

## Identification of Human and Mouse p19, a Novel CDK4 and CDK6 Inhibitor with Homology to p16<sup>ink4</sup>

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**The cell cycle in mammalian cells is regulated by a series of cyclins and cyclin-dependent kinases (CDKs). The G<sub>1</sub>/S checkpoint is mainly dictated by the kinase activities of the cyclin D-CDK4 and/or cyclin D-CDK6 complex and the cyclin E-CDK2 complex. These G<sub>1</sub> kinases can in turn be regulated by cell cycle inhibitors, which may cause the cells to arrest at the G<sub>1</sub> phase. In T-cell hybridomas, addition of anti-T-cell receptor antibody results not only in G<sub>1</sub> arrest but also in apoptosis. In searching for a protein(s) which might interact with Nur77, an orphan steroid receptor required for activation-induced apoptosis of T-cell hybridomas, we have cloned a novel human and mouse CDK inhibitor, p19. The deduced p19 amino acid sequence consists of four ankyrin repeats with 48% identity to p16. The human p19 gene is located on chromosome 19p13, distinct from the positions of p18, p16, and p15. Its mRNA is expressed in all cell types examined. The p19 fusion protein can associate *in vitro* with CDK4 but not with CDK2, CDC2, or cyclin A, B, E, or D1 to D3. Addition of p19 protein can lead to inhibition of the *in vitro* kinase activity of cyclin D-CDK4 but not that of cyclin E-CDK2. In T-cell hybridoma DO11.10, p19 was found in association with CDK4 and CDK6 *in vivo*, although its association with Nur77 is not clear at this point. Thus, p19 is a novel CDK inhibitor which may play a role in the cell cycle regulation of T cells.**

Apoptosis in immature T cells and T-cell hybridomas, which may relate to negative selection during T-cell development, can be initiated by signals through the T-cell receptor-CD3 complex (18, 32, 33). This process of activation-induced apoptosis (anti-CD3 apoptosis) consists of two distinct phases. The first phase is the cell cycle block at the G<sub>1</sub>/S transition; it is followed by the second phase, with the generation of apoptotic DNA ladders (18). The second phase requires extracellular calcium and can be inhibited by the immunosuppressive drug cyclosporin A (18). We and others have shown that Nur77 (NGFI-B) orphan steroid receptor is induced during anti-CD3 apoptosis through the calcium signals and it plays an essential role in the cell death process (17, 35). Dominant negative Nur77 can block apoptosis but not the interleukin 2 production of anti-CD3-treated T-cell hybridomas (35). Thus, Nur77 is involved in the second phase of anti-CD3 T-cell apoptosis.

As alluded to above, anti-CD3 death in T-cell hybridomas is also accompanied by a G<sub>1</sub> cell cycle block. In all organisms studied so far, cell cycle progression is mediated by cyclin-dependent kinases (CDKs) that consist of a catalytic subunit CDK and a regulatory subunit cyclin. In mammalian cells, cyclin E-CDK2 together with cyclin D-CDK4 and/or cyclin D-CDK6, which are active in the G<sub>1</sub> phase, control the G<sub>1</sub>-to-S transition (23, 24, 28, 31 and references therein). There are at least three different D-type cyclins, with T cells expressing cyclins D2 and D3 and two cyclin D-associating kinases, CDK4 and CDK6. One of their substrates is the retinoblastoma (Rb) protein, which, upon phosphorylation, releases the E2F transcription factor. E2F in turn activates genes that are required for the S phase (31). Cell cycle control in G<sub>2</sub>/M and S phases

is mediated by a different set of cyclins and CDKs. These are cyclin B-CDC2 and cyclin A-CDK2, which are active in G<sub>2</sub>/M and S phases, respectively. Their activities are required for cell entry into mitosis (23, 24, 28, 31).

Activity of these cyclin-CDK kinases is subjected to several levels of regulation. One of these is through the action of cell cycle inhibitors. Several of these inhibitors were isolated recently. p21 (also known as Waf1, Cip1, Sdi1, or CAP20) is transcriptionally regulated by p53 and processes leading to senescence (4, 5, 8, 22, 36). It can associate with G<sub>1</sub> cyclins as well as the mitotic cyclins and may play an important role in the assembly of the cyclin-CDK complexes and in DNA replication (16, 38). A closely related protein, p27, is implicated in G<sub>1</sub> phase arrest by transforming growth factor  $\beta$  (TGF- $\beta$ ), cAMP, and cell-cell contact (14, 26, 27, 34). It too can associate with a variety of cyclin-CDK kinases. In contrast, a different group of cell cycle inhibitors, p16<sup>ink4</sup> and p15<sup>ink4B</sup>, can associate only with the G<sub>1</sub> CDKs CDK4 and CDK6. Their inhibitory activity is restricted to the cyclin D-CDK4 and cyclin D-CDK6 kinases (7, 30). The predicted amino acid sequence of p16 and p15 contains four ankyrin repeats (7, 30). They are homologous to each other, with 44% identity in the first 50 amino acids and 97% identity in the last three ankyrin domains (7, 30). The genes encoding p16 and p15 are located on human chromosome 9p21, a site of frequent chromosomal deletions in many human tumor cell types (12, 21). Mutations in the p16 gene are frequently detected in primary melanoma cells (11, 13).

In searching for proteins interacting with Nur77 by a yeast two-hybrid screening strategy, we have cloned a novel cDNA, whose predicted amino acid sequence has extensive homology with that of the well-known p16 cell cycle inhibitor (30). Sequencing of the human and mouse cDNAs (whose product we designated p19) revealed 164- and 165-amino-acid open reading frames, respectively. As expected, p19 can only associate with CDK4 but not with CDK2, CDC2, or any of the cyclins

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(A, B, D1, D2 and D3). p19 protein can inhibit the kinase activity of cyclin D1-CDK4 but not that of cyclin E-CDK2. Furthermore, we showed that this protein associates with both CDK4 and CDK6 *in vivo* in T-cell hybridoma DO11.10. The human p19 gene maps to chromosome 19p13. Although its association with Nur77 is not clear at this point, p19 is nevertheless a novel CDK4 and CDK6 inhibitor which may play a role in the regulation of the cell cycle in T cells.

## MATERIALS AND METHODS

**Yeast two-hybrid system.** To generate a Gal4 fusion construct, a cDNA sequence corresponding to the DNA and ligand binding domains of mouse Nur77 was first amplified by PCR from the N10 plasmid containing Nur77 cDNA (in pKS-Bluescript). The primers used are T7 primer at the 3' end and a primer containing the Nur77 sequence starting from nucleotide 869 (8a). A *Bam*HI site was introduced at the 5' oligonucleotide. The resulting 1.5-kb fragment was first cloned into the *Bam*HI site of pSP72 (Promega). A 0.45-kb *Bam*HI-*Alw*NI (filled in) fragment including the DNA binding domain plus the A box was then subcloned into pAS-CYH1 at the *Bam*HI-*Sal*I (filled in) site. This fusion plasmid was used to screen a mouse peripheral blood T-cell library in pACT (a generous gift of S. Elledge) according to the published protocol (3).

**In vitro binding assay.** Ten micrograms of glutathione *S*-transferase (GST) or GST-p19 fusion proteins bound to glutathione-agarose beads was incubated with *in vitro*-translated <sup>35</sup>S-labeled cyclins or CDKs for 2 h at 4°C in 200 μl of binding buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.4], 150 mM NaCl, 10% glycerol, 0.05% Nonidet P-40). The beads were first washed four times with the binding buffer described above. They were then boiled and resolved on a 10% polyacrylamide gel.

**Immunoprecipitation.** DO11.10 cells were stimulated with 10 ng of PMA (phorbol 13-myristate 12-acetate) per ml plus 0.5 μM ionomycin (Sigma) at various time points as indicated in some of the figure legends. Cells were labeled with [<sup>35</sup>S]methionine (Translabel; ICN) in RPMI medium without methionine supplemented with 10% dialyzed fetal calf serum for 3 h. Immunoprecipitations were performed as described elsewhere (37). For sequential immunoprecipitation, the immunoprecipitates were boiled in IP buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 20 mM EDTA, 0.5% Nonidet P-40, and various protease inhibitors) with 2% sodium dodecyl sulfate (SDS) for 20 min. The resulting supernatant was diluted 1:25 in IP buffer, and the second immunoprecipitation was performed in the same way as in the first immunoprecipitation. Samples were resolved electrophoretically on 17.5% polyacrylamide gels.

**Propidium iodide assay.** DO11.10 cells were treated with PMA-ionomycin and sampled at various time points. Cells were harvested and washed twice with phosphate-buffered saline (PBS). The cell pellet was resuspended in 0.1 ml PBS and fixed with 0.9 ml of methanol at -70°C. Cells were washed twice in PBS and resuspended in 0.5 ml of RNase A (2 mg/ml) and 0.5 ml of propidium iodide (20 μg/ml). Samples were analyzed by flow cytometry with the Coulter EPICS XL machine.

**Plasmids.** Plasmids and baculoviruses for cyclin E and CDK2 were obtained from D. Morgan; plasmids and baculoviruses for CDK4 and cyclins D1, D2, and D3 were provided by C. Sherr; plasmids for CDC2, cyclin A, and cyclin B were a gift of B. Dynlacht. Plasmids for p16 and p21 were a gift of D. Beach. For mouse p19, a 1.1-kb *Xho*I insert from the yeast vector pACT was subcloned into the pSP72 plasmid (Promega) and used for *in vitro* transcription and translation experiments. Human p19 cDNA clones were isolated from a human thymus λgt11 library (Clontech) with mouse p19 as a probe under low-stringency conditions. The largest insert (1.3 kb) was then subcloned into the *Eco*RI site of the pSP72 plasmid (Promega). For *in vitro* transcription, the CDC2 plasmid was cut with *Eco*RV and transcribed with SP6 RNA polymerase, the CDK2 plasmid (in pSP72) was cut with *Hind*III and transcribed with T7 RNA polymerase, the CDK4 plasmid (in pSP72) was cut with *Hind*III and transcribed with T3 RNA polymerase, the cyclin A plasmid was cut with *Bam*HI and transcribed with SP6 RNA polymerase, the cyclin B plasmid was cut with *Hind*III and transcribed with SP6 RNA polymerase, the cyclin D1 plasmid was cut with *Bam*HI and transcribed with T7 RNA polymerase, the cyclin D2 plasmid was cut with *Xho*I and transcribed with T3 RNA polymerase, the cyclin D3 plasmid was cut with *Bam*HI and transcribed with T7 RNA polymerase, and the cyclin E plasmid was cut with *Bam*HI and transcribed with T7 RNA polymerase.

**Antibodies.** To generate polyclonal antibodies for mouse p19, we subcloned a *Bam*HI-*Bgl*II fragment of mouse p19 cDNA (in pACT) into the *Bam*HI site of pGex1N. Fusion protein was injected into New Zealand White rabbits at 2-week intervals. Rabbits were terminally bled after 5 injections. The antisera were first tested for their abilities to precipitate *in vitro*-translated mouse p19. The antisera were then precleared on a GST affinity column and purified on a GST-p19 affinity column. Affinity resins were made by conjugation of the corresponding bacterial fusion protein to the A20 gel matrix (Bio-Rad) according to the manufacturer's instructions. Antibodies for the CDK4 epitope (amino acids 282 to 303) and the CDK6 epitope (amino acids 306 to 326) were purchased from Santa Cruz Inc.

**Fluorescence in situ hybridization (FISH) experiment.** Bromodeoxyuridine-

synchronized, phytohemagglutinin-stimulated peripheral blood lymphocytes from a normal donor were used as a source of metaphase chromosomes. Genomic DNA in a cosmid vector was labeled with either digoxigenin-11-UTP or biotin-14-UTP and hybridized overnight at 37°C to fixed metaphase chromosomes according to published methods (25), except for the inclusion of 33 μg of highly reiterated human DNA self-annealed to Cot 1 per ml. Signals were detected by incubation of the slides with fluorescein-conjugated sheep antidigoxigenin antibodies and Texas red-conjugated avidin followed by counterstaining with 4',6-diamidino-2-phenylindole (DAPI) in antifade. Analyses of 20 hybridized chromosomes were made from digitally acquired merged images that were obtained with a charge-coupled device camera and a commercially available software package. Fluorescence microscopy was performed with Nikon Optiphot microscope.

**Kinase inhibition assay.** Insect Sf9 cells (10<sup>6</sup>) were infected with either cyclin D1 or CDK4 recombinant baculoviruses alone or were coinfecting with both viruses at a multiplicity of infection of 5. Cyclin E and CDK2 recombinant baculoviruses were also used to coinfect Sf9 cells. After 72 h, cells were resuspended in 500 μl of kinase buffer (50 mM HEPES [pH 7.5], 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.5 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate) plus 1 μg of antipain per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, and 0.1 mM phenylmethylsulfonyl fluoride (8, 36) and lysed by passage through a 26G1/2 needle six times. The cleared lysates were aliquoted and saved at -80°C.

For the kinase assays, 2 μl of insect cell lysates was mixed with various amounts of bacterially expressed GST, GST-p19, or GST-p16 (see Fig. 5) in 50 μl of kinase buffer and preincubated at 30°C for 30 to 40 min (36). This mixture was then added to the bacterially expressed GST-Rb large-pocket protein (0.5 μg; recombinant plasmid expressing GST-Rb was provided by J. Wang) immobilized on glutathione-agarose beads plus 50 μCi of [γ-<sup>32</sup>P]ATP in 50 μl of kinase buffer and was incubated at 30°C for 10 min. The phosphorylated proteins were separated by SDS-10% polyacrylamide gel electrophoresis and exposed to X-ray film.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the human and mouse p19 sequences are U20498 and U20497, respectively.

## RESULTS

**Cloning of a gene (p19) with homology to the p16 cell cycle inhibitor.** In searching for the protein(s) associating with Nur77 (2, 20), we have used a mouse Nur77 cDNA as a "bait" in a yeast two-hybrid screen. Plasmids encoding fusion proteins of the Gal4 DNA binding domain and various Nur77 protein domains were made and tested first for their abilities to activate a *lacZ* reporter gene under the control of several Gal4 DNA binding sites. Neither the Nur77 DNA binding domain (two zinc fingers and an A box) nor its C-terminal domain contains any transactivation activity. We used the Gal4-Nur77 DNA binding domain construct to screen a mouse peripheral blood T-cell cDNA library made in the appropriate plasmid as a fusion protein with the Gal4 activation domain. Screening was done as described elsewhere (3) by isolation of histidine-positive colonies and subsequent testing of them for expression of the *lacZ* gene. Clones that showed bait-dependent *lacZ* expression were chosen for further analysis. Several plasmids encoding proteins that can interact with the Nur77 DNA binding domain but not with a series of irrelevant proteins were obtained. Cotransfection of any of these plasmids with the Gal4-Nur77 fusion protein plasmid resulted in activation of the *lacZ* gene under Gal4 control. One of these clones contained an open reading frame with homology to the previously published human p16 and p15 cell cycle inhibitors (7, 12, 30), all of which consist of four ankyrin repeats (Fig. 1). We designated this gene p19 to reflect its protein molecular weight *in vitro* (after transcription and translation) and *in vivo* (detected by immunoprecipitation; see below). To make sure that the newly isolated cDNA is not simply a mouse homolog of human p16, we screened a human thymus cDNA library with the mouse p19 as a probe. The deduced human and mouse p19 protein sequences show 81% identity (Fig. 1). The human p19 is clearly different from p16, and its deduced sequence had 48% identity with that of human p16 over a stretch of 130 amino acids. The human p19 sequence is also different from the human p15 and p18 sequences (6, 7), indicating that p19 is a novel member of

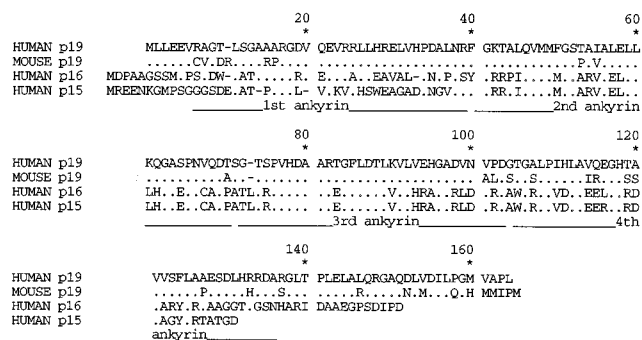


FIG. 1. The deduced amino acid sequences of human and mouse p19 and sequence comparison to human p16<sup>nk4</sup>. Amino acids are represented in single-letter code. Sequences have been aligned to show the highest homology by use of the MacVector program (Kodak). Dots represent identity; lines represent gaps in homology. The boundaries of the ankyrin repeats are shown.

the p16 cell cycle inhibitor family. Northern (RNA) blot analysis with mRNA from several cell lines showed that p19 mRNA is expressed in all cell types examined (Fig. 2). A transcript of approximately 1.4 kb can be found in pre-B-cell lines (1.8, 22D6, and WEHI231), macrophage cells (P388D1), T cells (AO4H5.3 and EL4), fibroblast cells (NIH 3T3 and LTK-), and erythroleukemia cells (MEL).

**The p19 gene is located on human chromosome 19p13.** Both the p16 and the p15 genes are located on human chromosome 9p21 (7, 12, 21), a site with frequent deletions in many types of cancerous cells. Mutations at the p16 gene can be seen in many primary melanoma cells (11, 13). In order to see whether the p19 gene is associated with any characteristic tumor-specific chromosomal abnormalities, we determined its chromosomal location. A human cosmid library (Stratagene) was screened with human p19 cDNA as a probe. Two overlapping cosmid clones (pCOS3A and pCOS4B) were obtained. They contain the p19 gene, as confirmed by Southern blot hybridization and by comparison of the restriction enzyme sites of the putative exons with that of the p19 cDNA. These cosmids were used in a FISH experiment (25) to map the chromosomal location of the human p19 gene. Human p19 is located on chromosome 19p13 (shown in green in Fig. 3), a region not characteristically involved in tumor-associated chromosomal abnormalities. This assignment was confirmed by using a previously mapped gene, DBP, which is located on chromosome 19q13 (shown in red in

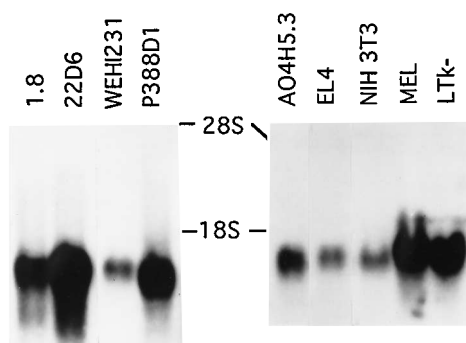


FIG. 2. Northern blot analysis of p19 mRNA expression in cell lines. 1.8, 22D6, and WEHI231 are pre-B-cell lines; P388D1 is a macrophage line; AO4H5.3 is a T-cell hybridoma; EL4 is a T-cell lymphoma; NIH 3T3 and LTK- are fibroblasts; MEL is an erythroleukemia line. The mouse p19 cDNA was used as a probe.

Fig. 3) (15). Thus, although p19 is highly homologous to p16, they are not located on the same chromosome.

**In vitro association of p19 with CDK4 but not with CDK2, CDC2, cyclin A, cyclin B, cyclin D, and cyclin E.** To investigate whether p19 behaves similarly to a cell cycle inhibitor in its ability to associate with components of the cell cycle machinery, we first expressed mouse p19 as a GST fusion protein in *Escherichia coli* and purified it with glutathione-agarose beads (we used the mouse p19 for all subsequent experiments). The p19 fusion protein was then used in an in vitro association assay with [<sup>35</sup>S]methionine-labeled proteins from in vitro transcription/translation of CDC2, CDK2, CDK4, and the various cyclins. As shown in Fig. 4, GST protein alone has no affinity for CDK4 but GST-p19 protein can associate with the in vitro-labeled CDK4 protein. The GST-p19 protein does not associate with either CDC2, CDK2, cyclin A, cyclin B, cyclin D1, cyclin D2, cyclin D3, or cyclin E (Fig. 4). Thus, p19 seems to be able to associate specifically with the G<sub>1</sub> CDKs, a property exhibited by the p18/p16/p15 but not p21/p27 cell cycle inhibitors.

**p19 can inhibit the cyclin D-CDK4 but not the cyclin E-CDK2 kinase activity.** The in vitro effect of p19 on CDK activity was then tested. The kinase activity was reconstituted in Sf9 insect cells by infection with baculoviruses encoding cyclin D and CDK4. As controls, Sf9 cells infected with a combination of cyclin E and CDK2 baculoviruses or from cells infected with either cyclin D or CDK4 alone were used. GST-Rb fusion protein was used as a kinase substrate. As expected, only coinfection of cyclin D and CDK4 baculoviruses resulted in extracts with kinase activity. Addition of increasing amounts of GST-p19 fusion protein but not GST alone led to inhibition of the cyclin D-CDK4 kinase activity (Fig. 5). Inhibition was not observed when the equivalent amount of GST-p19 was added to the kinase reaction mediated by cyclin E-CDK2 (Fig. 5). These data are consistent with the above finding that p19 can associate only with CDK4 and not with CDK2. As a comparison, GST-p16 was used to inhibit the kinase activity of cyclin D-CDK4 (Fig. 5). Hence, p19 is similar to p16 in its ability to inhibit the kinase activity of CDK4 but not that of CDK2.

**p19 associates with CDK4 and CDK6 in vivo.** To examine the state of p19 in vivo, we first generated rabbit antisera for the mouse p19. The antisera were purified on a GST-p19 affinity resin after the GST-specific antibodies had been removed with a GST affinity column. The purified antibodies were then used in immunoprecipitation experiments. To detect CDK4 and the related kinase CDK6, we also used the corresponding antibodies (Santa Cruz Inc). As DO11.10 T-cell hybridoma cells are arrested at G<sub>1</sub> phase starting at 5 h after stimulation with a combination of phorbol ester PMA and calcium ionophore ionomycin (Fig. 6), we examined the state of p19 at different time points after stimulation. Cells were labeled with [<sup>35</sup>S]methionine and stimulated with PMA and ionomycin for 0, 3, and 6 h. Whole-cell extracts were made from these cells, and immunoprecipitation was carried out with antibodies specific for CDK4, CDK6, and p19. Interestingly, similar patterns of immunoprecipitated proteins were observed at all time points (Fig. 7A). Immunoprecipitation with CDK4 yielded an abundant CDK4 protein itself (~34 kDa) that comigrated with in vitro-translated CDK4. Also coprecipitated was a lesser amount of a 19-kDa protein, which comigrated with in vitro-translated p19 (Fig. 7A). Inclusion of a competing CDK4 peptide in the immunoprecipitation experiment resulted in the disappearance of the 34- and 19-kDa proteins, indicating the specificities of the antibodies (Fig. 7A, lane 1). With anti-CDK6 antibodies, higher levels of coprecipitated 19-kDa pro-

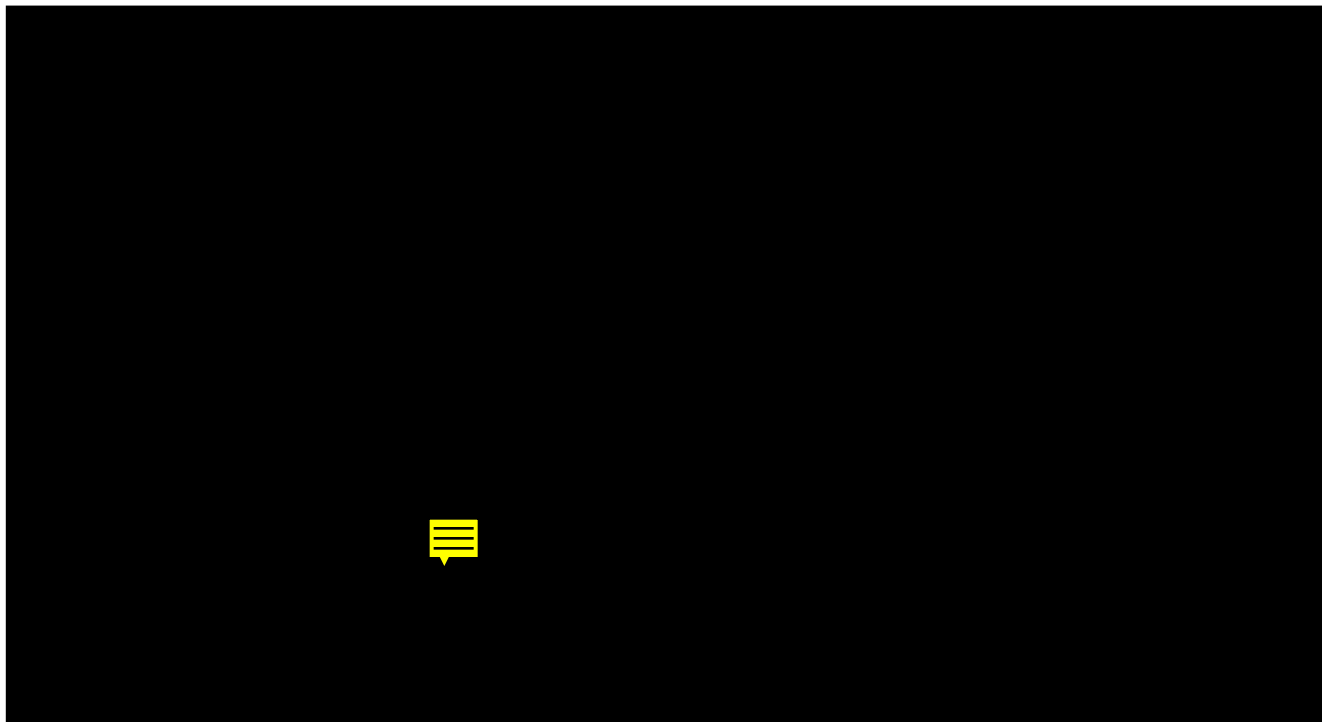


FIG. 3. Chromosomal mapping of human p19. Chromosomal localization of p19 to chromosome 19p13 by two-color FISH. Human genomic clones for *p19* (green) and *DBP*, which had previously been localized to chromosome 19q13 (red), were hybridized to human metaphase chromosomes from normal peripheral blood lymphocytes (magnification,  $\times 900$ ).

tein and an additional 16-kDa protein can be seen and again were caused to disappear by competition with CDK6 peptide (Fig. 7A). The 19-kDa protein again comigrated with the in vitro-translated p19 protein (Fig. 7A), whereas the 16-kDa protein comigrated with in vitro-translated p16 protein (data not shown), suggesting that they may correspond to the p19 and p16 cell cycle inhibitors, respectively. No p27, p21, or p15 inhibitor is evident here. With p19-specific antibody, a 19-kDa protein as well as 34- and 40-kDa proteins can be observed. These bands were diminished or eliminated when a competing GST-p19 protein was included in the reaction. It is clear from these experiments that both CDK4 and CDK6 proteins are present in molar excess relative to the level of the associating p19. The abundance of CDK4 and CDK6 may be due to the necessity for CDK proteins to associate with other proteins (e.g., cyclins and p16).

In order to ascertain the identity of the 19-kDa protein coprecipitated with the CDK6 antibodies, we performed a double immunoprecipitation experiment. We chose CDK6 because it was shown to be a major G<sub>1</sub> kinase in T cells (19). As shown in Fig. 7B, the second immunoprecipitation of the CDK6 immunocomplex with anti-p19 antibodies yielded the p19 protein. As a control, similarly purified rabbit antiserum specific for the Sp3 transcription factor or the preimmune serum was used. As expected, they did not bring down the 19-kDa protein. In a reciprocal experiment, anti-CDK6 antibodies precipitated a 40-kDa CDK6 protein from the anti-p19 immunocomplex (Fig. 7b). Thus, p19 associates with CDK6 protein in vivo. Similar experiments also showed that p19 associates with CDK4 in vivo (data not shown).

## DISCUSSION

Apoptosis and cell cycle are two fundamental processes in biology. Cell cycle regulation has been well characterized over

the last few years, whereas the molecular mechanism of apoptosis is still poorly understood, partly because different inducing agents may cause death through distinct cellular proteins. In radiation-induced apoptosis, for example, cell death is me-

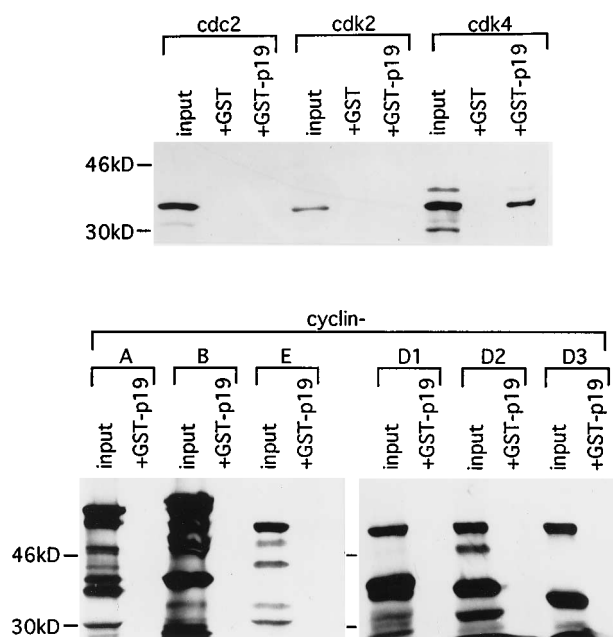


FIG. 4. p19 associates specifically with CDK4 in vitro. The indicated in vitro-translated cyclins or CDKs were incubated with either GST or GST-p19 proteins (10  $\mu$ g) on glutathione-agarose beads. Washed beads were analyzed on a 10% polyacrylamide gel. The inputs were loaded alongside for comparison.

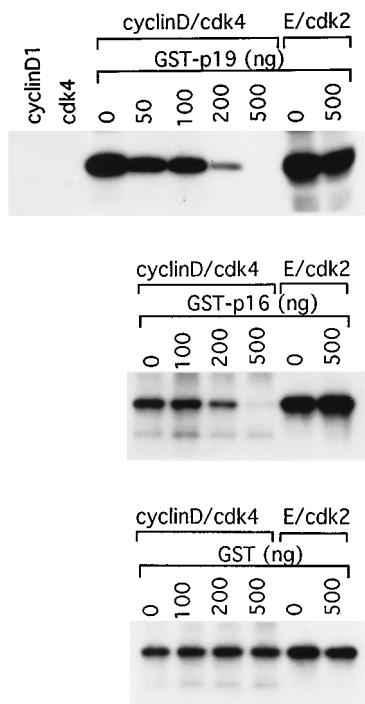


FIG. 5. p19 can inhibit the kinase activity of cyclin D1-CDK4 but not that of cyclin E-CDK2. Insect cell lysates containing cyclins, CDKs, or both were assayed for kinase activity by using bacterially expressed GST-Rb fusion protein as a substrate (see Materials and Methods). Prior incubation with increasing amounts of bacterially expressed GST-p19 decreased the cyclin D-CDK4 kinase activity (top panel) but not the cyclin E-CDK2 kinase activity. As controls, similar experiments were carried out with bacterially expressed human p16 (GST-p16, middle panel) (30) or GST (bottom).

diated by p53, which can transcriptionally regulate the level of p21 cell cycle inhibitor (4, 8). How p21 is related to the apoptotic function of p53 is not entirely clear at this moment, although the radiation-induced apoptosis is usually accompanied by a  $G_1$  arrest (1). In activation-induced apoptosis of T-cell hybridomas, which may mimic the process of negative selection in T-cell development, induction of Nur77 orphan steroid receptor is required (17, 35). Interestingly, when T-cell hybridomas are induced to die through the T-cell receptor signals, they are also arrested at the  $G_1$  state of the cell cycle (reference 18 and data presented in this paper). How  $G_1$  arrest and apoptosis are linked molecularly is an interesting question.

In searching for a protein(s) that may interact with Nur77 by yeast two-hybrid screening, we have isolated a novel mouse and human cell cycle inhibitor, p19. This p19 protein is similar to p16 and has all the properties of a cell cycle inhibitor. Increasing amounts of p19 protein in vitro can inhibit the kinase activity of CDK4 but not that of CDK2. Ectopic over-expression of p19 in NIH 3T3 cells leads to  $G_1$  arrest (9). This is consistent with the ability of p19 to associate with only the  $G_1$ -specific kinases, CDK4 and CDK6, but not with other cell cycle kinases or cyclins. We also showed that p19 can associate with CDK4 and CDK6 in vivo, although its level does not change appreciably when T cell hybridomas are arrested at the  $G_1$  phase through the T-cell receptor signals. This is similar to p16, for which regulation is still poorly understood. In contrast, the p15 protein level in keratinocytes increases dramatically in response to TGF- $\beta$ , leading to  $G_1$  arrest (7). Thus, if p19 participates in the  $G_1$  arrest of T cells, it may do so through posttranslational modification.

In cloning the p19 cDNA through a yeast two-hybrid screening strategy with the Nur77 DNA binding domain as a bait, we found that protein-protein interaction between p19 and Nur77 was relatively strong. The interaction survived the numerous tests used for the screening process. In vitro-translated full-length Nur77 protein, however, does not associate with the GST-p19 fusion protein (data not shown). Whether Nur77 associates with p19 in vivo has not been tested. Clear differences, however, are evident between in vitro-translated and in vivo Nur77 protein. Nur77 is phosphorylated in vivo and p19 may associate only with the phosphorylated Nur77 protein. It is also possible that the association between p19 and Nur77 may be weak and is detectable only by the yeast two-hybrid screen strategy. Interestingly, an analogous situation, such that a weak association between the transcription factor Pho4 and the cell cycle inhibitor Pho81 can be seen by use of the yeast two-hybrid strategy but not by coimmunoprecipitation with the corresponding antibodies, exists in yeast cells (10, 29). Pho81 contains four ankyrin repeats similar to that of the human p16/p19 cell cycle inhibitor family. In high-phosphate condition, the yeast Pho81 mediates interaction between the Pho4 transcription factor and Pho80-Pho85 cyclin-CDK complex (10, 29). Phosphorylation of Pho4 by the Pho85 CDK leads to inactivation of the Pho4 DNA binding activity. When phosphate starvation occurs, however, Pho81 inactivates the Pho80-Pho85 complex and dissociates from Pho4 at the same time. The resulting unphosphorylated Pho4 is an active protein (10, 29). Thus, the CDKs may directly modulate the activity of a transcription factor through interaction with the p16 family of cell cycle inhibitors. Hence, there could be an exciting possibility that the interaction between mouse p19 and Nur77, as observed in use of the yeast two-hybrid system, is functionally significant and not simply fortuitous. Speculatively, induction of Nur77 mRNA by either serum, nerve growth factor, or anti-T-cell receptor (TCR) antibodies may initially lead to an active phosphorylated protein. Death-associated activity of the Nur77 protein, however, is inactivated by a second phosphorylation event mediated by the CDK4-cyclin complex (or the CDK4-cyclin complex) through interaction with p19. Only when cells are arrested at  $G_1$  in anti-TCR treated cells will p19 dissociate itself from Nur77, along with inactivation of the CDK4-cyclin complex. The lack of a second phosphorylation event results in an active Nur77 protein, which is then involved in the apoptotic process of T-cell hybridomas. For thymocytes,

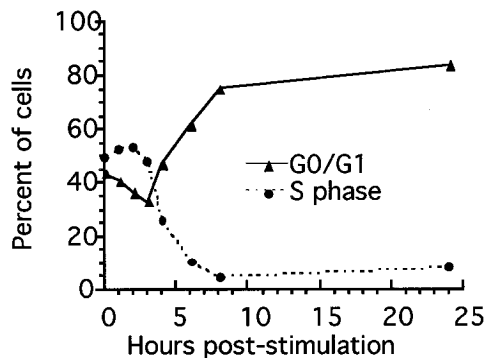


FIG. 6. Activation of DO11.10 T-cell hybridoma by PMA-ionomycin arrests cells at the  $G_1$  phase. DO11.10 cells were stimulated with 10 ng of PMA per ml and 0.5  $\mu$ M ionomycin, and an aliquot of the culture was analyzed for its cell cycle profile by propidium iodide staining at different times. The percentages of cells that were alive at various stages of the cell cycle were determined by a multicycle analysis program.

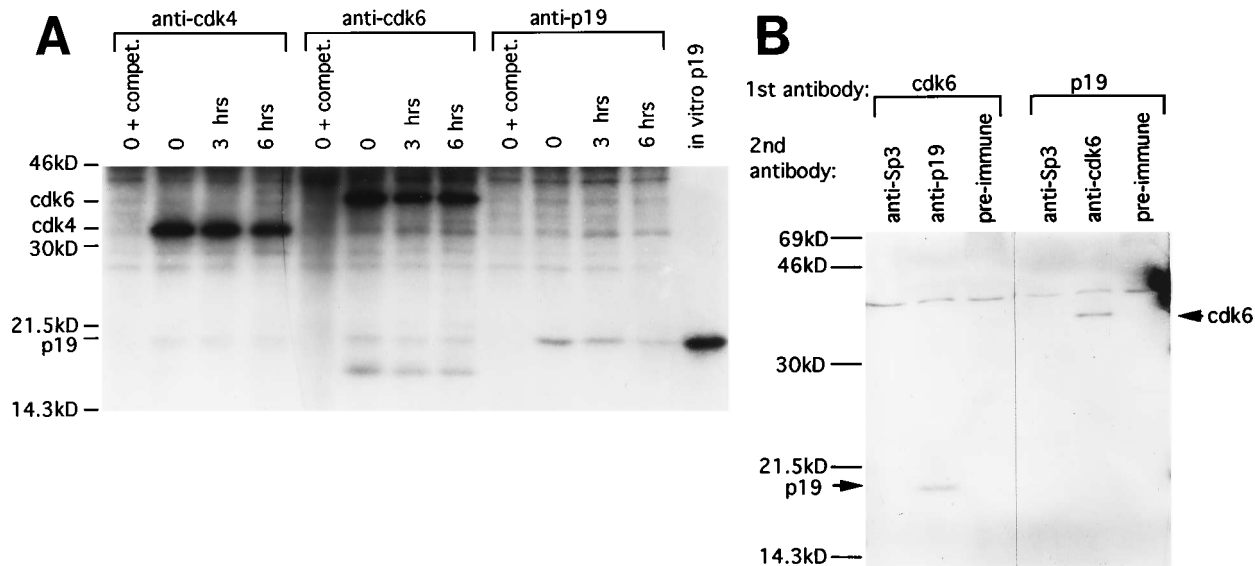


FIG. 7. p19 associates with CDK4 and CDK6 *in vivo*. (A) Immunoprecipitation of p19, CDK4, and CDK6. DO11.10 cells were stimulated with PMA-ionomycin for 0, 3, and 6 h. Specific competitors were added to the unstimulated extracts where indicated (0 + compet.). Cells were labeled with [<sup>35</sup>S]methionine for 3 h, and the immunoprecipitates were analyzed on a 17.5% polyacrylamide gel. (B) Double immunoprecipitation with p19 and CDK6. DO11.10 cells were labeled with [<sup>35</sup>S]methionine for 3 h and then precipitated with either anti-p19 or anti-CDK6 antibodies. The first immunoprecipitates were boiled in 2% SDS and then diluted for the second immunoprecipitation with the indicated antibodies. Portions of the immunoprecipitates from the first immunoprecipitation were also loaded as controls (data not shown). Samples were resolved on a 17.5% polyacrylamide gel.

which most of the cells are at the G<sub>0</sub>/G<sub>1</sub> state, mere expression of Nur77 can lead to apoptosis. This conjecture is based on a known biological system in yeasts and provides a molecular link between G<sub>1</sub> arrest and apoptosis in T cells.

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