Regulation of CDK7–Carboxyl-Terminal Domain Kinase Activity by the Tumor Suppressor p16INK4A Contributes to Cell Cycle Regulation

EIJI NISHIWAKI,‡ SARALINDA L. TURNER,‡ SUSANNA HARJU,§ SHIRO MIYAZAKI,† MASAHIDE KASHIWAGI,§ JAMES KOH, and HIROAKI SERIZAWA*†

Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160-7421,1 and Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont 05405-00682

Received 19 January 2000/Returned for modification 21 March 2000/Accepted 27 July 2000

The eukaryotic cell cycle is regulated by cyclin-dependent kinases (CDKs). CDK4 and CDK6, which are activated by D-type cyclins during the G1 phase of the cell cycle, are thought to be responsible for phosphorylation of the retinoblastoma gene product (pRb). The tumor suppressor p16INK4A inhibits phosphorylation of pRb by CDK4 and CDK6 and can thereby block cell cycle progression at the G1/S boundary. Phosphorylation of the carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II by general transcription factor TFIIH is believed to be an important regulatory event in transcription. TFIIH contains a CDK7 kinase subunit and phosphorylates the CTD. We have previously shown that p16INK4A inhibits phosphorylation of the CTD by TFIIH. Here we report that the ability of p16INK4A to inhibit CDK7-CTD kinase contributes to the capacity to induce cell cycle arrest. These results suggest that p16INK4A may regulate cell cycle progression by inhibiting not only CDK4-pRb kinase activity but also by modulating CDK7-CTD kinase activity. Regulation of CDK7-CTD kinase activity by p16INK4A thus may represent an alternative pathway for controlling cell cycle progression.

Cyclin-dependent kinases (CDKs) regulate cell cycle progression (references 13, 21, and 28 and references therein). CDK4 and CDK6 are activated by D-type cyclins and participate in controlling the G1-to-S phase transition by phosphorylating the retinoblastoma gene product (pRb). Phosphorylation of pRb induces remodeling of transcriptional repressor complexes at pRb-regulated genes and causes the release of transcription factors such as E2F. Free E2F can then activate the transcription of genes required for entering S phase (36, 41).

p16INK4A is a tumor suppressor gene product which binds CDK4 and inhibits CDK4-mediated phosphorylation of pRb (27). Overexpression of p16INK4A can block cell cycle progression through the G1-to-S phase boundary in a pRB-dependent manner (16, 19). Many p16INK4A mutants identified from human tumors have been shown to have defects in this activity (15, 16, 19, 20, 22, 31). These data suggest that the C4K inhibitory activity of p16INK4A is involved in regulating cell cycle progression through the G1/S boundary.

Koh et al. have described an interesting phenotype associated with a p16INK4A* mutant, G101W, that was originally identified in a familial melanoma kindred (14, 16). The G101W mutant was defective in inhibiting CDK4, although overexpression of the G101W mutant in an osteosarcoma cell line provoked cell cycle arrest at G1. In this mutant, the CDK4-pRb kinase-inhibitory activity of p16INK4A apparently does not correlate with the ability to induce cell cycle arrest in G1. When overexpressed, these results raise the possibility that an additional biochemical activity of p16INK4A might contribute to the ability to arrest cell cycle progression.

p15INK4B, p18INK4C, and p19INK4D are members of the p16INK4A gene family, and all have significant homology in their primary structures (11, 12). Like p16INK4A, the other INK4 family members can each bind and inhibit the activity of CDK4 and CDK6. Despite these similarities among the INK4 family members, only mutations in p16INK4A have been found to correlate with human tumors (15, 16, 19, 20, 22, 31, 39). These data suggest that the ability to inhibit pRb kinase activity may not be the sole determinant of the tumor suppressor activity of p16INK4A.

TFIIH is an essential factor for transcription by RNA polymerase II (RNA pol II). TFIIH is composed of nine subunits (2, 3, 40). CDK7, a kinase subunit of TFIIH, phosphorylates the carboxyl-terminal domain (CTD) of the largest subunit of RNA pol II in vitro (8, 23, 26, 29). The CTD is highly phosphorylated in vivo (reference 5 and references therein). Genetic data for the yeast Saccharomyces cerevisiae have suggested that phosphorylation of the CTD by KIN28, the kinase subunit of yeast TFIIH, is required for mRNA production and cell viability (35). These data suggest that phosphorylation of the CTD by TFIIH is required for transcription.

CyclinH, the obligate activating partner of CDK7, is also a subunit of TFIIH. CDK7 and cyclinH form a TFIIH subcomplex with MAT1, a component which stabilizes the association between cyclinH and CDK7 (7, 9, 32). Both TFIIH and the subcomplex composed of CDK7, cyclinH, and MAT1 can phosphorylate the threonine primary activation site of CDK2 and activate the histone H1 kinase activity of this enzyme (references 26 and 30 and references therein). To reflect this function, TFIIH and the cyclinH-CDK7-MAT1 subcomplex are called CDK-activating kinase (CAK). Genetic data for Drosophila have suggested that CAK activity by CDK7 regulates mitotic cell cycle progression (18).

We have recently reported that p16INK4A can specifically inhibit TFIIH-CTD kinase activity but that p16INK4A did not inhibit TFIIH-CDK2 kinase activity (25). Recombinant p16INK4A inhibited phosphorylation of the CTD by purified TFIIH or by recombinant CAK composed of CDK7, cyclinH, and MAT1. We define this novel biochemical function as...
CDK7-CTD kinase-inhibitory activity. Here we describe the cellular phenotypes of p16INK4A mutants defective for the ability to inhibit CDK7-CTD kinase, CDK4-pRb kinase, or both enzymes. In a transient overexpression system, we have found that CDK7-CTD kinase-inhibitory activity contributes to the ability of p16INK4A to induce cell cycle arrest. These results suggest that the CDK7-CTD kinase-inhibitory activity of p16INK4A may constitute a link between the basal transcription apparatus and regulation of cell cycle progression.

MATERIALS AND METHODS

Purification of recombinant Cdkn1A (rCdkn1A). Recombinant baculoviruses containing cDNAs of CDK7, cyclinH, and MAT1 were coinjected into Sf21 insect cells, and lysates from the infected cells were prepared using nitrogen cavitation, as described by Serizawa (25). The cell lysate containing ~13 mg of proteins was dialed against buffer C (20 mM HEPES-NaOH [pH 7.4], 10% glycerol, 1 mM dithiothreitol [DTT]), until it reached a conductivity equivalent to that of buffer C containing 50 mM KCl. The lysate was centrifuged at 35,000 rpm for 30 min in a Beckman type 50.2Ti rotor, and the supernatant was loaded onto a DEAE-5PW column (7.5 by 75 mm) (Toso-Haas) preequilibrated with buffer C containing 80 mM KCl. The column was washed using buffer C containing 80 to 1,000 mM KCl, and eluted at 1 ml/min using a 60-ml linear gradient from 80 to 1,000 mM KCl in buffer C. One-milliliter fractions were collected. Active fractions, which eluted at approximately 200 mM KCl, were pooled (fraction I). The column was washed using buffer C containing 50 mM KCl, and the flowthrough fraction was collected and pooled. The flowthrough fraction was centrifuged at 35,000 rpm for 30 min, and was loaded onto a heparin-5PW column (5.5 by 50 mm) (Toso-Haas) preequilibrated with buffer C containing 80 mM KCl. The column was washed using buffer C containing 80 to 1,000 mM KCl, and eluted at 1 ml/min using a 60-ml linear gradient from 80 to 1,000 mM KCl in buffer C. One-milliliter fractions were collected. Active fractions, which eluted at approximately 200 mM KCl, were pooled (fraction II). The column was washed using buffer C containing 50 mM KCl, and the flowthrough fraction was collected and pooled. The flowthrough fraction was centrifuged at 35,000 rpm for 30 min, and was loaded onto a SP-5PW column (5 by 50 mm) (Toso-Haas) preequilibrated with buffer A (10 mM Tris-HCl [pH 7.9], 5 mM MgCl2, 10% glycerol, 0.5 mM DTT) containing 50 mM KCl. The column was washed using buffer A containing 50 mM KCl, and eluted at 0.3 ml/min using a 10-ml linear gradient from 50 to 300 mM KCl in buffer C. Fractions (0.3 ml) were collected. Active fractions were eluted at approximately 200 mM KCl.

Expression constructs of His-p15, His-chimera 1, His-chimera 2, His-R24P, His-L31R, and His-G101W. The primary structures of p16INK4A and p15INK4B sequences of the constructs were determined. The recombinant proteins used in the phosphorylation reaction mixtures contained 50 mM HEPES-NaOH (pH 7.5), 1 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% (v/v) glycerol. Phosphorylated proteins were analyzed by electrophoresis in SDS–10% polyacrylamide gels. 32P incorporated into GST-CTD was detected by autoradiograms.

Phosphorylation of pRb kinase activity in cell lysates and CDK4-pRb kinase-inhibitory activities of p16INK4A mutants and the wild type at 28°C were shown to be equivalent to those at 30°C (data not shown).

Western blotting analysis. Western blotting experiments were carried out as described previously (25). Antibodies against CDK7, cyclinH, MAT1, p16INK4A, and p15INK4B were obtained from Santa Cruz Biotechnology. These molecules were detected by chemiluminescence using horseradish peroxidase-conjugated secondary antibodies (Amersham).

Protein quantitation. The recombinant proteins prepared from E. coli lysates were assayed by Bio-Rad according to the manufacturer’s instruction, with bovine serum albumin as the standard. Protein quantitation of TFIIH and rCAK was based on the A280 values of the preparations (25).

Cell cycle analysis using flow cytometry. Cell cycle analysis was carried out essentially as described previously (16). U2OS cells were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (FBS). Plasmids encoding a membrane-bound derivative of EGFP (I-EGFP; Clontech), wild-type p16INK4A, or the p16INK4A mutants under the control of the cytomegalovirus promoter (pCDNA3.1 [Invitrogen]) were cotransfected using the Effectene transient transfection kit (Qiagen) in the presence of FBS by following the manufacturer’s instructions. After 36 h, the cells were harvested using trypsin. The harvested cells were washed once in culture media containing 25 mM HEPES and 5 mM EDTA-phosphate-buffered saline (PBS) and then were fixed in 75% ethanol overnight at 4°C. The fixed cells were treated with RNase A and stained with propidium iodide (PI) using standard conditions. The fixed and stained cells were then analyzed using a Coulter EPICS XL analytical flow cytometer. Flow cytometry data were generated from a minimum of 80,000 cells per sample. A gate was set to select green fluorescent protein (GFP)-positive cells with a signal intensity at least 20 times greater than that in the untransfected cells. The PI signal was used as an indicator of DNA content. The gates were set using the WinList and ModFit analytical software packages (Verity Software).

Confocal microscopy. HeLa cells plated onto glass coverslips were washed one time in PBS and then were fixed in 75% ethanol overnight at −20°C. The coverslips were then incubated in 50 mM HEPES–0.5 M LiCl, 0.1 M NaCl, 10 mM MgCl2, 10% glycerol, 0.5 mM DTT (L-PBS) solution for 20 min at room temperature. The fixed cells were treated with RNase A and stained with propidium iodide (PI) using standard conditions. The fixed and stained cells were then analyzed using a Coulter EPICS XL analytical flow cytometer. Flow cytometry data were generated from a minimum of 80,000 cells per sample. A gate was set to select green fluorescent protein (GFP)-positive cells with a signal intensity at least 20 times greater than that in the untransfected cells. The PI signal was used as an indicator of DNA content. The gates were set using the WinList and ModFit analytical software packages (Verity Software).

Cell cycle analysis using flow cytometry. Cell cycle analysis was carried out essentially as described previously (16). U2OS cells were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (FBS). Plasmids encoding a membrane-bound derivative of EGFP (I-EGFP; Clontech), wild-type p16INK4A, or the p16INK4A mutants under the control of the cytomegalovirus promoter (pCDNA3.1 [Invitrogen]) were cotransfected using the Effectene transient transfection kit (Qiagen) in the presence of FBS by following the manufacturer’s instructions. After 36 h, the cells were harvested using trypsin. The harvested cells were washed once in culture media containing 25 mM HEPES and 5 mM EDTA-phosphate-buffered saline (PBS) and then were fixed in 75% ethanol overnight at 4°C. The fixed cells were treated with RNase A and stained with propidium iodide (PI) using standard conditions. The fixed and stained cells were then analyzed using a Coulter EPICS XL analytical flow cytometer. Flow cytometry data were generated from a minimum of 80,000 cells per sample. A gate was set to select green fluorescent protein (GFP)-positive cells with a signal intensity at least 20 times greater than that in the untransfected cells. The PI signal was used as an indicator of DNA content. The gates were set using the WinList and ModFit analytical software packages (Verity Software).

Confocal microscopy. HeLa cells plated onto glass coverslips were washed one time in PBS and then were fixed in 75% ethanol overnight at −20°C. The coverslips were then incubated in 50 mM HEPES–0.5 M LiCl, 0.1 M NaCl, 10 mM MgCl2, 10% glycerol, 0.5 mM DTT (L-PBS) solution for 20 min at room temperature. The fixed cells were treated with RNase A and stained with propidium iodide (PI) using standard conditions. The fixed and stained cells were then analyzed using a Coulter EPICS XL analytical flow cytometer. Flow cytometry data were generated from a minimum of 80,000 cells per sample. A gate was set to select green fluorescent protein (GFP)-positive cells with a signal intensity at least 20 times greater than that in the untransfected cells. The PI signal was used as an indicator of DNA content. The gates were set using the WinList and ModFit analytical software packages (Verity Software).

CDK7-CTD KINASE INHIBITOR ACTIVITY BY p16INK4A 7727
His-p16 and His-p15 stained by Coomassie blue (CBB). Recombinant proteins were analyzed in an SDS–13.5% PAGE. Arrowheads, His-p16 and His-p15 were purified as described in Materials and Methods. These from rat liver. This preparation of TFIIH was previously shown (25). (c and d) antibodies against CDK7, cyclinH, and MAT1, as indicated. (b) TFIIH purified stain), by phosphorylation assays with GST-CTD, and by Western blotting with analyzed by SDS–13.5% polyacrylamide gel electrophoresis (PAGE) (silver column, as described in Materials and Methods. The column fractions were eluted in a heparin HPLC column. The CTD phosphorylation eern blots (Fig. 1a) show that CDK7, cyclinH, and MAT1 co- was purified to near homogeneity using conventional high- was overexpressed in E. coli and was extensively purified using nickel affinity and phenyl-Sepharose columns (Fig. 1d). Phosphorylation of GST-CTD by purified TFIIH and by purified rCAK was carried out in the presence of increasing amounts of His-p15. In contrast to His-p16, purified His-p15 did not inhibit the phosphorylation of GST-CTD (Fig. 2a, lanes 4 to 6 and 10 to 12). To confirm the CDK4-inhibitory activities of the His-p16 and His-p15 preparations, phosphorylation of GST-pRb by cyclinD-CDK4 complexes was assayed in the presence of these proteins. GST-pRb was phosphorylated in lysates prepared from insect cells coinfected by baculovirus constructs containing CDK4 and cyclinD, but phosphorylation of GST-pRb in lysates prepared from insect cells infected by a baculovirus construct containing either CDK4 alone or cyclinD alone was undetectable (data not shown). Addition of either His-p15 or His-p16 inhibited phosphorylation of GST-pRb by cyclinD-cyclin D-CDK4 CDK4 kinase under conditions where CDK4 and CTD (GST-CTD) by the purified rCAK was carried out in the presence of increasing amounts of His-p16. The addition of His-p16 inhibited phosphorylation of GST-CTD by rCAK under conditions where rCAK was limiting (Fig. 2a, lanes 7 to 9). In control experiments, similar inhibitory activities of His-p16 were detected in the phosphorylation of CTD by purified TFIIH (Fig. 2a, lanes 1 to 3). These results suggest that His-p16 inhibits phosphorylation of GST-CTD not only by purified TFIIH but also by purified rCAK composed of CDK7, cyclinH, and MAT1.

Recombinant p15INK4B does not inhibit phosphorylation of CTD by TFIIH and rCAK. The CDK inhibitors p16INK4A and p15INK4B have significantly similar primary structures (12) (Fig. 3), and both are able to inhibit phosphorylation of pRb by CDK4 (12). We examined whether p15INK4B could inhibit CTD phosphorylation by TFIIH and rCAK. A recombinant containing six histidine residues (His-p15) was overexpressed in E. coli and was extensively purified using nickel affinity and phenyl-Sepharose columns (Fig. 1d). Phosphorylation of GST-CTD by purified TFIIH and by purified rCAK was carried out in the presence of increasing amounts of His-p15. In control experiments, similar inhibitory activities of His-p15 and CTD (GST-CTD) by the purified rCAK was carried out in the presence of increasing amounts of His-p16. The addition of His-p16 inhibited phosphorylation of GST-CTD by rCAK under conditions where rCAK was limiting (Fig. 2a, lanes 7 to 9). In control experiments, similar inhibitory activities of His-p16 were detected in the phosphorylation of CTD by purified TFIIH (Fig. 2a, lanes 1 to 3). These results suggest that His-p16 inhibits phosphorylation of GST-CTD not only by purified TFIIH but also by purified rCAK composed of CDK7, cyclinH, and MAT1. Recombinant p16INK4A inhibits phosphorylation of CTD by TFIIH and by rCAK. We previously demonstrated that recombinant p16INK4A containing six histidine residues (His-p16) inhibits phosphorylation of the CTD by highly purified TFIIH (25). We sought to determine whether His-p16 could inhibit phosphorylation of the CTD by isolated rCAK composed of CDK7, cyclinH, and MAT1. Baculovirus constructs containing cDNAs encoding these three molecules were coinfected into SF21 insect cells and were overexpressed therein. The rCAK was purified to near homogeneity using conventional high-performance liquid chromatography (HPLC) columns. Western blots (Fig. 1a) show that CDK7, cyclinH, and MAT1 co-eluted in a heparin HPLC column. The CTD phosphorylation activity in these column fractions coeluted with the three proteins. The silver-stained gel of these column fractions revealed proteins corresponding to CDK7, cyclinH, and MAT1; however, MAT1 was not stained by silver as strongly as CDK7 and cyclinH. His-p16 was overexpressed in E. coli and was purified to near homogeneity by nickel affinity and phenyl-Sepharose columns (Fig. 1c). Phosphorylation of a fusion protein of GST

---

**RESULTS**

Recombinant p16INK4A inhibits phosphorylation of CTD by TFIIH and by rCAK. We previously demonstrated that recombinant p16INK4A containing six histidine residues (His-p16) inhibits phosphorylation of the CTD by highly purified TFIIH (25). We sought to determine whether His-p16 could inhibit phosphorylation of the CTD by isolated rCAK composed of CDK7, cyclinH, and MAT1. Baculovirus constructs containing cDNAs encoding these three molecules were coinfected into SF21 insect cells and were overexpressed therein. The rCAK was purified to near homogeneity using conventional high-performance liquid chromatography (HPLC) columns. Western blots (Fig. 1a) show that CDK7, cyclinH, and MAT1 co-eluted in a heparin HPLC column. The CTD phosphorylation activity in these column fractions coeluted with the three proteins. The silver-stained gel of these column fractions revealed proteins corresponding to CDK7, cyclinH, and MAT1; however, MAT1 was not stained by silver as strongly as CDK7 and cyclinH. His-p16 was overexpressed in E. coli and was purified to near homogeneity by nickel affinity and phenyl-Sepharose columns (Fig. 1c). Phosphorylation of a fusion protein of GST

---

**FIG. 1.** Purified rCAK, TFIIH, recombinant His-p16, and recombinant His-p15. (a) Purification of rCAK. The rCAK was fractionated in a heparin-SPW column, as described in Materials and Methods. The column fractions were analyzed by SDS–13.5% polyacrylamide gel electrophoresis (PAGE) (silver stain), by phosphorylation assays with GST-CTD, and by Western blotting with antibodies against CDK7, cyclinH, and MAT1, as indicated. (b) TFIIH purified from rat liver. This preparation of TFIIH was previously shown (25). (c and d) His-p16 and His-p15 were purified as described in Materials and Methods. These recombinant proteins were analyzed in an SDS–13.5% PAGE. Arrowheads, His-p16 and His-p15 stained by Coomassie blue (CBB).

---

**FIG. 2.** His-p15 does not inhibit CDK7-CTD kinase under conditions where His-p16 inhibited this kinase. (a) Phosphorylation of GST-CTD by purified TFIIH and rCAK. We previously demonstrated that recombinant p16INK4A containing six histidine residues (His-p16) inhibits phosphorylation of the CTD by highly purified TFIIH (25). We sought to determine whether His-p16 could inhibit phosphorylation of the CTD by isolated rCAK composed of CDK7, cyclinH, and MAT1. Baculovirus constructs containing cDNAs encoding these three molecules were coinfected into SF21 insect cells and were overexpressed therein. The rCAK was purified to near homogeneity using conventional high-performance liquid chromatography (HPLC) columns. Western blots (Fig. 1a) show that CDK7, cyclinH, and MAT1 co-eluted in a heparin HPLC column. The CTD phosphorylation activity in these column fractions coeluted with the three proteins. The silver-stained gel of these column fractions revealed proteins corresponding to CDK7, cyclinH, and MAT1; however, MAT1 was not stained by silver as strongly as CDK7 and cyclinH. His-p16 was overexpressed in E. coli and was purified to near homogeneity by nickel affinity and phenyl-Sepharose columns (Fig. 1c). Phosphorylation of a fusion protein of GST
activity was limiting (Fig. 2). These results suggest that the preparation of His-p15 used in GST-CTD phosphorylation assays was as active as His-p16 in inhibiting phosphorylation of GST-pRb by CDK4, although it was unable to inhibit phosphorylation of GST-CTD by TFIIH and rCAK. These results suggest that His-p15 does not inhibit phosphorylation of GST-CTD by purified TFIIH and rCAK.

Chimeras of p16INK4A and p15INK4B inhibit phosphorylation of CTD by TFIIH and rCAK. The structural similarity of p16INK4A and p15INK4B is concentrated in the middle regions of these proteins, as shown in Fig. 3a. The amino-terminal region containing amino acid residues 1 through 43 of p16INK4A, however, is quite divergent from the amino-terminal region of p15INK4B. To test whether the nonhomologous region of p16INK4A is involved in CDK7-CTD kinase-inhibitory activity, we constructed recombinant chimeric proteins of p16INK4A and p15INK4B that exchanged these nonhomologous regions. Expression constructs containing the chimeras depicted in Fig. 3b were generated by PCR techniques. Chimera 1 has the nonhomologous region of p16INK4A at the amino terminus and the homologous region of p15INK4B at the carboxyl terminal. Chimera 2 has the nonhomologous region of p15INK4B at the amino terminus and the homologous region and the carboxyl-terminal 20 amino acid residues of p16INK4A at the carboxyl terminus. These recombinant chimeric proteins each contain six histidine residues at their amino termini (His-chimera 1 and His-chimera 2). The proteins were overexpressed in E. coli, and were purified to near homogeneous activity using nickel affinity and phenyl-Sepharose beads (Fig. 3c). The chimeric proteins were then tested for their ability to inhibit the CTD kinase activity of purified TFIIH and rCAK. Phosphorylation of GST-CTD by purified TFIIH and rCAK was assayed in the presence of increasing amounts of His-chimera 1 and His-chimera 2 under conditions where wild-type His-p16 was able to fully inhibit this kinase activity. His-chimera 1 inhibited phosphorylation of GST-CTD by both purified TFIIH and rCAK, whereas His-chimera 2 did not inhibit phosphorylation of GST-CTD by purified TFIIH and rCAK (Fig. 4). The intensities and profiles of His-chimera 1 and His-p16 inhibition of CDK7-CTD kinase were similar (Fig. 2a and 4a) (25). In CTD phosphorylation reactions, addition of 20 pmol or more of His-p16 or His-chimera 1 saturated the inhibition of CDK7-CTD kinase, reducing CTD phosphorylation by 60 to 70% (data not shown). These results suggest that the nonhomologous region of p16INK4A exchanged in these chimeric proteins is involved in the CDK7-CTD kinase-inhibitory activity of p16INK4A.

To confirm that His-chimera 1 and His-chimera 2 retained the ability to inhibit CDK4, phosphorylation of pRb by cyclinD-CDK4 complexes was carried out in the presence of these chimeric proteins under conditions where His-p16 and His-p15 showed full inhibition. Addition of His-chimera 1 or His-chimera 2 inhibited phosphorylation of pRb by CDK4 (Fig. 4). These results suggest that the recombinant chimeras used here preserved their CDK4-inhibitory activity and that the conserved regions of p16INK4A and p15INK4B are responsible for CDK4-pRb kinase-inhibitory activity.

**Phenotypes of p16INK4A mutants in the inhibitory activities for CDK7-CTD kinase and CDK4-pRb kinase.** To examine whether the CDK7-CTD kinase-inhibitory activity of p16INK4A could affect cell cycle progression, we generated p16INK4A mutants using PCR techniques and saturation mutagenesis and

![Figure 3](image3.png)

**FIG. 3.** Preparation of chimera 1 and chimera 2. (a) Amino acid alignment of p16INK4A and p15INK4B showing structural similarities. The amino acid alignment was carried out using ClustalW, as described previously (34). Vertical lines indicate amino acid identity, colons indicate conservative substitutions, and single dots indicate nonconservative substitutions. (b) Chimera 1 and chimera 2. (c) His-chimera 1 and His-chimera 2 were expressed and purified as described in Materials and Methods. Purified His-chimera 1 and His-chimera 2 were analyzed by SDS-13.5% polyacrylamide gel electrophoresis and were stained using Coomassie blue (CBB).

![Figure 4](image4.png)

**FIG. 4.** His-chimera 1, but not His-chimera 2, inhibits CDK7-CTD kinase. (a) Phosphorylation of GST-CTD by purified TFIIH and rCAK was carried out as described in Materials and Methods. His-chimera 1 and His-chimera 2 (4 pmol each) were added in the kinase reactions shown in lanes 2, 5, 8, and 11; 8 pmol of these recombinant proteins was added in the kinase reactions shown in lanes 3, 6, 9, and 12. The reactions shown in lanes 1, 4, 7, and 10 had no chimeric inhibitors. Arrowheads, GST-CTD phosphorylated by purified TFIIH and rCAK. (b) Phosphorylation of GST-pRb in insect cell lysates containing CDK4 and cyclinD was carried out as described in Materials and Methods. Purified His-chimera 1 at 0.05, 0.1, and 0.5 pmol was added in the phosphorylation reactions shown in lanes 6 to 8, respectively. The reactions shown in lanes 1 and 5 had neither inhibitor. Arrowheads, phosphorylated GST-pRb.
His-R24P, His-L31R, and His-G101W were added in the phosphorylation by rCAK in the presence of His-p16 and His-G101W. Phosphorylation of GST-CTD was carried out using rCAK, as described in the text. Four (lanes 2, 6, 10, and 14) and 0.1 (lanes 3, 7, 11, and 15, 0.5 pmol (lanes 4, 8, 12, and 16) of His-p16, His-R24P, His-L31R, and His-G101W were added in the phosphorylation reactions. The reactions in lanes 1, 5, 9, and 13 did not have these p16INK4A proteins. Arrowheads, phosphorylated GST-CTD and GST-pRb. (c) The inhibition of pRb phosphorylation by CDK4 in the presence of His-p16 and His-L31R. Phosphorylation of GST-pRb in insect cell lysates containing CDK4 and cyclinD was carried out as described in Materials and Methods. Phosphorylation reaction mixtures contained 0.05 (lanes 2, 6, 10, and 14), 0.1 (lanes 3, 7, 11, and 15), and 0.5 pmol (lanes 4, 8, 12, and 16) of His-p16, His-R24P, His-L31R, and His-G101W. The reactions shown in lanes 1, 5, 9, and 13 contained no recombinant proteins. Arrowheads, phosphorylated GST-pRb.

Next, to determine the ability of the p16INK4A mutants to inhibit pRb phosphorylation by CDK4, phosphorylation of GST-pRb was carried out in the presence of increasing amounts of His-R24P, His-L31R, His-G101W, and His-p16 under conditions in which CDK4 is limiting. GST-pRb was phosphorylated by lysates prepared from insect cells coinfected by baculovirus constructs containing CDK4 and cyclinD, but no GST-pRb kinase activity was detectable in lysates prepared from insect cells infected by a baculovirus construct containing either CDK4 or cyclinD alone (data not shown). Addition of His-p16 and His-L31R to the lysates containing CDK4-cyclinD complexes inhibited phosphorylation of GST-pRb; however, addition of His-G101W and R24P did not inhibit this kinase activity in parallel experiments (Fig. 5c). The IC50 of His-R24P and His-G101W were 10 times greater than those of His-p16 and His-L31R (Table 1), and the IC50 of His-G101W was equivalent to that reported by Yang et al. (37). Clearly, His-R24P and His-G101W do not inhibit pRb phosphorylation by CDK4 under conditions where His-p16 and His-L31R efficiently inhibit CDK4 activity. These biochemical data indicate that His-R24P is defective in the inhibition of both CTD and pRb phosphorylation, that His-L31R is defective in the inhibition of only the CDK7-CTD kinase, and that His-G101W is defective in the inhibition of only the CDK4-pRb kinase.

**Phenotypes of p16 mutants in inducing cell cycle arrest when overexpressed.** To assay for the ability of the p16 mutants to induce cell cycle arrest, expression constructs encoding wild-type p16 and the mutants R24P, L31R, and G101W were transiently transfected into the human osteosarcoma cell line U2OS. An expression construct encoding GFP was cotransfected as a marker to identify successfully transfected cells. Cells were harvested, and DNA content as indicated by PI staining intensity was assayed by flow cytometry, gating on GFP-positive cells in order to restrict analysis to the transfected-cell population (16). Cell cycle distribution was calculated by analyzing the histogram profiles using the ModFit software package (Verity). Overexpression of mutants L31R and G101W increased cell populations at G0-G1 phase as well as the wild type (Fig. 6 and Table 1). The results from the overexpression of the G101W mutant confirm previously published data (16). The population of cells in the G0-G1 phase of cell cycle did not significantly change when an expression construct containing R24P was transfected (Fig. 6 and Table 1). Western blots indicate that each of these mutant proteins was overexpressed in transiently cotransfected cells (Fig. 6). These results suggest that the L31R and G101W mutants are

**TABLE 1. Phenotypes of p16INK4A mutants**

| p16INK4A allele | IC50 (pmol) for: | % Accumulation of G1 cells |
|-----------------|------------------|--------------------------|
|                 | rCAK             | CDK4                     |                          |
| R24P            | >40              | >0.8                     | 53.2                     |
| L31R            | >40              | 0.06–0.08                | 72.5                     |
| G101W           | -15              | >0.8                     | 62.8                     |
| Wild type       | -20              | 0.04                     | 73.8                     |
| Mock transfection |                  |                         | 48.6                     |

*IC50 values and G0-G1 cell populations were estimated from results shown in Fig. 5 and 6 and from extensive titration using recombinant mutant and wild-type proteins. Phosphorylated GST-CTD and GST-pRb were detected by autoradiogram and were quantified using a two-dimensional densitometer (Molecular Dynamics).
Fig. 7a, p16INK4A could be detected in CDK7 immunoprecipitates from experiments using calf thymus extracts (data not shown). Western blots was significantly less. Similar results were obtained where p16INK4A could effectively inhibit these kinases (Fig. 2). These results suggest that the CDK7-CTD kinase-inhibitory activity of p16INK4A is at least partially regulated by human cancer (15, 16, 19, 20, 22, 31, 38, 39). These data suggest that p16INK4A has a specific function in human oncogenesis. We have identified a new biochemical activity associated with p16INK4A, TFIH-CDK7 kinase-inhibitory activity (25). The present study focused on the structural similarity of p16INK4A and p15INK4B and compared the CDK-inhibitory activities of these molecules. p15INK4B did not inhibit phosphorylation of GST-CTD by TFIH and rCAK under conditions where p16INK4A could effectively inhibit these kinases (Fig. 2). These results suggest that the CDK7-CTD kinase-inhibitory activity of p16INK4A may be specific and unique among the INK4 proteins and may correlate with its tumor suppressor activity. To further confirm the interaction between CDK7 and p16INK4A in cells, confocal microscopy analysis was performed. The affinity-purified polyclonal rabbit anti-CDK7 was detected with an ALEXA 488-conjugated antiserum secondary antibody, and the protein G-purified monoclonal anti-p16 antibody was detected with an ALEXA 488-conjugated antiserum secondary antibody. As shown in Fig. 7b, CDK7 and p16INK4A can be detected in close proximity within the nuclei of the cells (each pixel area is 147 nm², and the depth of field is approximately 1.5 μm). p16INK4A but not CDK7 is also visible in the cytoplasm of the cells. These results indicate that CDK7 and p16INK4A are predominantly localized to the same subcellular compartment and can be detected in close proximity to one another in the nuclei of HeLa cells.

**DISCUSSION**

We have identified a region in the amino terminus of p16INK4A required for inhibition of CDK7-CTD kinase activity and have shown that the inhibitory activities of p16INK4A to the CDK7-CTD kinase and CDK4-pRb kinase reside in distinct structural domains. We have identified three p16INK4A mutants that display different phenotypes in inhibiting CDK7-CTD kinase and CDK4-pRb kinase and in mediating cell cycle arrest. These mutants are R24P, L31R, and G101W, and their phenotypes, as summarized in Table 1, are as follows: (i) the R24P mutant fails to inhibit both CDK7 and CDK4 and does not induce G1 arrest when overexpressed; (ii) the L31R and G101W mutants are defective in inhibiting CDK7 and CDK4, respectively, but both mutants can still induce G1 arrest. These results suggest that the activities shown by p16INK4A for the inhibition of both CDK4-pRb kinase and CDK7-CTD kinase may contribute to the ability of p16INK4A to induce G1 arrest when over expressed. CDK7-CTD kinase activity may therefore contribute to progression through the cell cycle at the G1-to-S phase boundary.

**Regions in p16INK4A involved in the inhibition of CDK7-CTD kinase and CDK4-pRb kinase.** p16INK4A and p15INK4B have been shown to inhibit phosphorylation of pRb by CDK4 and are involved in regulating the G1-to-S phase transition (12, 16, 19, 27). Despite the facts that p16INK4A and p15INK4B have significant homology in their primary structures and that both inhibit phosphorylation of pRb by CDK4 and CDK6, genetic studies suggest that only mutations of p16INK4A closely correlate with human cancer (15, 16, 19, 20, 22, 31, 38, 39). These data suggest that p16INK4A has a specific function in human oncogenesis. We have identified a new biochemical activity associated with p16INK4A, TFIH-CDK7 kinase-inhibitory activity (25). The present study focused on the structural similarity of p16INK4A and p15INK4B and compared the CDK-inhibitory activities of these molecules. p15INK4B did not inhibit phosphorylation of GST-CTD by TFIH and rCAK under conditions where p16INK4A could effectively inhibit these kinases (Fig. 2). These results suggest that the CDK7-CTD kinase-inhibitory activity of p16INK4A may be specific and unique among the INK4 proteins and may correlate with its tumor suppressor function.

The region of highest homology between p16INK4A and p15INK4B is located in the middle portion of p16INK4A. The amino-terminal regions of p16INK4A and p15INK4B are not homologous (Fig. 3). Experiments using recombinant chimeric proteins in which the nonhomologous regions of p16INK4A and p15INK4B were exchanged indicate that the amino-terminal region of p16INK4A is involved in inhibiting phosphorylation of pRb by CDK7, whereas the homologous region is involved in inhibiting phosphorylation of pRb by CDK4 (Fig. 4). These

**Interaction of p16INK4A with CDK7 in vivo.** Our previous data suggested that recombinant p16INK4A can form a complex with purified TFIIH and recombinant CDK7 in vitro (25). To examine whether p16INK4A associates with CDK7 in cells, we prepared crude cell lysates and assayed for p16INK4A-CDK7 complexes by coimmunoprecipitation (Fig. 7a). Crude lysates prepared from HeLa cells were incubated with an antibody against CDK7, and the immunocomplexes were precipitated using protein A-Sepharose beads. Proteins in the supernatants and protein A-bound precipitates were probed in parallel on Western blots using an antibody against CDK7. Our previous studies suggest that only mutations of p16INK4A closely correlate with human cancer (15, 16, 19, 20, 22, 31, 38, 39). These data suggest that p16INK4A has a specific function in human oncogenesis. We have identified a new biochemical activity associated with p16INK4A, TFIH-CDK7 kinase-inhibitory activity (25). The present study focused on the structural similarity of p16INK4A and p15INK4B and compared the CDK-inhibitory activities of these molecules. p15INK4B did not inhibit phosphorylation of GST-CTD by TFIH and rCAK under conditions where p16INK4A could effectively inhibit these kinases (Fig. 2). These results suggest that the CDK7-CTD kinase-inhibitory activity of p16INK4A may be specific and unique among the INK4 proteins and may correlate with its tumor suppressor function.

The region of highest homology between p16INK4A and p15INK4B is located in the middle portion of p16INK4A. The amino-terminal regions of p16INK4A and p15INK4B are not homologous (Fig. 3). Experiments using recombinant chimeric proteins in which the nonhomologous regions of p16INK4A and p15INK4B were exchanged indicate that the amino-terminal region of p16INK4A is involved in inhibiting phosphorylation of pRb by CDK7, whereas the homologous region is involved in inhibiting phosphorylation of pRb by CDK4 (Fig. 4). These
results suggest that there are distinct boundaries separating the two different kinase-inhibitory activities in p16INK4A.

X-ray crystal structure studies indicate that p16INK4A is composed of four ankyrin repeats (24). The ankyrin repeats in p16INK4A are thought to form helix-turn-helix structures (33). The first ankyrin repeat at the amino terminus of p16 INK4A overlaps with the region of nonhomology between p15 INK4B and p16INK4A (Fig. 3). The R24P and L31R mutations reside in this region, and both proteins were shown to be defective in inhibiting CDK7-CTD kinase (Fig. 5). The helix-turn-helix structure of the first ankyrin repeat may therefore be involved in inhibiting phosphorylation of the CTD by CDK7, and the rest of the ankyrin repeats in p16INK4A may be involved in inhibiting phosphorylation of pRb by CDK4. The fourth ankyrin repeat has been suggested to be involved in binding CDK4 and may be important for inhibiting CDK4 (10). The R24P mutant was defective in inhibiting CDK4-pRb kinase, although the mutation lies outside the homologous region suggested to be involved in inhibition of CDK4-pRb kinase by p16INK4A. It may be that the mutation alters not only the structure of the nonhomologous region but also the overall structure of p16INK4A, including the homologous region. Such structural changes might result in the R24P mutant displaying defective inhibition of both CDK7-CTD kinase and CDK4-pRb kinase.

Whereas His-p16 inhibited CTD phosphorylation by CDK7 by approximately 60 to 70% (Fig. 2) (25), it resulted in almost complete inhibition of pRb phosphorylation by CDK4. The inhibition of CDK4-pRb kinase and the inhibition of CDK7-CTD kinase by p16INK4A were measured under different conditions. Furthermore, rCAK and TFIIH contain MAT1, whereas CDK4 has no subunit equivalent to MAT1, precluding direct comparison of their different inhibitory intensities. The intensities of these inhibitory activities under physiological conditions will be addressed in future studies. However, it is possible that differential inhibition of CDK7-CTD kinase and CDK4-pRb kinase by p16INK4A could contribute to modulation of cell cycle regulation.

Phenotypes of p16INK4A mutants R24P, L31R, and G101W. It has been suggested that p16INK4A plays an important role in a signaling pathway regulating entry into S phase from the G1 phase of the cell cycle (references 28, 36, and 41 and references...
tion of CDK7-CTD kinase activity may contribute to cell cycle regulation at the G1-to-S phase. G1 CDK-pRb kinase-inhibitory activity. However, in several CDK4, cyclinD, and GST-pRb, to David Morgan for baculovirus con-
struct of p15INK4B, to Mary Patterson for helpful discussions and
construct of p16INK4A closely correlate with human oncogenesis (39).
Many p16INK4A mutants derived from human tumors have lost G1 CDK-pRb kinase-inhibitory activity. However, in several G101W mutant has a defect in inhibiting
p16INK4A mutants, the biochemical phenotypes with respect to p16, cdk4, and pRb immunohistochemistry in glioblas-
tors such as E2F form a complex with pRb in quiescent cells,
phosphorylation by G1 CDKs and negatively regulates cell
cycle progression at the G1 phase. A wide variety of p16INK4A
mutations have been identified from human tumors (15, 16, 19,
20, 22, 31, 38, 39), and it has been suggested that mutations of
p16INK4A closely correlate with human oncogenesis (39).

ACKNOWLEDGMENTS

We are grateful to Hitoshi Matsushima for cDNA constructs of CDK4, cyclinD, and GST-pRb, to David Morgan for baculovirus con-
structs of CDK7, cyclinH, and MAT1, to David Beach for a cDNA
construct of p15INK4B, to Mary Patterson for helpful discussions and
reading the manuscript, and to Cristina Ward for editing and scientific
comments. This work was supported by research grants from the American
Cancer Society and the American Heart Association, Heartland Affil-
iate, to H.S., and by a research grant from the V Foundation to J.K.
S. was a Kansas Health Foundation Scholar.

REFERENCES

1. Burns, K. L., Ueki, S. L., Jhung, J. Koh, and D. N. Louis. 1998. Molecular

gene expression. We are grateful to Hitoshi Matsushima for cDNA constructs of CDK4, cyclinD, and GST-pRb, to David Morgan for baculovirus constructs of CDK7, cyclinH, and MAT1, to David Beach for a cDNA construct of p15INK4B, to Mary Patterson for helpful discussions and reading the manuscript, and to Cristina Ward for editing and scientific comments. This work was supported by research grants from the American Cancer Society and the American Heart Association, Heartland Affiliate, to H.S., and by a research grant from the V Foundation to J.K. S. was a Kansas Health Foundation Scholar.

REFERENCES

1. Burns, K. L., Ueki, S. L., Jhung, J. Koh, and D. N. Louis. 1998. Molecular
gene expression. We are grateful to Hitoshi Matsushima for cDNA constructs of CDK4, cyclinD, and GST-pRb, to David Morgan for baculovirus constructs of CDK7, cyclinH, and MAT1, to David Beach for a cDNA construct of p15INK4B, to Mary Patterson for helpful discussions and reading the manuscript, and to Cristina Ward for editing and scientific comments. This work was supported by research grants from the American Cancer Society and the American Heart Association, Heartland Affiliate, to H.S., and by a research grant from the V Foundation to J.K. S. was a Kansas Health Foundation Scholar.

REFERENCES

1. Burns, K. L., Ueki, S. L., Jhung, J. Koh, and D. N. Louis. 1998. Molecular
gene expression. We are grateful to Hitoshi Matsushima for cDNA constructs of CDK4, cyclinD, and GST-pRb, to David Morgan for baculovirus constructs of CDK7, cyclinH, and MAT1, to David Beach for a cDNA construct of p15INK4B, to Mary Patterson for helpful discussions and reading the manuscript, and to Cristina Ward for editing and scientific comments. This work was supported by research grants from the American Cancer Society and the American Heart Association, Heartland Affiliate, to H.S., and by a research grant from the V Foundation to J.K. S. was a Kansas Health Foundation Scholar.

REFERENCES

1. Burns, K. L., Ueki, S. L., Jhung, J. Koh, and D. N. Louis. 1998. Molecular
gene expression. We are grateful to Hitoshi Matsushima for cDNA constructs of CDK4, cyclinD, and GST-pRb, to David Morgan for baculovirus constructs of CDK7, cyclinH, and MAT1, to David Beach for a cDNA construct of p15INK4B, to Mary Patterson for helpful discussions and reading the manuscript, and to Cristina Ward for editing and scientific comments. This work was supported by research grants from the American Cancer Society and the American Heart Association, Heartland Affiliate, to H.S., and by a research grant from the V Foundation to J.K. S. was a Kansas Health Foundation Scholar.

REFERENCES

1. Burns, K. L., Ueki, S. L., Jhung, J. Koh, and D. N. Louis. 1998. Molecular
gene expression. We are grateful to Hitoshi Matsushima for cDNA constructs of CDK4, cyclinD, and GST-pRb, to David Morgan for baculovirus constructs of CDK7, cyclinH, and MAT1, to David Beach for a cDNA construct of p15INK4B, to Mary Patterson for helpful discussions and reading the manuscript, and to Cristina Ward for editing and scientific comments. This work was supported by research grants from the American Cancer Society and the American Heart Association, Heartland Affiliate, to H.S., and by a research grant from the V Foundation to J.K. S. was a Kansas Health Foundation Scholar.

REFERENCES

1. Burns, K. L., Ueki, S. L., Jhung, J. Koh, and D. N. Louis. 1998. Molecular
gene expression. We are grateful to Hitoshi Matsushima for cDNA constructs of CDK4, cyclinD, and GST-pRb, to David Morgan for baculovirus constructs of CDK7, cyclinH, and MAT1, to David Beach for a cDNA construct of p15INK4B, to Mary Patterson for helpful discussions and reading the manuscript, and to Cristina Ward for editing and scientific comments. This work was supported by research grants from the American Cancer Society and the American Heart Association, Heartland Affiliate, to H.S., and by a research grant from the V Foundation to J.K. S. was a Kansas Health Foundation Scholar.

REFERENCES
32. Tassan, J. P., M. Jaquenoud, A. M. Fry, S. Frutiger, G. J. Hughes, and E. A. Nigg. 1995. In vitro assembly of a functional human CDK7-cyclin H complex requires MAT1, a novel 36 kDa RING finger protein. EMBO J. 14:5608–5617.
33. Tevelev, A., I. J. Byeon, T. Selby, K. Ericson, H. J. Kim, V. Kraynov, and M. D. Tsai. 1996. Tumor suppressor p16INK4A: structural characterization of wild-type and mutant proteins by NMR and circular dichroism. Biochemistry 35:9475–9487.
34. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.
35. Valay, J. G., M. Simon, M. F. Dubois, O. Bensaude, C. Facca, and G. Faye. 1995. The KIN28 gene is required both for RNA polymerase II mediated transcription and phosphorylation of the Rpb1p CTD. J. Mol. Biol. 249:535–544.
36. Weinberg, R. A. 1995. The retinoblastoma protein and cell cycle control. Cell 81:323–330.
37. Yang, R., A. F. Gombart, M. Serrano, and H. P. Koehler. 1995. Mutational effects on the p16INK4a tumor suppressor protein. Cancer Res. 55:2503–2506.
38. Yarbrough, W. G., R. A. Buckmire, M. Bessho, and E. T. Liu. 1999. Biologic and biochemical analyses of p16(INK4a) mutations from primary tumors. J. Natl. Cancer Inst. 91:1569–1574.
39. Zarwala, M., E. Liu, and Y. Xiong. 1996. Mutational analysis of the p16 family cyclin-dependent kinase inhibitors p15INK4b and p18INK4c in tumor-derived cell lines and primary tumors. Oncogene 12:451–455.
40. Zawel, L., and D. Reinberg. 1995. Common themes in assembly and function of eukaryotic transcription complexes. Annu. Rev. Biochem. 64:533–561.
41. Zhu, L., G. R. Enders, C. L. Wu, M. A. Starz, K. H. Moberg, J. A. Lees, N. Dyson, and E. Harlow. 1994. Growth suppression by members of the retinoblastoma protein family. Cold Spring Harbor Symp. Quant. Biol. 59:75–84.