Amplify *p15*, exon 1
P15-2F	5′-CCTTAAATGGCTCCACCTGC-3′	Amplify *p15*, exon 2
P15-2R	5′-CGTTGGCAGCCTTCATCG-3′	Amplify *p15*, exon 2
P15-S15	5′-GAAAGAAGGGAAGAGTGTCG-3′	Sequence *p15*, exon 1
P15-S4	5′-CAAGTCCACGGGCAGACG-3′	Sequence *p15*, exon 2
P16-S9	5′-GGAGCACGATGGAGCCG-3′	RT-PCR *p16* cDNA
TNT-P16	5′-CCCCAAGCTTAATACGACTCACTATAGGGAGACCACCATGGAGCCGGCGGCG-3′	*In vitro* transcription and translation of *p16*
TNT-P15	5′-CCCCAAGCTTAATACGACTCACTATAGGGAGACCACCATGCGCGAGGAGAAC-3′	*In vitro* transcription and translation of *p15*

[a] These primers have been described previously (20).
[b] P16-S5 was also used to sequence exon 2 of the *p15* gene.

To test whether p15 associates with cyclin-dependent kinases, Cdks and cyclins were expressed in Sf9 cells and metabolically labeled with [^{35}S]methionine (14). To generate Sf9 cell extracts, ~2 × 10^6 cells were lysed in 0.2 ml of 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM NaF, 0.1 mM sodium vanadate, and 5 μg/ml of leupeptin and antipain; and the solution was brought to 150 mM NaCl prior to centrifugation. For *in vitro* binding, 10μl of cell extract were incubated with 2 μl of *in vitro* translated *p15* or *p16* for 10 min. Immunoprecipitations were performed after addition of 150 μl binding buffer (50 mM Tris-HCl, pH 8; 150 mM NaCl; 2 mM EDTA; 1 mM DTT; 10.5% NP40, 10 mM NaF; 5 μg/ml leupeptin and antipain), 1 mg of protein A-Sepharose, and about 5 μg of the appropriate antibody. Immune complexes were washed three times with 1 ml of binding buffer prior to electrophoresis through 13% SDS-polyacrylamide gels.

Anti-Cdk2 and anti-Cdk4 antibodies were purchased from Santa Cruz Biologicals. Anti-Cdk6 and baculovirus Cdk6 were a generous gift of Drs. M. Meyerson and E. Harlow. For expression of Cdk5HA in SF9 cells, the Cdk5HA coding sequence (generously provided by Dr. L. H. Tsai) was cloned into pVL1393 and viruses generated using Baculogold (Pharmacia). Other baculoviruses were provided by Drs. C. Sherr and D. Morgan.

RT-PCR Analysis of *p15* and *p16* Expression. Gene expression was determined by PCR amplification using primers P16-S9/P16-S13 for the *p16* gene and primers P15-1F/P15-2R for the *p15* gene (Table 1). The integrity of RNA samples was evaluated by control PCR amplifications of human *Cdk4* or human *p27* cDNA fragments.[4]

Results

Deletions of *p16* in Brain Tumors. We began our analysis by searching for *p16* deletions in a panel of brain tumors xenografted in nude mice. We chose to examine xenografts for a variety of reasons: (*a*) many of the previously described alterations in *p16* involved homozygous deletions (20–29). Such deletions are difficult to demonstrate in primary tumor samples because of contaminating nonneoplastic cells. The xenografts, in contrast, contain virtually no nonneoplastic human cells and deletion analysis is unambiguous; (*b*) human tumor xenografts provide an accurate representation of the genetic abnormalities found in the original tumor at both the cytogenetic and molecular genetic levels (38, 39). The conditions allowing tumor growth in nude mice appear to mimic those found in the human host and do not in general select for rare genetic variants, as sometimes occurs during tissue culture.

Fifty-seven brain tumor xenografts and 25 colorectal tumor xenografts were examined for deletions of exon 1 of *p16* using a PCR-based assay. The tumor specificity of the deletions that were found was striking. Twenty-six of 38 GM xenografts had homozygous deletions of the sequence tested (Fig. 1*A*, *top*), but 0 of 19 other brain tumors (14 medulloblastomas, 5 ependymomas) and 0 of 25 colorectal cancers had such deletions. All PCR reactions were repeated at least once to confirm the results. The integrity of the DNA templates was also controlled through positive amplification of a *Cdk4* genomic fragment which revealed similar signals in all samples (Fig. 1*A*, *bottom*).

Homozygous Deletions of *p16* Gene in Primary Tumors. There has been a debate about whether *p16* deletions occur primarily during *in vitro* passage of human tumor cell lines rather than in the primary tumors *in vivo* (26–29). To address this question with respect to GM, we analyzed DNA from four primary tumors from which xenografts exhibited deletions. These four cases were chosen solely because the primary tumors had little stromal or inflammatory component. PCR analysis of genomic DNA showed very weak *p16* signals from each of these tumors (Fig. 1*B*, *top*). Although PCR is not a quantitative technique, controls showed it was unlikely that the *p16* gene was present in more than a small fraction of the cells. The amount of the *p16* PCR product in these tumors was less than 3% of that observed in reactions containing an equal amount of DNA from normal cells. Similar results were obtained for the other two *p16* exons. In contrast, the *Cdk4* PCR product from each of these four primary tumor samples was equivalent to that obtained with DNA from normal cells (Fig. 1*B*, *bottom*). Thus, *p16* deletions most likely occurred in the original tumors from which the xenografts were derived (see "Discussion").

Intragenic Mutations of *p16*. The high fraction of GM tumors with *p16* deletions, coupled with the tumor specificity of such deletions, suggested that *p16* was indeed the "target" of the deletion events. The "target" refers to the critical gene that lies within the deleted region and the absence of which provides the growth advantage allowing selection of cells in which the deletion occurred. If *p16*

[4] J. Pietenpol, unpublished data.

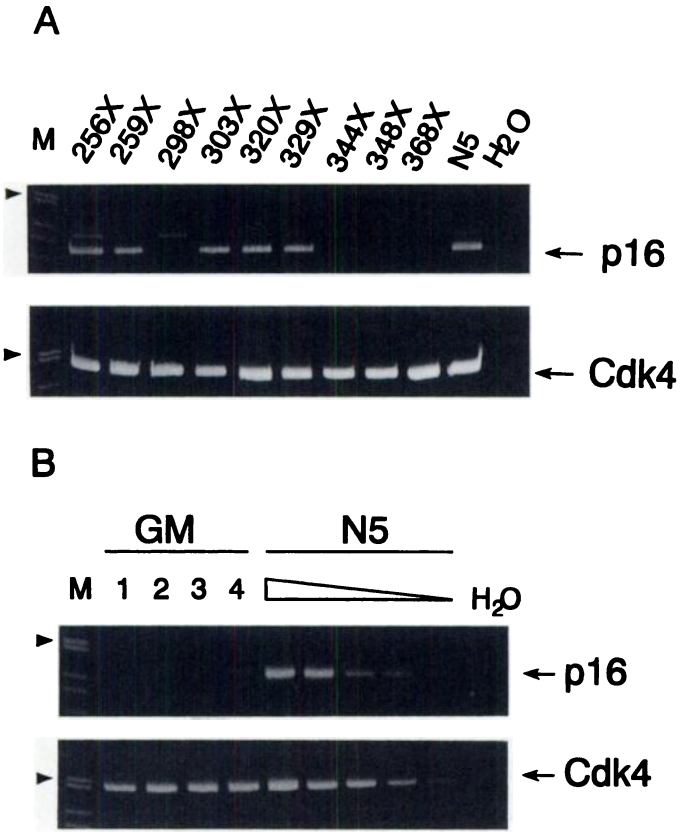

Fig. 1. Homozygous deletions of *p16* gene in GM. Deletions were assessed by PCR as described in the text. In *A*, tumors 298X, 344X, 348X, and 368X exhibited homozygous deletion of *p16* gene exon 1 while tumors 256X, 259X, 303X, 320X, and 329X did not (*top*). All samples amplified equivalently using primers specific for the *CDK4* genomic fragment (*bottom*). In *B*, homozygous deletions of p16 exon 3 were apparent in primary tumors H368 (*GM1*), H409 (*GM2*), H450 (*GM3*), and H457 (*GM4*). Normal genomic DNA (*N5*) was diluted at ratios of 1, ½, ⅛, 1/32, and 1/128 of the amount used in tumor samples as indicated. The *lane* marked H_2O contained no template and served as the negative control. The genes examined are indicated by *arrows*. *Arrowheads*, position of the 506/516-base pair size markers from the 1-kilobase ladder (*M* lane; Life Technologies, Inc). A nonspecific product of amplification is observed in *A* just above the *p16* signal.

were the target, one would expect to find additional GM tumors in which *p16* was inactivated through mechanisms other than homozygous deletion. Indeed, multiple mechanisms for inactivation are found in other tumor suppressor genes (40), and subtle intragenic mutations of *p16* have been found in other human tumor types (20–23). We therefore determined the sequence of the entire coding region in the 12 GM tumors without homozygous deletions of *p16* exon 1. PCR was used to amplify each of the three coding exons and the resultant products were purified and sequenced directly (see "Materials and Methods"). No mutations were found in any of the 12 samples, although we did observe polymorphisms that have been described previously (24–26).

Mapping of the Deleted Region. The absence of intragenic mutations in the GM without deletions suggested that there might be a gene other than *p16* that was the target of the 9p21 deletions. To evaluate this possibility, we examined 8 sequence-tagged sites within an 80-kilobase region encompassing *p16*. These included segments of each of the three exons of *p16*, three sequence-tagged site markers centromeric to *p16*, and two segments of *p15*, a gene with a 3′ end approximately 20 kilobases centromeric to *p16* (Ref. 20 and described in more detail below). The results of these analyses are recorded in Fig. 2. Twenty-three of the GM tumors had homozygous deletions of all tested markers, while 10 tumors had no deletion of any marker. Five tumors lost only a subset of the 8 markers. Tumor 392X had a deletion that included all of *p16* and exon 2 of *p15*. Tumor 298X had a deletion of all three exons of *p16*, but none of the five other markers. Tumor 263X retained exons 2 and 3 of *p16*, but not other markers, while tumors 256X and 329X retained all three exons of *p16*, but had deletions of more centromeric markers. Three tumors had centromeric deletions while two tumors had telomeric deletions. The small segment just upstream of exon 1 of *p16* and bordered by marker R2.3 contained the region of overlap, *i.e.*, a region that was consistently deleted in all tumors with an identifiable deletion.

***p15* Sequence and Function.** The results of Fig. 2 suggested that another gene immediately centromeric to *p16* might be the actual target of the deletion observed. The most obvious candidate for such a target was *MTS2*. A fragment of this gene was reported by Kamb *et al.* (20), discovered through its sequence similarity to *p16*. It was not clear, however, whether the reported sequences were part of an expressed gene, the product of which was functional, rather than a pseudogene or other evolutionary relic.

To address these questions about *MTS2*, we derived its cDNA sequence and corresponding intron exon organization. Through PCR and direct cloning, an ORF encoding an 138-amino acid protein was found (Fig. 3). The predicted molecular weight of this protein was 14.7 kilodaltons, and the gene encoding this protein will henceforth be referred to as *p15*. The presumptive initiating methionine in this ORF was preceded by an in-frame stop codon (Fig. 3*A*, *underlined*). The first two exons of *p15* were arranged in a pattern similar to that of *p16*. The open reading frame of *p15* ended within exon 2, however, while that of *p16* continued with 16 additional amino acids in the second exon and 4 amino acids in a third exon (20, 21). A high degree of similarity between the amino acid sequences of these genes was evident throughout their ORFs (Fig. 3*B*).

In order to determine whether p15, like p16, could interact with Cdks, *in vitro* translated proteins were generated as described in "Materials and Methods." The ^{35}S-labeled proteins were incubated with extracts from Sf9 cells infected with baculoviruses expressing Cdk or cyclins and immunoprecipitated with antibodies to Cdks. Comparable amounts of p15 and p16 were bound to Cdk4 and Cdk6 but not to cyclin D2 (Fig. 4, *A* and *B*). In contrast, neither p15 nor p16 bound to Cdk2 or Cdk5, or their cyclin complexes (Fig. 4*C*).

Mutational Analysis of *p15*. If *p15* were the target of the 9p21 deletions in GM, one would expect to find subtle intragenic mutations of this gene in tumors, as described for *p16*. Of the 38 GM tumor

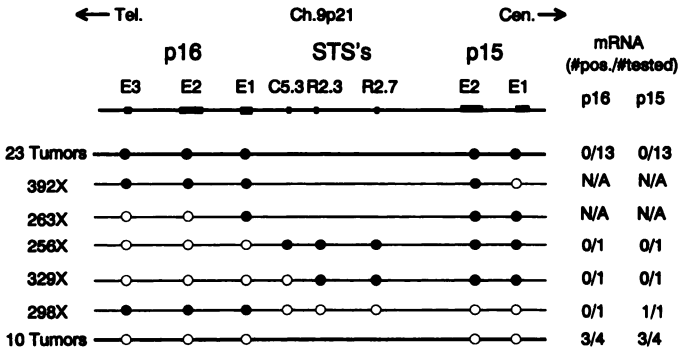

Fig. 2. Deletion mapping of the *p16* region in 38 GM. The map of the markers was based on the data in Ref. 20. The map is not drawn to scale but the entire region shown is approximately 80 kilobases and is contained within the P1 clone 2420 (See "Materials and Methods"). ● and ○, markers tested in each sample. ●, homozygous loss of the marker; ○, retention of the marker. Twenty-three GM had homozygous deletions of all markers tested, while ten GM retained all markers. Tumors with partial deletions are individually shown. The two rightmost columns indicate the *p16* and *p15* gene expression patterns. The fractions refer to the number of samples expressing each gene divided by the total number of samples tested for each class. *N/A*, not analyzed; *Tel.*, teleomere; *Cen.*, centromere; *STS's*, sequence-tagged sites.

A

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5'ctggcctcccgcgatcacagcggacaggggcggagcctaagg
gggtgggagacgccggccccttggcccagctgaaaacggaattct
ttgccggctggctccccactctgccagagcgaggcggggcagtga
ggactccgcgacgcgtccgcaccctgcggccagagcggctttgag
ctcggctgcgtccgcgctaggcgctttttcccagaagcaatccag
gcgcgcccgctggttcttgagcgccaggaaaagcccggagctaac
gaccggccgctcggccactgcacggggccccaagccgcagaagga
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agtttacggccaacggtggattatccgggccgctgcgcgtctggg
ggctgcggaATGCGCGAGGAGAACAAGGGCATGCCCAGTGGGGGC
GGCAGCGATGAGGGTCTGGCCAGCGCCGCGGCGCGGGGACTAGTG
GAGAAGGTGCGACAGCTCCTGGAAGCCGGCGCGGATCCCAACGGA
GTCAACCGTTTCGGGAGGCGCGCGATCCAGgtagctggggcccca
gggcctcgcggcaggggcgcgcgaacgcgggcgcggcctcggcg
gatcggggctggaacctagatcgcgatgtagatttgtacaggagt
ctacgttggcgaggtgtgcattcacggtaaacagc-3'
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B

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p15  greenkgmpsggg DEG S  L K Q  G D  40
p16  epaagssmep-- ADW T  R E A  M L  38

p15  GV RF  A    80
p16  AP SY  P    78

p15                120
p16                118

p15  R  G  T T d--------------------  138
p16  L  R  A A gtrgsnharidaaegpsaipd  156
```

Fig. 3. Sequence of the *p15* gene. *A*. The deduced coding sequence for the first exon of *p15* is shown in *upper case* while the presumptive 5′ noncoding region and a portion of intron 1 are shown in *lower case. Arrowhead,* the 5′ end of the rapid amplification of cDNA ends clone. The first 115 nucleotides before the *arrowhead* and the intron 1 sequences were obtained by direct sequencing of the P2420 P1 clone. The in-frame stop codon upstream of the open reading frame is *underlined.* The sequence of *p15* exon 2 is given by Kamb *et al.* (20). *B,* protein sequence alignment of *p15* and *p16.* The alignment was done using the MACAW program (Version 2.03). *Arrowhead,* junction between the two exons.

xenografts studied, 27 had deletions of 1 or both exons of *p15* (Fig. 2). DNA from the 11 tumors without *p15* deletions were used as a template to determine the sequence of *p15,* but no mutations or polymorphisms within the coding region were identified.

Expression of *p15* and *p16* in Normal Tissues and GM. RT-PCR was used to determine the expression patterns of full length *p16* and *p15* in normal and tumor tissues. The expressed product of *p16* gene was observed in Hela cells, but barely detectable or absent in other tissues (Fig. 5*A, top*). In contrast, *p15* expression was easily detected in most of the tissues tested (Fig. 5*A, bottom*). Examples of expression patterns for *p15* and *p16* in GM xenografts are shown in Fig. 5*B* and summarized in Fig. 2. In 13 tumor xenografts with deletion of both genes, no expression could be detected, as expected (Figs. 2 and 5*B*). In contrast, three of four tumors without any observed deletion in the region expressed both *p15* and *p16.* Two tumors (256X and 329X) in which *p15* was deleted and *p16* was present did not express either *p15* and *p16.* In the one tumor (298X) with a *p16* deletion but retaining *p15* sequences, *p15* but not *p16* was expressed. In all cases, the integrity of cDNA was assessed with control reactions using primers for other genes, as shown in Fig. 5*B* for *p27.*

Discussion

The results described above lead to several conclusions. First, two related genes reside within a small portion of chromosome 9p21, and these genes are homozygously deleted in over two-thirds of GM tumors. There is remarkable specificity in the deletions, because they were not found in brain tumors other than GM. The brain tumor specificity adds to the specificity observed in tumors of the gastrointestinal system. Xenografts from pancreatic cancers often contain *p16* deletions (23), but those from colorectal cancers do not (this study). Our data on the primary tumors strongly suggest that these deletions arise *in vivo* in the human host and that cells with the deletions clonally expand to comprise the major cell type within the tumor. If the deletions we observed were present in only a small fraction of the primary tumor cells, which then selectively proliferated during xenografting, or if the deletions occurred during xenografting, we would not have obtained the results shown in Fig. 1*B.* It is also important to

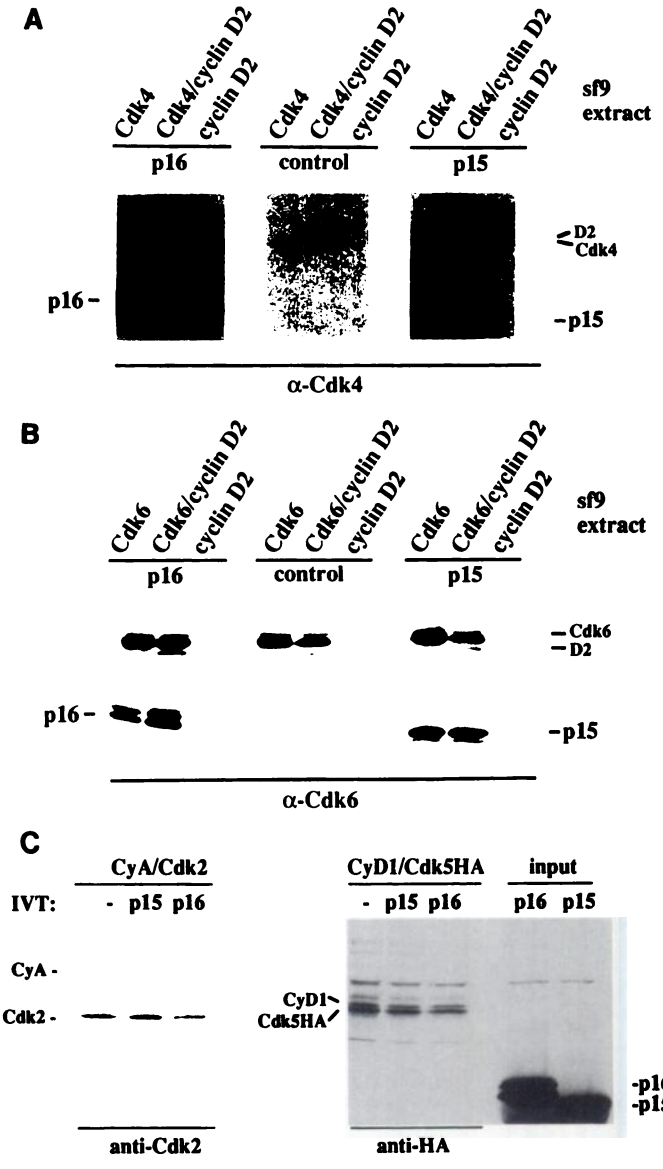

Fig. 4. Association of p16 and p15 with Cdk/cyclin (*Cy*) complexes. The indicated Sf9 extracts were incubated with ^{35}S-labeled p16, p15, or a mock translation lysate, and Cdk4 (*A*), Cdk6 (*B*), or Cdk2 (*C*) and Cdk5 (*C*) immunoprecipitates were collected using corresponding anti-Cdk antibodies as described in "Materials and Methods." The molar ratio of Cdks to p16 and p15 was 10:1. Immune complexes were analyzed by SDS-PAGE and dried gels were subjected to autoradiography. The positions of p15, p16, Cdk, and cyclin polypeptides are indicated.

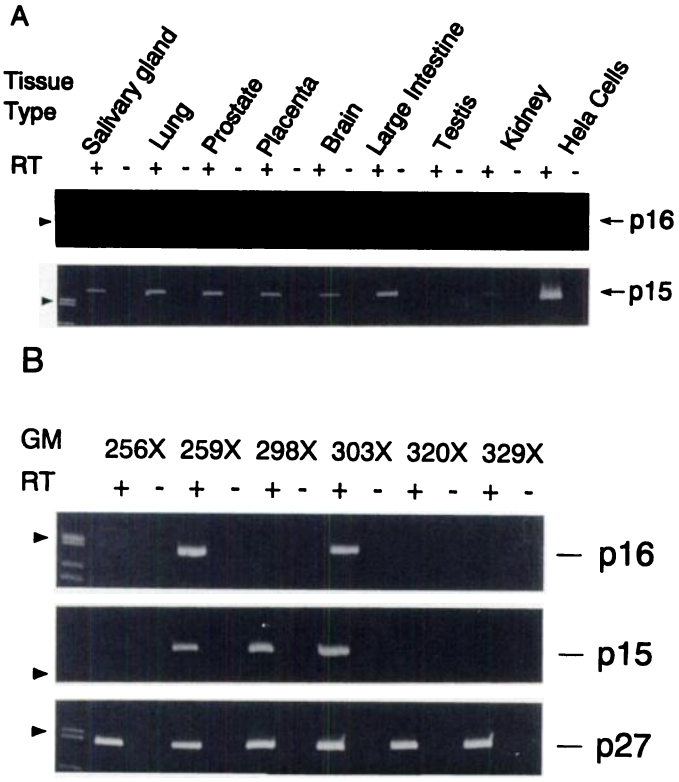

Fig. 5. Expression of the *p16* and *p15* genes. RT-PCR was used to examine the expression of *p16* and *p15* genes in normal tissues (*A*) and GMs (*B*). RNA samples were incubated in reverse transcription reactions with (+) or without (-) reverse transcriptase and the resultant cDNA was used to PCR amplify the gene products of interest (*arrows*). *Arrowheads*, position of the 506/516-base pair size marker.

note that the majority of GM tumors can be xenografted in nude mice (30). Therefore, these xenografts do not represent a small fraction of GM that have the capacity to grow in nude mice but should be representative of GM in general.

The second point concerns the functional analysis of *p15*. This gene is expressed at a much higher level and in a broader range of tissues than *p16*, and the encoded protein has an equivalent capacity to bind Cdk4. This binding, like that of p16, is more restricted than the other Cdk inhibitors thus far identified (p21 and p27). In the case of p21 and p27, association with Cdks were greatly enhanced by cyclins.[5] In contrast, both p15 and p16 associated tightly with the Cdk subunits in the absence of a cyclin (Fig. 4, *A* and *B*). Our sequence of *p15* and the demonstration of its Cdk-binding activity confirm the independent studies by Hannon and Beach who additionally made the intriguing observation that p15 is transcriptionally induced following treatment of cells with transforming growth factor β and can functionally inhibit, as well as bind to, Cdk4 complexes (41).

Finally, there is the question of the "target" gene responsible for the deletions in GM. The most persuasive evidence for a given gene being the target of such deletions is provided when (*a*) the gene is within the smallest common region of deletion in the tumors and (*b*) the gene is subtly altered by intragenic mutation in tumors without homozygous deletion. For *p15* and *p16*, the second of these criteria is not met, inasmuch as no intragenic mutations of either gene has been identified in GM. However, the mapping studies shown in Fig. 2 demonstrate that the smallest common region of deletion lies between *p15* and *p16*, strongly suggesting that a gene in this region is the target. Several explanations of these results can be considered: (*a*) in some tumors, *p16* is the target, and in others it is *p15*; (*b*) there is a third gene, lying between exon 1 of *p16* and the marker R2.3, that is the target, and deletions of *p16* and *p15* are inconsequential; (*c*) two genes are involved but neither of these are *p16* or *p15*. One is telomeric to *p16* and the other centromeric to *p15*. The apparent overlap between the deletions in different tumors is coincidental; (*d*) *p16* is the target and *p15* is irrelevant. This is consistent with the observation that in the two tumors (256X and 329X) where *p16* coding sequences were not included in the deleted region, no full length *p16* RNA was made, suggesting that the deletions affected the regulatory region of *p16*. The same was not true for *p15*, because one tumor (298X) was found to have a deletion adjacent to *p15*, but nonmutant *p15* was still expressed; (*e*) the deletion has a double target, *i.e.*, both *p16* and *p15*.

In our view, the last explanation is the most likely one. It is consistent with several observations; both *p16* and *p15* had analogous biochemical activities and both genes were expressed in three of four GM tumors without deletions of the region, suggesting that they both play a role in cell cycle control in glial cells. The hypothesis explains why the most common genetic alteration in the region is a homozygous deletion which almost always inactivates both genes. However, this hypothesis does not explain all observations. For example, one might not expect tumor 298X to have expressed normal *p15* if this hypothesis were true, although it remains possible that the *p15* gene product in this tumor was inactivated posttranscriptionally. Further experiments which address the effects of p15 and p16 on GM growth, as well as experiments in mice with "knockouts" of one or both genes, should provide additional evidence to refute or support this "double target" conjecture.

[5] J. W. Harper *et al.*, submitted for publication.

Acknowledgments

The authors thank T. Gwiazda for help in preparation of the manuscript. We thank Drs. N. Nicolaides for advice on the P1 clone and J. Pietenpol for human *p27* primers.

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