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Isolation and Characterization of p19^{INK4d}, a p16-related Inhibitor Specific to CDK6 and CDK4

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Cyclin-dependent kinases 4 and 6 are complexed with many small cellular proteins in vivo. We have isolated cDNA sequences, INK4d, encoding a 19-kDa protein that is associated with CDK6 in several hematopoietic cell lines. p19 shares equal similarity and a common ancestor with other identified inhibitors of the p16/INK4 family. p19 interacts with and inhibits the activity of both CDK4 and CDK6 and exhibits no detectable interaction with the other known CDKs. p19 protein is present in both cell nuclei and cytoplasm. The p19 gene has been mapped to chromosome 19p13.2, and the level of its mRNA expression varies widely between different tissues. In contrast to p21 and p27 whose interaction with CDK subunits is dependent on or stimulated by the cyclin subunit, the interaction of p19 and p18 with CDK6 is hindered by the cyclin protein. Binary cyclin D1-p18/p19 or cyclin D1-CDK6 complexes are highly stable and cannot be dissociated by excess amounts of cyclin D1 or p19/p18 proteins, suggesting that p16 inhibitors and D cyclins may interact with CDKs 4 and 6 in a competing or potentially mutually exclusive manner.

INTRODUCTION

The eukaryotic cell cycle is regulated by the sequential formation, activation, and subsequent inactivation of a series of structurally related serine/threonine protein kinases, CDKs (cyclin-dependent kinases) (see two recent reviews in Sherr, 1994; Hunter and Pines, 1994). The enzymatic activity of a CDK is regulated by at least three different mechanisms: cyclin binding and activation, subunit (inhibitory and activating) phosphorylation, and inhibition by a CDK inhibitor. In mammalian cells, there exists at least two distinct families of CDK inhibitors, represented by two prototype CDK inhibitors p21 and p16. p21 (also variously known as CIP1, WAF1, SDI1, CAP20, PIC1, and CDKN1), first identified in normal human fibroblasts as a component of quaternary cyclin D-CDK complexes that also contain proliferating cell nuclear antigen (PCNA; Xiong *et al.*, 1992), is a potent inhibitor of most, if not all, cyclin-CDK enzymes (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a). The p21 family of CDK inhibitors currently contains three related genes, p21, p27^{Kip1} (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994), and p57^{Kip2} (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). Both p27 and p57 share significant amino acid homology with p21 in their amino-terminal region and, like p21, interact with and inhibit the activity of a variety of cyclin-CDK complexes (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994; Lee *et al.*, 1995; Matsuoka *et al.*, 1995). In addition to inhibiting the kinase activity of cyclin-CDK enzymes, p21 can also regulate the cell cycle through two other separate

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mechanisms: by interacting with PCNA through a carboxyl-terminal domain in the absence of cyclin and CDK proteins to inhibit PCNA-dependent DNA replication (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994; Chen *et al.*, 1995; Luo *et al.*, 1995; Nakanish *et al.*, 1995; Warbrick *et al.*, 1995), and by blocking the activating phosphorylation of CDKs by CDK-activating kinase (CAK; Kato *et al.*, 1994a; Aprelikova *et al.*, 1995). Expression of the p21 gene can be induced by a wide range of cell growth regulatory signals, including the DNA damage and tumor suppressor p53, the antimitogenic cytokine growth factor transforming growth factor (TGF)-β, cellular senescence, and cell differentiation, supporting a broad biological role for the p21 CDK inhibitor (reviewed by Sherr and Roberts, 1995).

Four genes have been isolated as members of the p16 family of CDK inhibitors as follows: p16 (also known as INK4a, MTS1, CDK4I, and CDKN2; Serrano *et al.*, 1993; Kamb *et al.*, 1994; Nobori *et al.*, 1994), p15 (also known as MTS2, INK4b, and p14 (Guan *et al.*, 1994; Hannon and Beach, 1994; Kamb *et al.*, 1994), p18 (INK4c; Guan *et al.*, 1994; Hirai *et al.*, 1995), and p19 (this study). Two members of this family, p16 and p15, are homozygously deleted at a high frequency in a wide variety of human tumor-derived cell lines (Kamb *et al.*, 1994; Nobori *et al.*, 1994) and several specific types of primary tumors (reviewed in Sherr, 1994; Hunter and Pines, 1994), indicating a tumor suppression function of p16. The major, if not only, inhibitory targets of the p16 family are CDK4 and CDK6 (Serrano *et al.*, 1993; Xiong *et al.*, 1993b; Guan *et al.*, 1994; Hannon and Beach, 1994). Their preferential association with D-type cyclins, substrate preference, and the timing of the onset of their kinase activity have strongly implicated both CDK4 and CDK6 as physiological kinases for the retinoblastoma susceptibility gene product pRB, whose growth-suppressing activities are known to be down-regulated by cell cycle-dependent phosphorylation (for a recent review, see Sherr, 1994). Ectopic expression of p18 or p16 suppresses cell growth with a correlated dependence on endogenous wild-type pRb (Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995; Sherr and Roberts, 1995), suggesting a potential mechanism by which members of the p16 family inhibit cell growth, inhibiting the activity of CDK6 and CDK4 kinases, thereby preventing the phosphorylation of pRb, and keeping pRb in its active growth-suppressing state. To elucidate the mechanism by which the activities of CDK4 and CDK6 are regulated during the cell cycle and cell growth, we have isolated and characterized a novel CDK4 and CDK6 specific inhibitor, p19, and further investigated the interaction of the two families of CDK inhibitors and their CDK targets.

MATERIALS AND METHODS

Cell Culture and Immunochemistry Procedures

ML1 (a human myeloid leukemia cell line), CEM (a human acute lymphoblastic leukemia cell line, ATCC CCL 119), and Jurkat (a human acute T cell leukemia cell line, ATCC TIB 153) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Most of the antibodies used and procedures for [^{35}S]methionine metabolic labeling, immunoprecipitation, and SDS-PAGE have been described previously (Xiong *et al.*, 1993b; Guan *et al.*, 1994; Jenkins and Xiong, 1995).

Rabbit polyclonal anti-human p19 peptide antibody was raised against a synthetic peptide CDLVDILQGHMVAPL with the underlined region corresponding to the carboxyl-terminal region of human p19. Two additional rabbit polyclonal antibodies were raised against full length human p19 protein–one against a purified native GST-p19 fusion protein and the other against p19 denatured by SDS-PAGE. All three were subsequently affinity purified. The antibodies against human p19 do not exhibit detectable cross-reactivity with any other members of the p16 family in immunoprecipitation or immunoblotting, nor do they cross-react with any other known cell cycle proteins.

Nucleic Acid Procedures

To isolate p16-/p18-related genes, three degenerate oligonucleotides were synthesized based on the amino acid sequences conserved between p16 (Serrano *et al.*, 1993), p14 (Guan *et al.*, 1994; Hannon and Beach, 1994), and p18 (Guan *et al.*, 1994). The nucleotide sequences for the 5′ primers, A and B, are CGGAATTC-ACNGCNGCNGCN(A,C)GNGG encoding AAARG, and CGGAA-TTC(C,G)CN(A,C,T)TNCA(A,G)GTNATG encoding ALQVM, respectively. The 3′ primer is CGGAATTCAGNGT(A,G)-TCNA(A,G)-(A,G)AANCC, which encodes GFLDT. An *Eco*R I restriction site was introduced into each primer (underlined sequence) for the convenience of subsequent subcloning. A pair of primers were used to amplify potential p16-/p18-related sequences from cDNA templates prepared from human B cells, leukocytes, testis, and brain by polymerase chain reaction (PCR) for 35 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. The PCR products were resolved on a 2.5% Nusieve (FMC) agarose gel. DNA fragments with approximately 150–250 bp were purified using Geneclean (Bio 101, La Jolla, CA) and used as templates for a second round of PCR. PCR products were again resolved on a 3% Nusieve agarose gel and DNA fragments of approximately 150 bp were gel purified and subcloned into the *Eco*R I site of M13 mp19 for sequencing. Of 64 M13 subclones sequenced, one (derived from leukocytes) was found to contain an insert that encodes a potential p16-/p18-related gene, and this 150-bp insert was then labeled by random primer labeling methods and used as a probe to screen a human spleen cDNA library. Of 10 positive cDNA clones isolated from this screen, the longest one, S2, was completely sequenced.

For the Northern analysis, 2 μg poly(A)$^+$ RNA was isolated from different human tissues, resolved on a 1.2% agarose gel, and transferred to a nylon membrane (Clontech, Cambridge, UK). The 1.3-kb DNA fragment of full length human or mouse p19 cDNA was labeled by the random priming labeling method and used as a probe for Northern hybridization. The same blot was stripped and re-hybridized with a 0.6-kb DNA fragment corresponding to the coding region of human p18 that detects only two major mRNA transcripts and with a 2-kb full length human p21 cDNA fragment as probes (Xiong *et al.*, 1993a; Guan *et al.*, 1994).

Kinase Assay

Reconstitution of active CDK4 and CDK6 kinases was performed by methods similar to those previously described (Kato *et al.*, 1994b). Glutathione *S*-transferase (GST) fusion proteins containing human cyclin D2, CDK4, and CDK6 were constructed by placing the entire

coding region of each sequence into pGEX-KG, transforming into *Escherichia coli* strain TG1, and purifying the resultant GST fusion proteins following published procedures (Guan and Dixon, 1991). Two micrograms of purified GST-cyclin D2 protein were mixed with 2 μg of purified GST-CDK4 or GST-CDK6. The mixtures were incubated with cell lysate (50 μl) derived from 10^6 exponentially growing Jurkat cells that were lysed in a buffer containing 80 mM sodium-β-glycerophosphate, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4, 15 mM $MgCl_2$, 0.3 mM sodium orthovanadate, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml trypsin inhibitor, 2 μg/ml pepstatin, 2 μg/ml chymostatin, 10 μg/ml aprotinin, and 2 mM dithiothreitol. Cells were briefly sonicated and centrifuged at 4°C for 10 min at maximum speed in a microcentrifuge. ATP was added to the mixture of GST fusion proteins and Jurkat cell lysate at a final concentration of 1 mM. The reaction was incubated at room temperature for 1 h and centrifuged for 5 min. The activated GST-cyclin D2/GST-CDK4 or CDK6 was recovered from the mixture on glutathione agarose beads and eluted in 50 μl of kinase assay buffer (50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM ATP) containing 10 mM glutathione. One-fourth (approximately 0.5 μg) of the activated CDK4 or CDK6 enzymes was used to phosphorylate 1 μg of purified GST-Rb C-terminal fusion protein in a final volume of 20 μl with addition of 10 μCi [γ-^{32}P]ATP at 30°C for 1 h. The reaction was terminated by adding 20 μl of 2× SDS sample buffer, and one-half of the reaction was analyzed by SDS-PAGE followed by autoradiography. CDC2 and CDK2 kinase activities were assayed on anti-CDC2 and anti-CDK2 immunoprecipitates derived from Jurkat cells as described previously (Guan *et al.*, 1994). To assay for the inhibitory activity of p19, the full length p19 coding region was subcloned into pGEX-KG, and GST-p19 fusion protein was purified as described (Guan and Dixon, 1991). Purified GST, GST-p19, or control buffer was added to the activated CDK4-cyclin D2 or CDK6-cyclin D2 before the addition of GST-Rb substrate and [γ-^{32}P]ATP.

Chromosomal Localization

Approximately 2 μg of DNA bearing a 1.3-kb full length p19 cDNA insert was nick translated and hybridized to human metaphase chromosomes as described previously (Guan *et al.*, 1994). Additionally, the biotinylated p19 cDNA probe was co-hybridized to human metaphase spreads with a digoxigenin-labeled Alu consensus 77 mer (GM009; Matera and Ward, 1992). After hybridizing overnight, the slides were washed in 50% formamide/2× SSC at 37°C, followed by washes in 1× SSC at 60°C. The p19 probe was detected with fluorescein isothiocyanate (FITC)-conjugated avidin, while Alu oligo was detected with rhodamine-conjugated anti-digoxigenin. The cytogenetic location in band 19p13, determined by R banding, was confirmed and further localized to 19p13.2 by averaging FL pter measurements from four metaphase spreads (range 0.16–0.23; median 0.2).

Transfection and Immunofluorescence Staining

Transfection of Swiss 3T3 cells with pCMV-p19 was performed using Lipofectamine methods (Life Technologies, Gaithersburg, MD). Swiss 3T3 cells cultured on coverslips in 24-well culture plates were washed twice with 1 ml of ice-cold phosphate-buffered saline (PBS) and fixed for 10 min at room temperature in 1 ml of 3% formaldehyde diluted in PBS followed by 1 ml of a 1:1 mixture of methanol and acetone for 5 min with gentle shaking. After washing three times with 1 ml PBS, the coverslips were incubated for 60 min with the two different affinity-purified anti-p19 antibodies (anti-p19 and anti-GST-p19, both 1:100 dilution) in 0.2 ml of PBS supplemented with 1% bovine serum albumin, 1 mg/ml thyroglobulin, and 0.1% saponin (Sigma, St. Louis, MO), and then washed four times with PBS plus 0.1% saponin. The coverslips were incubated with anti-rabbit IgG Texas Red conjugate (1:100) for 45 min in the dark in 0.2 ml of PBS containing 0.1% saponin and 1% bovine serum albumin and washed four times, 2 min each with 1 ml of PBS plus 0.1% saponin followed by two washes with PBS, 2 min each. 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Sigma) was added in the second wash to stain nuclei. Finally, the coverslips were mounted with 4 μl of buffered glycerol (10% PBS, 0.1% *p*-phenylenediamine, and 90% glycerol) on a glass slide, sealed with nail polish, and examined by fluorescence microscopy (Zeiss, Thornwood, NY).

In Vitro Translation and In Vitro Binding Assay

In vitro translation and in vitro binding assays were carried out as described previously (Guan *et al.*, 1994). For in vitro binding experiments, each mixing step was 60 min at room temperature. The mixtures were immunoprecipitated in 200 μl of NP-40 lysis buffer (the same as that used in immunoprecipitation) with anti-CDK6 (Guan *et al.*, 1994). For the dissociation experiment shown in Figure 8D, 1 μl of purified p19 protein diluted with extraction buffer (50 mM NaCl/50 mM Tris-HCl, pH 7.5/1 mM EDTA/10% glycerol) to different concentrations, as indicated on the top of each lane, was added to the mixture after the mixing of in vitro–translated CDK6 with cyclin D1 for 20 min at room temperature.

RESULTS

Cloning of p19

We and others have previously found that CDK4 and CDK6 associate with, in addition to $p16^{INK4a/MTS1}$, many small cellular proteins, and have isolated cDNA sequences encoding $p14/p15^{MTS2/INK4b}$ and p18 (Guan *et al.*, 1994; Hannon and Beach, 1994; Li *et al.*, 1994b). Most of these small CDK4- and CDK6-associated proteins are likely to encode novel members of the p16 family of CDK inhibitors. Degenerate oligonucleotides were designed based on regions conserved among the p15, p16, and p18 sequences, and used as primers to amplify cDNA sequences from several different cell lines in an attempt to isolate genes encoding novel p16- and p18-related CDK4 and CDK6 inhibitors (see MATERIALS AND METHODS). One PCR fragment identified in cDNA prepared from human leukocytes encodes a potentially novel p16-related molecule and was used as a probe to screen a human spleen cDNA library to obtain the full length sequence. The longest lambda cDNA clone, S2, was completely sequenced and found to contain a 1254-bp insert with a short poly A tail preceded by a putative polyadenylation signal (Figure 1A). Conceptual translation of this clone revealed an apparent full-length open reading frame with 166 amino acid residues starting at nucleotide 140 preceded by an in-frame termination codon present 54 bp 5′ to the initiating methionine. The predicted molecular weight of this protein is 17,689 Da, but its apparent molecular weight as seen in SDS-PAGE is 19 kDa (p19, see Figure 3A). p19 shares similar protein sequence identity with the other three previously identified CDK4- and CDK6-specific inhibitors: 40% protein sequence identity to $p16^{INK4a/MTS1}$ over a 154-amino acid region, 44% to $p14/p15^{MTS2/INK4b}$ over a 133-amino acid region, and

A

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CACGAGGGAGAGCAGCGCACGCGGGTGCACCGCGGCCGCGCCCCGGGAGGGCTGTTCGGGCCAGCGCCCGCCGGCTGCTCCGCGCTGACA   90

GCGCCGGGCTGGGGCGGGGCGGGGGGCTTTGCAGGCCGCCAGTGTCGACATGCTGCTGGAGGAGGTTCGCGCCGGCGACCGGCTGAGTGG  180
                                                  M  L  L  E  E  V  R  A  G  D  R  L  S  G   14

GGCGGCGGCCCGGGGCGACGTGCAGGAGGTGCGCCGCCTTCTGCACCGCGAGCTGGTGCATCCCGACGCCCTCAACCGCTTCGGCAAGAC  270
  A  A  A  R  G  D  V  Q  E  V  R  R  L  L  H  R  E  L  V  H  P  D  A  L  N  R  F  G  K  T   44

GGCGCTGCAGGTCATGATGTTTGGCAGCACCGCCATCGCCCTGGAGCTGCTGAAGCAAGGTGCCAGCCCCAATGTCCAGGACACCTCCGG  360
  A  L  Q  V  M  M  F  G  S  T  A  I  A  L  E  L  L  K  Q  G  A  S  P  N  V  Q  D  T  S  G   74

TACCAGTCCAGTCCATGACGCAGCCCGCACTGGATTCCTGGACACCCTGAAGGTCCTAGTGGAGCACGGGGCTGATGTCAACGTGCCTGA  450
  T  S  P  V  H  D  A  A  R  T  G  F  L  D  T  L  K  V  L  V  E  H  G  A  D  V  N  V  P  D  104

TGGCACCGGGGCACTTCCAATCCATCTGGCAGTTCAAGAGGGTCACACTGCTGTGGTCAGCTTTCTGGCAGCTGAATCTGATCTCCATCG  540
  G  T  G  A  L  P  I  H  L  A  V  Q  E  G  H  T  A  V  V  S  F  L  A  A  E  S  D  L  H  R  134

CAGGGACGCCAGGGGTCTCACACCCTTGGAGCTGGCACTGCAGAGAGGGGCTCAGGACCTCGTGGACATCCTGCAGGGCCACATGGTGGC  630
  R  D  A  R  G  L  T  P  L  E  L  A  L  Q  R  G  A  Q  D  L  V  D  I  L  Q  G  H  M  V  A  164

CCCGCTGTGATCTGGGGTCACCCTCTCCAGCAAGAGAACCCCGTGGGGTTATGTATCAGAAGAGAGGGGAAGAAACACTTTCTCTTCTTG  720
  P  L  *  166

TTTCTCCTGCCCACTGCTGCAGTAGGGGAGGAGCACAGTTTGTGGCTTATAGGTGTTGGTTTTGGGGGTGTGAGTGTTTGGGGGACGTTT  810

CTCATTTGTTTTTCTCACTCCTTTTGGTGTGTTGGACAGAGAAGGGCTCCTGCAGGCCACAGCCACCTAAACGGTTCAGTTTCTTCTGCG  900

CCTCAGGCTGCTGGGGCCTCAGACGAGACCCAAGGGCAGAGCATTTAAGAGTGAAGTCATGACCTCCAGGGAGCCTAGAAGCTGGTGGCC  990

CTTGCCCGGCCTGTGCTCAGAGACCTGAAGTGTGCACGTTGCTTCAGGCATGGGGGGTGGGGGGAGCGTCCCAAATCAATAAGAAGGTAG 1080

AATGAGTTATGAGTTATTCATATTCTGTTGGAAGCTTGTTTTCCAGTCTCTTGTACAGCGTTTTAAAAGAAATGGATTCTATTTATTATG 1170

CTTTATTGGAAAAAATGTTGTAATAATTTAATGTTTTTACCCATTAAATTAAGACTTGTGCATGATCCAAAAAAAAAAAAAAAA       1254
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B

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p19          MLLEEVRAGDRLSGAAARGDVQEVRRLLHRELVHPDALNRFGKTALQVMMFGSTA
p18            MAEPWGNELASAAARGDLEQLTSLLQ-NNVNVNAQNGFGRTALQVMKLGNPE
p16      MDPAAGSSMEPSADWLATAAARGRVEEVRALLE-AGALPNAPNSYGRRPIQVMMMGSAR
p15    MREENKGMPSGGGSDEGLASAAARGLVEKVRQLLE-AGADPNGVNRFGRRAIQVMMMGSAR

p19    IALELLKQGASPNVQD-TSGTSPVHDAARTGFLDTLKVLVEHGADVNVPDGTGALPIHLA
p18    IARRLLLRGANPDLKD-RTGFAVIHDAARAGFLDTLQTLLEFQADVNIEDNEGNLPLHLA
p16    VAELLLLHGAEPNCADPATLTRPVHDAAREGFLDTLVVLHRAGARLDVRDAWGRLPVDLA
p15    VAELLLLHGAEPNCADPATLTRPVHDAAREGFLDTLVVLHRAGARLDVRDAWGRLPVDLA

p19    VQEGHTAVVSFL--AAESDLHRRDARGLTPLELALQRGAQDLVDILQGHMVAPL 166
p18    AKEGHLRVVEFLVKHTASNVGHRNHKGDTACDLARLYGRNEVVSLMQANGAGGATNLQ 168
p16    EELGHRDVARYLRAAAGGTRG-SNHARIDAAEGPSDIPD 154
p15    EERGHRDVAGYLRTATGD 138
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C

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                N  R  F  G  K  T  A  L  Q                    V  M  M  F  G  S  T
p19: cDNA       AACCGCTTCGGCAAGACGGCGCTGCAG                  GTCATGATGTTTGGCAGCACC
p19: Genomic    AACCGCTTCGGCAAGACGGCGCTGCAGGTGAG....TCAAACAGGTCATGATGTTTGGCAGCACC

                N  G  F  G  R  T  A  L  Q                    V  M  K  L  G  N  P
p18: cDNA       AATGGATTTGGAAGGACTGCGCTGCAG                  GTTATGAAACTTGGAAATCCC
p18: Genomic    AATGGATTTGGAAGGACTGCGCTGCAGGTTGG....TTTTCCAGGTTATGAAACTTGGAAATCCC

                N  S  Y  G  R  R  P  I  Q                    V  M  M  M  G  S  A
p16: cDNA       AATAGTTACGGTCGGAGGCCGATCCAG                  GTCATGATGATGGGCAGCGCC
p16: Genomic    AATAGTTACGGTCGGAGGCCGATCCAGGTGGG....TCTGGCAGGTCATGATGATGGGCAGCGCC

                N  R  F  G  R  R  A  I  Q                    V  M  M  M  G  S  A
p15:cDNA        AACCGTTTCGGGAGGCGCGCGATCCAG                  GTCATGATGATGGGCAGCGCC
p15:Genomic     AACCGTTTCGGGAGGCGCGCGATCCAGGTCTG....TCTGGCAGGTCATGATGATGGGCAGCGCC
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Figure 1. DNA and protein sequence of the human p19 gene. (A) Nucleotide and amino acid sequences of human p19. Numbers for both nucleotides and amino acids are given, and the stop codon is indicated by an asterisk. (B) Amino acid sequence comparison of p19 with the other three p16 family genes, p14/p15$^{MTS2/INK4b}$ (Guan *et al.*, 1994; Hannon and Beach, 1994), p16 (Serrano *et al.*, 1993), and p18 (Guan *et al.*, 1994). The complete coding regions of the four inhibitor proteins were aligned, and amino acid residues identical in all four sequences are in bold. Several small gaps were introduced as represented by hyphens. (C) An intron interrupts all four isolated INK4 genes at the same position. Partial characterization of the genomic structure of p19 by DNA sequencing and comparison with p19 cDNA revealed an intron located at a position corresponding to nucleotides 280–281 of the p19 cDNA presented in Figure 1A. The coding regions of the other three INK4 genes, p15, p16, and p18 are also interrupted by an intron at the identical position (Guan *et al.*, 1994; Kamb *et al.*, 1994). The accession number for this sequence is U40343.

43% to p18 over a 165-amino acid region (Figure 1B). As first noticed in p16, p19 also contains tandem ankyrin repeats like other members of the family (Serrano *et al.*, 1993). In addition, like p18 (Guan *et al.*, 1994), p19 also shares significant protein sequence identity with the Notch genes, with 35% identity over the 107 residues to the human Notch homologue, TAN1, protein. The functional significance of the protein sequence similarity between the Notch gene products and the p16 family of CDK inhibitors, in partic-

ular p18 and p19, remains unclear. After submission of this study, two articles appeared that reported the isolation of both the murine and human p19 CDK inhibitor (Chan *et al.*, 1995; Hirai *et al.*, 1995). The p19 protein sequence as presented in Figure 1A is nearly identical to that reported by Chan *et al.* (1995) except in two places, at amino acid residues 11 and 12 (AG**DR**LS versus AG**T**-LS) and at residue 159 (IL**Q**GH versus IL**P**GH). In both discrepant areas, the sequence presented in Figure 1A is identical to the genomic sequence determined from a clone isolated from a human placenta genomic library (our unpublished results). Human p19 is very closely related to mouse p19 (Chan *et al.*, 1995; Hirai *et al.*, 1995). Following the genetic nomenclature first used for the p16 gene (Serrano *et al.*, 1993), p19 can be designated as INK4d.

Using the p19 cDNA fragment as the probe, we have isolated lambda clones containing the p19 gene from a human placental genomic DNA library and partially determined the genomic structure of p19 (Figure 1C). DNA sequencing and comparison with the p19 cDNA revealed an intron located at a position corresponding to nucleotides 280–281 of the p19 cDNA presented in Figure 1A. The coding regions of the p15, p16, and p18 genes are also interrupted by an intron at the identical position (Guan *et al.*, 1994). These results demonstrate that all four isolated INK4 genes were evolved from a common ancestor, and thereby constitute a family of evolutionarily related genes.

p19 Specifically Interacts with CDK4 and CDK6

To assess the specificity of p19's interaction with known CDKs, six CDK proteins were in vitro translated with [^{35}S]methionine labeling and mixed with similarly in vitro–translated p19 protein. The mixtures were then immunoprecipitated with antisera specific to each CDK and resolved by SDS-PAGE. p19 was coprecipitated with CDK4 and CDK6, but not with the other CDK proteins (Figure 2B). Reciprocally, in vitro–translated, [^{35}S]-labeled CDK proteins were mixed with excess amounts of purified GST-p19 fusion protein and recovered from the different mixtures on glutathione-agarose beads. Consistently, CDK4 and CDK6, but not the other CDK proteins, were bound to and recovered with the GST-p19 protein (Figure 2C).

In Vivo Association of p19 with CDK6

To establish the in vivo association of p19 with CDK4 and CDK6 and to determine whether p19 encoded the CDK6-associated, p18-related p20 protein previously identified in several human leukemia cell lines (Guan *et al.*, 1994), [^{35}S]methionine-labeled lysates were prepared from ML1 human myeloid leukemia cells and immunoprecipitated with antibodies specific to CDK4, CDK6, and p19 (Figure 3A). The CDK6-associated 20-kDa polypeptide comigrated exactly with in vitro–translated p19 protein (our unpublished results) and with p19 protein immunoprecipitated by the anti-p19 antibody (Figure 3A). When the p20 band from the anti-CDK6 immunoprecipitate was excised from an SDS-polyacrylamide gel, partially digested with V8

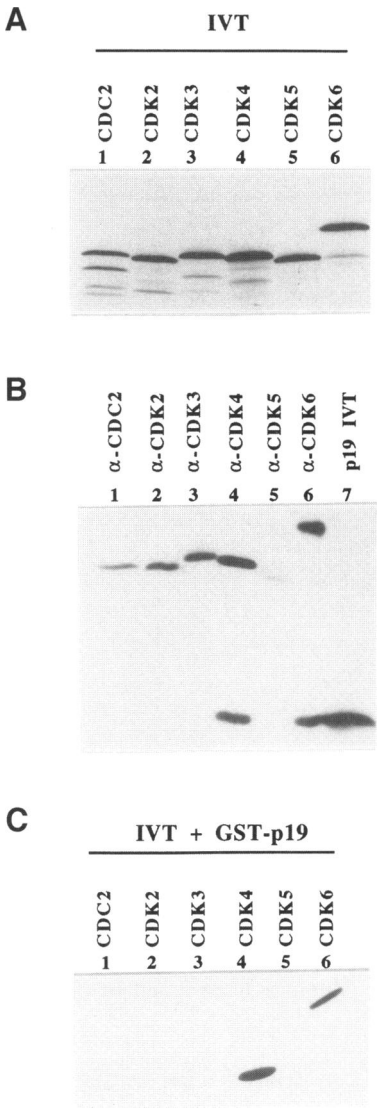

Figure 2. Interaction of p19 with CDKs. (A) The six CDK proteins were synthesized in vitro in rabbit reticulocyte lysate with [^{35}S]methionine labeling and adjusted to an equivalent amount. (B) Equal amounts of each of the six in vitro–translated CDK proteins were mixed with similarly in vitro–translated p19 protein. The mixtures were immunoprecipitated with antibody specific to each CDK protein and precipitates were resolved by SDS-PAGE followed by autoradiography. (C) Purified GST-p19 fusion proteins were incubated with equal amounts of each of the six different in vitro–translated [^{35}S]methionine-labeled CDK proteins and recovered from the different mixtures on glutathione-agarose beads. Proteins bound to GST-p19 were resolved by SDS-PAGE followed by autoradiography. Control GST proteins did not bind to any CDK proteins (our unpublished observation).

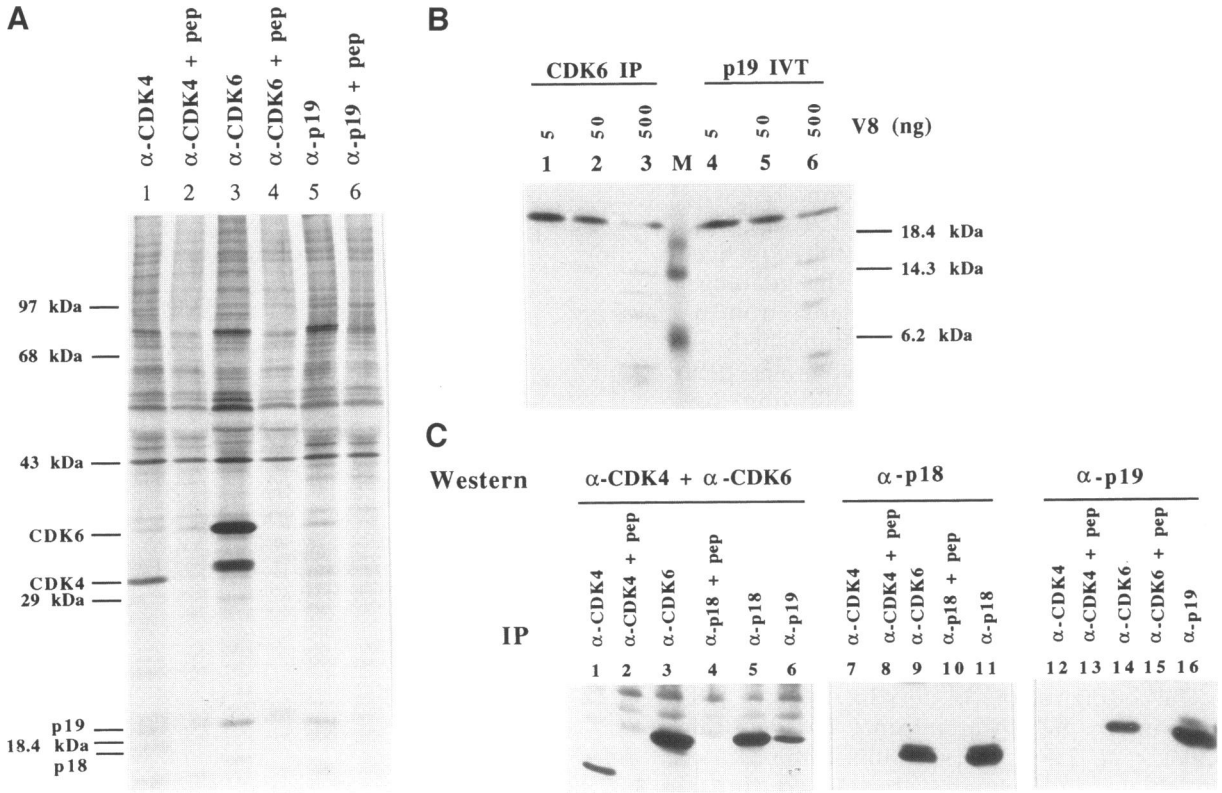

Figure 3. In vivo association of p19 with CDK6. (A) [^{35}S]methionine-labeled lysates were prepared from a myeloid leukemia cell line, ML1. Lysates were immunoprecipitated with anti-CDK4, anti-CDK6, and anti-p19 antibodies, with or without pre-incubation with a competing antigen peptide. The immunoprecipitated polypeptides were resolved on a 15% SDS-polyacrylamide gel. The mobility of protein molecular weight standards (Life Technologies) and relevant proteins are indicated. (B) Comparison of V8 proteolysis patterns of p19 proteins derived from anti-CDK6 immunoprecipitate (lanes 1–3) and in vitro translation (lanes 4–6). (C) Coupled immunoprecipitation and immunoblotting. ML1 cell lysates were immunoprecipitated with antibodies as indicated. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with four different antibodies as indicated on the top of each panel.

protease, and compared with similarly V8-digested in vitro–translated p19 (Figure 3B), they displayed identical proteolysis patterns, confirming the in vivo association of p19 with CDK6. To maintain consistency with the molecular weight identification reported after the submission of this manuscript (Chan *et al.*, 1995; Hirai *et al.*, 1995), the 20-kDa protein is hereafter referred to as p19. In the over twenty cell lines that have been examined, we primarily detected p19 in hematopoietic cell lines in association with CDK6.

As further confirmation of the in vivo association of p19 with CDK6 and to assess the relative contribution of different INK4 proteins to the inhibition of CDK4/6 in a given cell type, we performed coupled immunoprecipitation-immunoblotting (IP-Western) experiments in ML1 cells. ML1 cells expressed a much higher level of CDK6 than CDK4 (Figure 3C, lanes 1 and 3), comparable levels of p18 and p19 (Figure 3C, lanes 11 and 16), and p16 and p15 were undetectable (our unpublished results). There was no detectable interaction between CDK4 and p18 or CDK4 and p19 in ML1 cells as determined by either [^{35}S]methionine-labeling immunoprecipitation or IP-Western (Figure 3). Approximately three times more p18 than p19 was present in CDK6 immunoprecipitates (Figure 3C, lanes 5 and 6). Reciprocally, there is more CDK6 protein co-precipitated with p18 than with p19 (Figure 3C, lanes 9 and 14; note that all three panels of Figure 3C were derived from a single experiment and the same exposure). These results suggest that p18 may have a higher affinity for, and is therefore a more potent inhibitor of, CDK6 than p19 in cells such as ML1 that express similar levels of both proteins.

p19 Inhibits CDK4 and CDK6 Kinase Activity

To directly test whether p19 inhibits the kinase activity of CDK6 and CDK4, we reconstituted the kinase activity of both CDK6 and CDK4 (see MATERIALS AND METHODS). Purified GST-CDK4 or GST-CDK6 protein was mixed with purified GST-cyclin D2 protein in vitro. The mixtures of CDK4- and CDK6-cyclin

D2 were activated by incubating with Jurkat cell lysate and used to assay p19 inhibitory activity using purified GST-Rb fusion protein as a substrate (see MATERIALS AND METHODS). Purified GST-p19 protein (Figure 4A, lanes 3 to 7), but neither control buffer (our unpublished results) nor GST protein (Figure 4, lane 8), effectively inhibits the kinase activity of CDK6 in a dosage-dependent manner. Although p19 was not detected in association with CDK4 in vivo in ML1 cells (Figure 3), it inhibited CDK4 kinase activity in vitro with a similar potency as it inhibited CDK6 (Figure 4B). Forty nanograms of p19 protein or less was sufficient to completely inhibit the kinase activity reconstituted from approximately 100 ng CDK4-cyclin D2 and CDK6-cyclin D2, presumably because only a fraction of the CDK4/6-cyclin D2 complexes was activated. The technical difficulties involved in determining the active portion of the recombinant proteins expressed and purified from bacteria prevented us from determining the stoichiometry of p19's inhibitory interaction with its target kinases and the relative affinity of p19 and other INK4 proteins for CDK4 and CDK6. Consistent with the in vitro binding assays (Figure 2), p19 has no detectable effect on the kinase activity of either immunoprecipitated CDC2 or CDK2 (Figure 4C). CDC2 and CDK2 associate with multiple cyclins (cyclin A, B1, B2, Ds, and E) to constitute several distinct cyclin-CDK enzymes. These data demonstrate that p19 is an inhibitor of CDK4- and CDK6.

Tissue Specificity of p19 mRNA Expression

Northern blot analysis was carried out to determine the expression of p19 mRNA in sixteen different human tissues. Under high stringency conditions, the full length p19 cDNA probe detected a single band of approximately 1.4 kb (Figure 5). For comparison, the same blot was striped and rehybridized with a p18 or p21 probe. Unlike p21 and p27 whose mRNAs accumulate in most human tissues with much less variation (Polyak *et al.*, 1994; Guan *et al.*, 1994; Figure 5), the level of p19 and p18 mRNAs varied significantly between different tissues. The highest level of p19 mRNA was observed in thymus, peripheral blood leukocytes, and brain, and moderate levels were present in spleen, testis, skeletal muscle, and heart. At the other end of the spectrum, p19 mRNA was undetectable in prostate, ovary, small intestine, colon, liver, kidney, and pancreas. Tissue-specific expression was also seen for p18 (Guan *et al.*, 1994; Figure 5), but with a pattern distinctly different from that of p19, particularly in testis and ovary tissues.

Chromosomal Localization of p19

Using the 1.3-kb full length p19 cDNA as a probe, we have determined its chromosomal location by fluorescence in situ hybridization (FISH) to 19p13.2 (Figure 6,

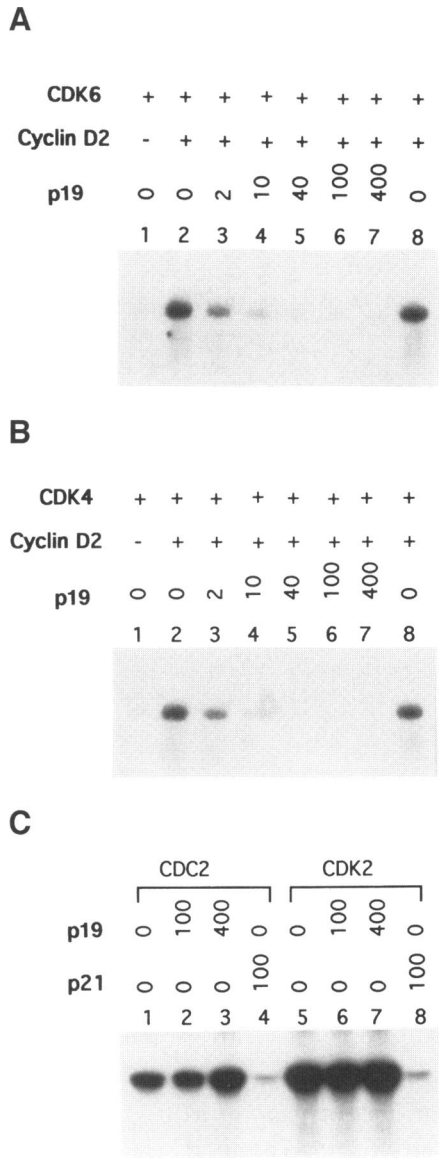

Figure 4. p19 inhibits the kinase activity of CDK4 and CDK6. (A) Inhibition of CDK6-cyclin D2 by p19. The purified recombinant GST-p19 fusion protein (in nanograms) was added to the activated CDK6-cyclin D2 to assay for its inhibitory activity. Purified GST-Rb fusion protein was used as a substrate of CDK6-cyclin D2 kinase. The kinase reaction was resolved on a 10% SDS gel, and transferred to a nitrocellulose filter, followed by autoradiography (20-min exposure). (B) Inhibition of CDK4-cyclin D2 kinase activity by p19. The purified recombinant GST-p19 fusion protein (in nanograms) was added to the activated CDK4-cyclin D2 to assay for its inhibitory activity. (C) p19 does not inhibit CDC2- and CDK2-associated kinase activity. Equal amounts of CDC2 or CDK2 enzymes were immunoprecipitated from Jurkat cells with an anti-CDC2 or anti-CDK2 antisera and assayed for kinase activity using histone H1 as a substrate in the presence (lanes 2–4) or absence (lane 1) of purified inhibitor proteins. Both CDC2 and CDK2 kinase activity was completely inhibited by 100 ng of purified p21 CDK inhibitor protein (lane 4), but not by up to 400 ng of purified GST-p19 (lanes 2 and 3).

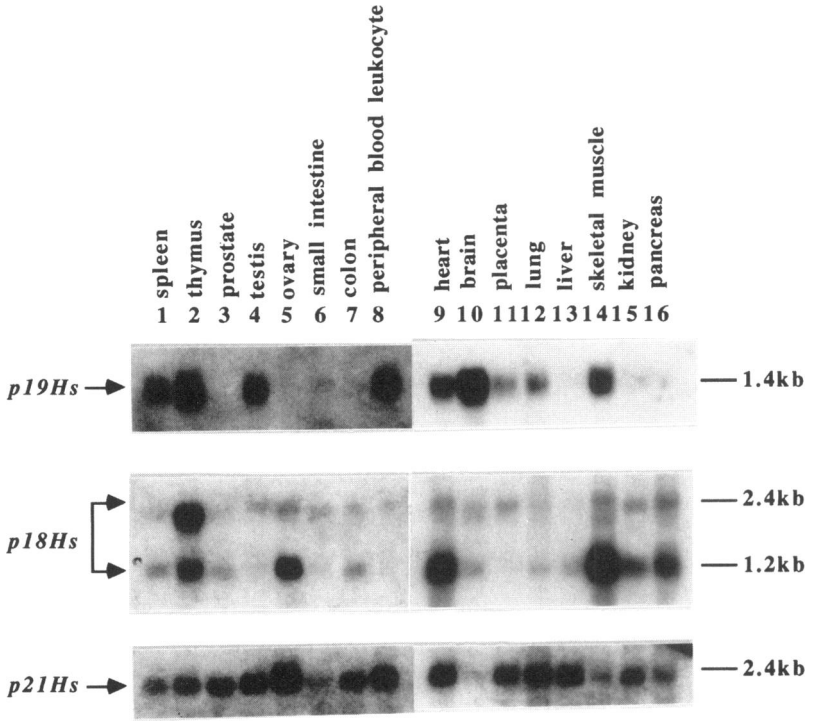

Figure 5. Expression of p19 mRNA in different tissues. Two micrograms of poly(A)$^+$ RNA from 16 different human tissues as indicated at the top of each lane were hybridized with a 1.3-kb probe derived from full length p19 cDNA. A 1.4-kb discrete band was detected by this probe as indicated by an arrow. The same blot was stripped and re-hybridized with a p18 and a p21 probe.

A–C). Abnormalities in the distal arm of chromosome 19p13 have been reported in several human tumors, in particular in ovarian cancer (Kiechle *et al.*, 1994) and leukemia (Huret *et al.*, 1993). Considering the recent discoveries that p16 and p15, both homologues of p19, are deleted or mutated in several specific types of primary tumors (see reviews in Sherr, 1994; Hunter and Pines, 1994), the possibility that p19 might encode a tumor suppressor is clearly worth exploration.

Subcellular Localization of p19

Using affinity-purified antibody raised against a denatured form of p19 protein, we performed indirect immunofluorescence to determine the subcellular localization of p19 in Swiss 3T3 cells (see MATERIALS AND METHODS). This analysis revealed a low level of p19 present in the entire cell (Figure 7, panel 1A). To confirm this observation, we transiently transfected 3T3 cells with a p19 overexpression plasmid (pCMV-p19) and immunostained the transfected cells with the same anti-p19 antibody. The same distribution of p19 across the whole cell was observed (Figure 7, panel 2A), similar to the endogenous p19 protein. To further confirm this observation, a different affinity-purified anti-p19 antibody that was raised against the native form of a GST-p19 fusion protein was used to immunostain pCMV-p19–transfected 3T3 cells (Figure 7, panel 3A). Again, the same p19 distribution pattern across the whole cell was observed. In pCMV-p19–transfected cells immunostained with both antibodies, we noticed a lower intensity of p19 staining in the nucleus. This would suggest that p19 protein may be predominantly present in the cytoplasm, a possibility that remains to be confirmed by further study such as analysis by confocal microscopy. Staining of pCMV-p19–transfected and –untransfected 3T3 cells with the pre-immune sera of either of the antibodies or with the antibodies themselves after pre-incubation with antigen protein did not display any signal (Guan, unpublished observations). A similar observation has also been made that p16 is localized in both the cytosol and nucleus (Okamoto *et al.*, 1994; Lukas *et al.*, 1995; Serrano *et al.*, 1995). Distinct from p16 and p19, cyclin D1 was found to be predominantly localized to the nucleus (e.g., Baldin *et al.*, 1993); Figure 7, panel 4A), raising the possibility that the different pattern of cellular localization of D-type cyclins and INK4 proteins may contribute in part to the lack of ternary cyclin D-CDK4/6-p16 inhibitor complexes in vivo.

It is worth noting that in the transfection-immunostaining experiments all p19 positive cells were single cells and did not form multi-cell clusters. Because the immunofluorescence staining was performed 48 h after transfection, 3T3 cells should have had sufficient time to complete at least two rounds of cell division and form clusters containing more than one cell. This observation indicates that the ectopic expression of p19 inhibits the cell cycle.

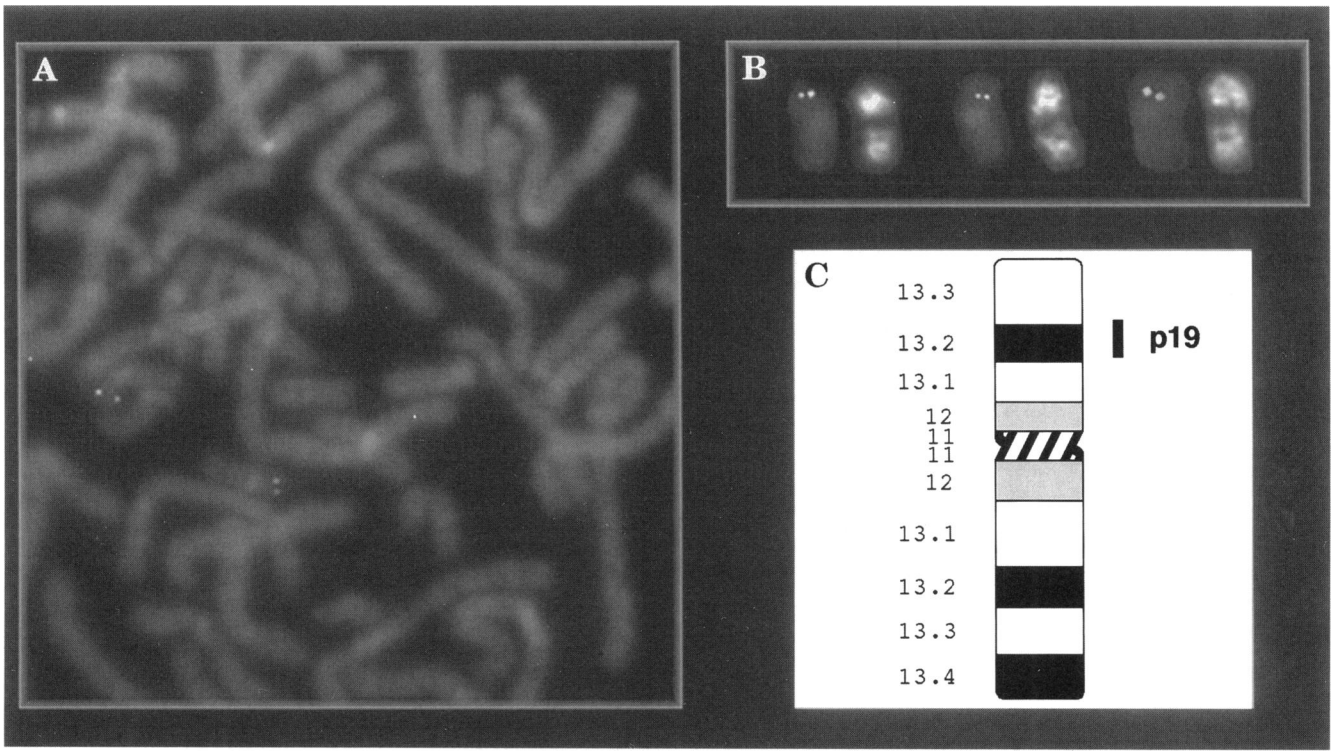

Figure 6. Chromosomal location of p19. (A) After detection of the p19 cDNA probe using FITC-conjugated avidin, the chromosomes were counterstained with DAPI. The metaphases were imaged using a cooled charge-coupled device camera, and the source images were merged using Gene Join on an Apple Macintosh computer. (B) Three different chromosomes 19, at a higher magnification, show hybridization of p19 to 19p13. The left hand image of each pair represents the DAPI-stained chromosomes merged with the avidin-FITC signal. The right hand image of each pair shows the same chromosomes stained with rhodamine-conjugated anti-digoxigenin. The Alu oligo was used to generate an R-banding pattern that unambiguously localized the p19 hybridization signals to chromosome 19p13. (C) An ideogram of chromosome 19. The bar to the right represents the range of p19 signals.

Cyclins Have Different Effects on the Two CDK Inhibitor Families' Binding to CDKs

Members of the p16 family (p15, p16, p18, and p19) and members of the cyclin D family (D1, D2, and D3) can both be detected in CDK4 and CDK6 immunocomplexes. However, reciprocal immunoprecipitations, using antibodies specific to p16 inhibitors or D cyclins, have yet to detect in vivo interactions between p16 family inhibitors and cyclin D family members. Furthermore, coupled IP-Western experiments in ML1 cells using antibodies against p18, p19, and cyclins D1, D2, and D3 revealed no p16 family inhibitors in the cyclin D immunocomplexes and reciprocally no D cyclins in the inhibitor immunocomplexes (Jenkins and Xiong, unpublished results). These observations suggest that these two families of proteins may interact with CDK4/6 in a mutually exclusive manner in vivo. In contrast, both p21 and p27 can be detected in CDK and cyclin immunocomplexes, indicating the formation of CDK-cyclin-p21/p27 ternary complexes. Using in vitro–assembled CDK6-cyclin D1 complexes, we further investigated the interaction of both families of CDK inhibitors with CDK proteins.

Assembly of CDK4/CDK6 with D cyclins using bacterially expressed and purified proteins has been found to be inefficient and requires additional incubation with mammalian cell lysate or coexpression in insect cells (Kato *et al.*, 1994b). CDK4 or CDK6 and cyclin D complexes, however, can be efficiently assembled using proteins in vitro translated in rabbit reticulocyte lysate (Figure 8). Equal amounts of in vitro–translated, [^{35}S]methionine-labeled CDK6 protein were mixed with increasing amounts of similarly in vitro–translated, [^{35}S]methionine-labeled cyclin D1, followed by the addition of one of two representative proteins from each of the two CDK inhibitor families. The mixtures were then immunoprecipitated with an antibody specific to CDK6 and resolved by SDS-PAGE. p18 and p19 can each efficiently form binary complexes with CDK6 in the absence of cyclin D1 protein (Figure 8A, lanes 1 and 7). Preincubation with increasing amounts of cyclin D1 protein resulted in a decreased association of p18 or p19 to CDK6 (Figure 8A, lanes 2–6 and lanes 8–12). These observations indicated that INK4 proteins interact preferentially with the unbound CDK6 subunit and that the forma-

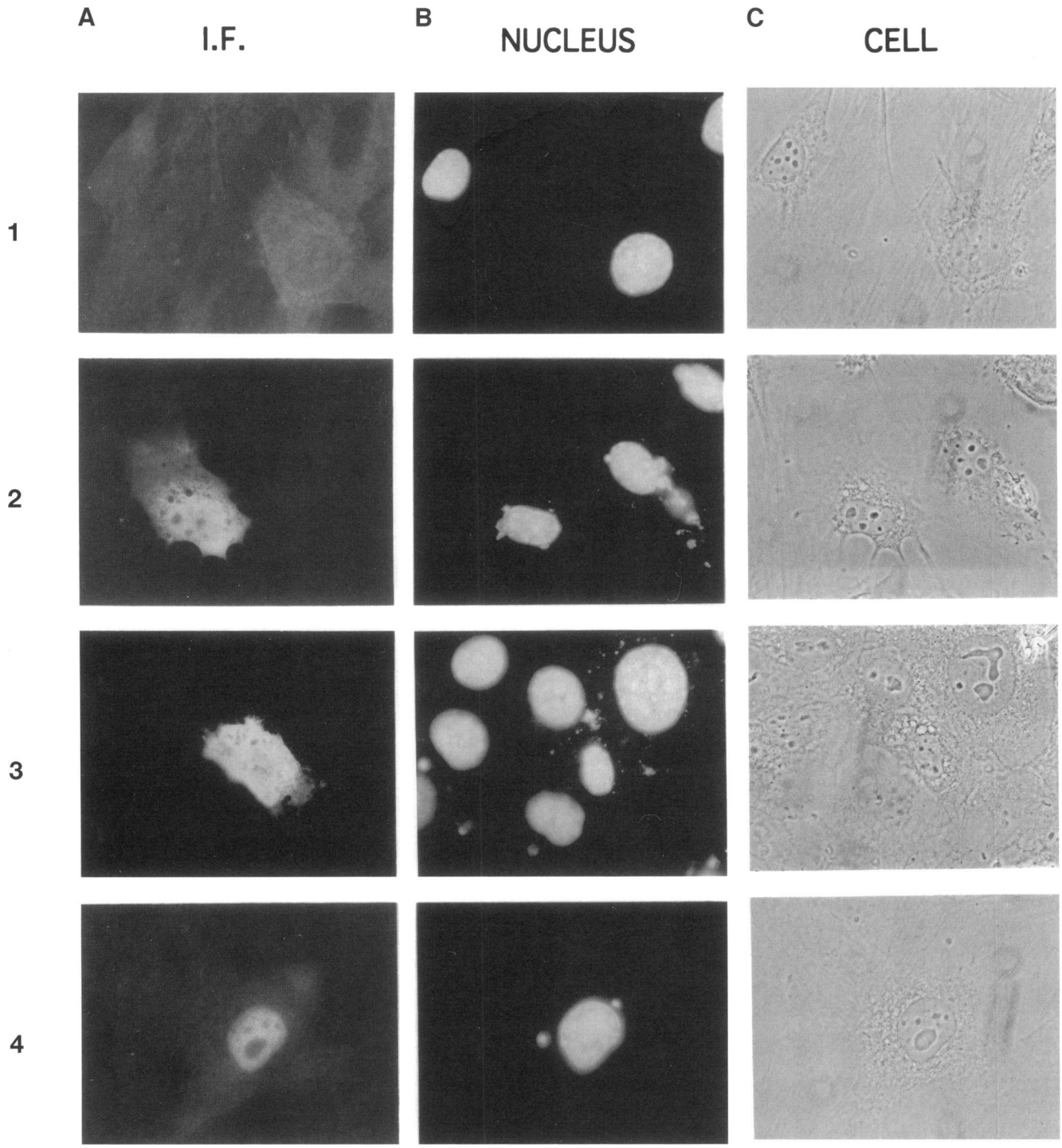

Figure 7. Subcellular localization of p19. (1A) Immunofluorescence staining of untransfected Swiss3T3 cells with affinity purified anti-p19 antibody; (2A) Immunofluorescence staining of pCMV-p19–transfected Swiss3T3 cells with affinity-purified anti-p19 antibody; (3A) Immunofluorescence staining of pCMV-p19–transfected Swiss3T3 cells with affinity-purified anti-GST-p19 antibody; (4A) Immunofluorescence staining of untransfected Swiss3T3 cells with affinity-purified anti-cyclin D1 antibody. Column B (DAPI staining for the nucleus) and column C (phase contrast of cells) correspond to the same field as column A (immunofluorescence staining). I.F. denotes immunofluorescence.

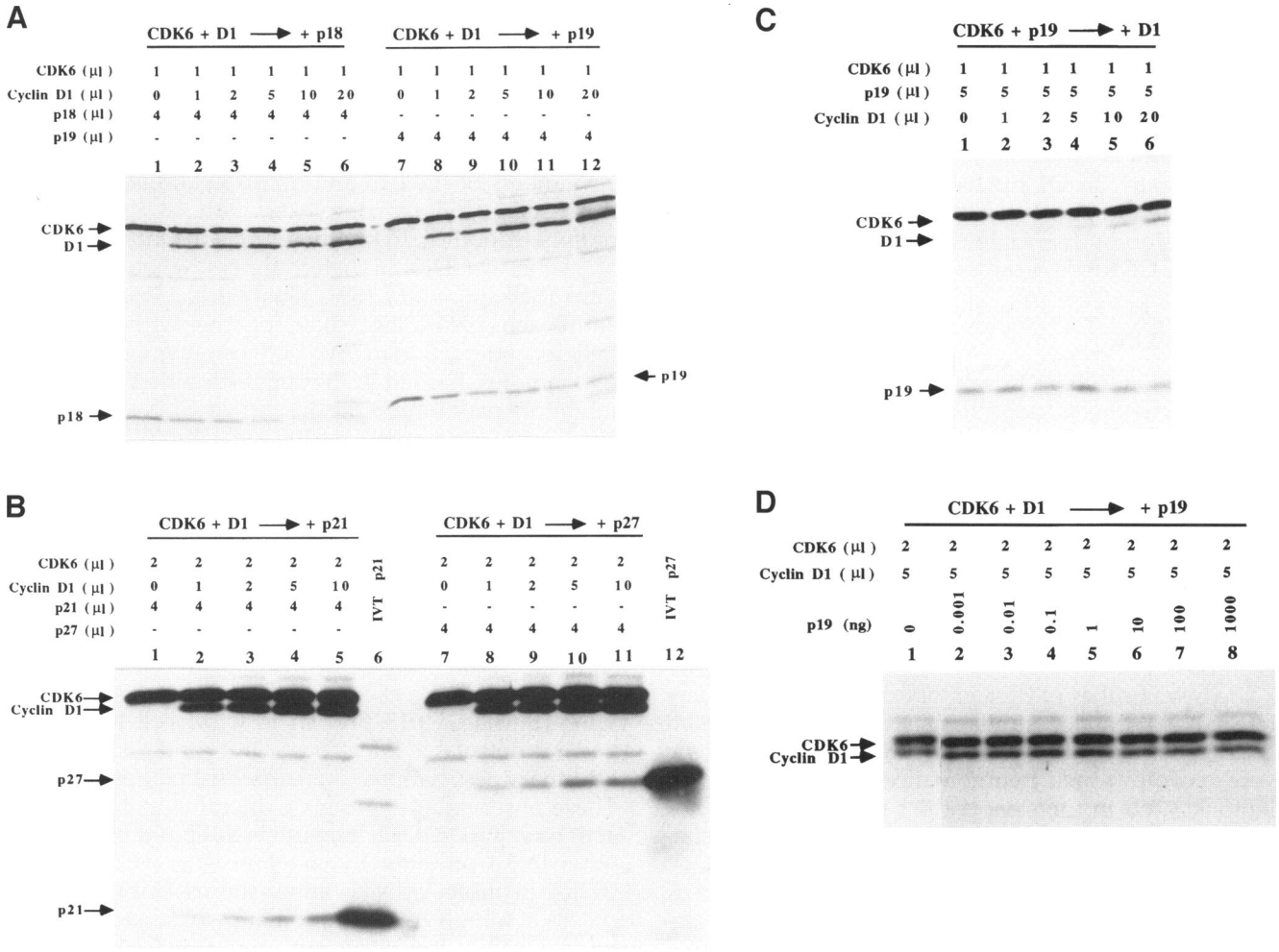

Figure 8. Opposite effects of cyclin on the binding of the two families of inhibitors to CDK. After sequential incubation of appropriate proteins, all mixtures were immunoprecipitated with anti-CDK6 antibody and precipitates were resolved by SDS-PAGE followed by autoradiography. (A) CDK6 was pre-incubated with increasing amounts of cyclin D1 protein followed by the addition of a set amount of p18 (lanes 1–6) or p19 (lanes 7–12). All proteins were in vitro translated with [^{35}S]methionine labeling. (B) CDK6 protein was mixed with equal amounts of p21 (left) or p27 (right) protein in the absence (lanes 1–7) or presence of increasing amounts of cyclin D1 protein. All proteins were in vitro translated with [^{35}S]methionine labeling. (C) Preassembled CDK6-p19 binary complexes were incubated with an increasing amount of cyclin D1. CDK6 complexes were recovered by immunoprecipitation with an antibody specific to CDK6 and resolved by SDS-PAGE. (D) Preassembled CDK6-cyclin D1 binary complexes using in vitro–translated [^{35}S]methionine-labeled CDK6 and cyclin D1 proteins were mixed with an increasing amounts of bacterially produced and purified p19 protein. CDK6 complexes were recovered by immunoprecipitation with an antibody specific to CDK6 and resolved by SDS-PAGE.

tion of cyclin D1-CDK6 complex interferes with or prevents this interaction. In contrast, in the absence of the cyclin subunit, neither p21 nor p27 bind to CDK6 efficiently. Addition of cyclin D1 protein stimulated the binding of p21 and p27 to CDK6 or CDK6-cyclin D1 complexes (Figure 8B). This is consistent with recent findings that addition of an increasing amount of cyclin greatly enhanced the association of p21, p27, and p57^{Kip2} with CDK2 (Harper *et al.*, 1995; Matsuoka *et al.*, 1995). These results demonstrated an opposite effect of cyclin D protein on the association of the two families of inhibitors with CDK proteins.

p19 Does Not Disrupt Cyclin D1-CDK6 Association

We next determined whether INK4 proteins and D cyclins could dissociate each other from CDK4/6. Preassembled CDK6-p19 binary complexes were incubated with increasing amounts of cyclin D1 protein and CDK6 complexes were recovered by immunoprecipitation with an antibody specific to CDK6 and resolved by SDS-PAGE (Figure 8C). Addition of an excess amount of cyclin D1 protein did not have any detectable effect on the preassembled CDK6-p19 binary complex. Reciprocally, addition of an excess

amount of p19 protein expressed and purified from *E. coli*, up to 1 μg, did not dissociate preassembled CDK6-cyclin D1 binary complexes either (Figure 8D). The same results were also obtained for p18 in which addition of up to 1 μg of p18 protein expressed and purified from *E. coli* did not dissociate already assembled CDK6-cyclin D1 binary complexes (our unpublished results). These results indicate that p19 and cyclin D1 do not dissociate each other's binary association with CDK6.

DISCUSSION

In mammalian cells, a number of cell cycle regulatory proteins have been identified that physically interact with cyclin or CDK proteins (see reviews by Hunter and Pines, 1994; Sherr, 1994; Sherr and Roberts, 1995). Most of these CDK-interacting proteins encode inhibitory proteins and they fall into two distinct classes: the p21 family and the p16 family. In this report, we isolated and characterized a novel gene encoding a CDK4- and CDK6-specific inhibitor, $p19^{INK4d}$, that belongs to the expanding p16/INK4 family of CDK inhibitors.

The two families of CDK inhibitors do not share any detectable sequence similarity and all four isolated INK4 genes including the newly identified p19 gene were evolved from a common ancestor. In addition, as demonstrated in this report the two families of CDK inhibitors interact with CDKs via distinct mechanisms—while the cyclin subunit stimulates the association of p21 and p27 with CDKs or cyclin-CDK complexes to form ternary p21/p27-CDK-cyclin association, it impedes p19 and p18 (and most likely other members of this family as well) from binding to CDKs. The cyclin-dependent binding of p21 to CDK provides a plausible explanation for the observation that although both p21 and CDC2 proteins are expressed during S phase, they do not become associated until the subsequent expression of cyclin B1 protein and the formation of CDC2-cyclin B1 complexes after the cells have entered the G2 phase (Li *et al.*, 1994a). These findings suggest that there may be a sequential change in the composition of CDK complexes during the cell cycle: CDKs start as monomeric subunits, assemble into catalytically inactive p21-CDK-cyclin complexes, and then convert to active enzymes as the level of cyclin continues to increase and overcomes the threshold of p21 inhibition. p21 and its two relatives, p27 and p57, are all capable of interacting with multiple CDK-cyclin complexes, pointing to the possibility that they may further regulate the activity of these CDK kinases in a cell cycle–dependent manner by shuffling from one CDK enzyme to another. Cyclin-stimulated or cyclin-dependent binding of p21 and p27 to CDKs suggests that the accumulation of cyclins is critical for this shuffling, since CDK protein is generally present in excess throughout the cell cycle, and by itself is apparently not sufficient to release p21 or p27 from—thus potentially activating—existent cyclin-CDK-p21/p27 complexes. The state of a p21- or p27-inhibited CDK kinase may thus be dependent on and linked to the synthesis of the next cyclin in the ordered sequence.

Members of the p16 and cyclin D families can both form stable binary complexes with CDK6. Purified protein from the p16 family inhibits the kinase activity of cyclin D-CDK4/6 heterodimers when added in vitro (Serrano *et al.*, 1993; Guan *et al.*, 1994; Hannon and Beach, 1994). This, taken together with the failure to detect interaction in vivo between members of these two families, has led to the suggestion that p16 family inhibitors may inhibit the activity of CDK4 and CDK6 kinases by disrupting cyclin D-CDK4/6 association (e.g., Guan *et al.*, 1994). Contrary to this prediction, binary CDK6-cyclin D complexes assembled in vitro are highly stable and cannot be dissociated by excess amounts of p19 (Figure 8D) or p18 (our unpublished results). Although the in vitro setting may lack some modifications occurring in vivo that could affect the interaction between inhibitors and cyclin D-CDK4/6 complexes, this seems unlikely to be the case, since the addition of large excess amounts of purified or in vitro–translated p18 protein to CEM cell lysate also had no detectable effect on CDK4- and CDK6-cyclin D1 binary associations (our unpublished observation). These observations present a dilemma as to how the active cyclin D-CDK6 enzyme is inhibited by p19 and other INK4 proteins. By co-expressing cyclin D2 and CDK4 in insect cells to allow the assembly of complexes at a high concentration and subsequently incubating with a large amount of purified p19 protein, Hirai *et al.* (1995) detected a small amount of p19 protein in cyclin D2 immunoprecipitates concomitant with inhibition of cyclin D2-CDK4 kinase activity by p19, indicating the formation of p19-CDK6-cyclin D2 ternary complexes (Hirai *et al.*, 1995). It remains to be determined, however, whether this represents the primary mechanism of inhibition by p16 family members in vivo.

These in vivo observations and the in vitro studies suggest two different mechanisms for p16 inhibitors to control the activity of CDK4 and CDK6 both before and after their activation. The preferential association of p19 and p18 with unbound CDK6 and the interference of D cyclins and p16 inhibitors with each other's CDK association (Figure 8, C and D) indicate that p16 inhibitors and D cyclins may interact with CDK6 in a competing or potentially mutually exclusive manner. Thus, the temporal order of p16 inhibitor and D-type cyclin expression during the cell cycle may play a key role in regulating the activity of CDK4 and CDK6 as it will determine which binary complexes, active cyclin D-CDK4/6 or inactive p16-CDK4/6, will be assembled first, and consequently be preferentially main-

tained. This would provide an inhibitory mechanism for p16 family proteins not dependent on the ternary complexes as yet unobserved in vivo. Once they have already been activated, holo CDK4/6-cyclin D enzymes may be inactivated by a p16 inhibitor either through direct inhibition—the formation of catalytically inactive ternary p16-cyclin D-CDK4/6 complexes—or indirectly, through continuous loss of cyclin D from the ternary complexes as the result of cyclin D's short half-life with the consequent accumulation of binary CDK4/6-p16 inhibitor complexes that may prevent further binding to and activation of CDK4/6 by newly synthesized cyclin D.

The p16/INK4 family of CDK inhibitors is expanding rapidly. In addition to four genes that have already been isolated, several additional CDK4- and CDK6-associated proteins have also been identified that are likely to encode p16-related genes (Guan *et al.*, 1994; Hannon and Beach, 1994; Li *et al.*, 1994b; our unpublished observation). While these genes share a common biochemical property in inhibiting the kinase activity of two closely related CDK enzymes, the transcriptional regulation of each member of this family is quite different. The p16 gene is transcriptionally repressed by the retinoblastoma susceptibility gene product, pRb, and thus forms a feedback regulatory loop (Li *et al.*, 1994b). The level of $p14/p15^{MTS2/INK4B}$ mRNA is dramatically increased shortly after the treatment of human keratinocytes with the anti-proliferative agent TGF-β (Hannon and Beach, 1994). So far, we have not observed any effect on the expression of either p18 or p19 mRNA by the inactivation of p53 or pRb function, or by TGF-β treatment (our unpublished observation). The expression of both p18 and p19 mRNAs, however, exhibits remarkably distinct tissue specificity (Guan *et al.*, 1994; Figure 5), suggesting that p19 and p18 may play a role in inducing and/or maintaining cell differentiation. Two critical aspects of cell differentiation are permanent withdrawal from the active cell division cycle and the prevention of terminally differentiated cells from re-entering the cell cycle. CDK4 and CDK6 are two major CDK enzymes that are activated during, and required for the G1 progression of the cell cycle, most likely by phosphorylating and thus downregulating the growth-suppressing activity of pRb. Activation of a particular INK4 family gene expression by a specific differentiation signal such as p19 in hematopoietic cells or p18 in muscle cells could effectively arrest G1 cell cycle progression to initiate differentiation. Alternatively, but not mutually exclusively, accumulation of a high level of an INK4 inhibitor may prevent terminally differentiated cells from re-entering the cell cycle through the same mechanism.

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