Cyclin-dependent Kinase Inhibitor p16\textsuperscript{INK4A} Inhibits Phosphorylation of RNA Polymerase II by General Transcription Factor TFIIH\textsuperscript{*}

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The cell cycle is regulated by various protein kinases, including cyclin-dependent kinases (CDKs). D-type CDKs, CDK4, and CDK6, phosphorylate retinoblastoma protein and are believed to regulate through the G1 phase of the cell cycle. CDK inhibitor p16\textsuperscript{INK4A} has been characterized as binding CDK4 and CDK6 and as inhibiting phosphorylation of retinoblastoma protein by these CDKs. Thus p16\textsuperscript{INK4A} is implicated in regulating the cell cycle at the G1 phase. The largest subunit of RNA polymerase II (pol II) contains an essential C-terminal domain (CTD). General transcription factor TFIIH, which contains CDK7, phosphorylates the CTD of the largest subunit of RNA pol II in vitro. The CTD phosphorylation is shown to be involved in transcriptional regulation in vivo and in vitro. Phosphorylation of RNA pol II CTD by TFIIH is thought to play an important role in transcriptional regulation. Here we report that p16\textsuperscript{INK4A} associates with RNA pol II CTD and TFIIH. p16\textsuperscript{INK4A} inhibited the CTD phosphorylation by TFIIH. These findings suggest that p16\textsuperscript{INK4A} may regulate transcription via CTD phosphorylation in the cell cycle.

EXPERIMENTAL PROCEDURES

Preparation of Insect Cell Lysates—Sf21 cells were infected by recombinant baculoviruses containing cDNA sequences of p16\textsuperscript{INK4A}, CDK4, and CDK2. The infected cells were harvested after 48 h, were washed with phosphate-buffered saline, and were burst in homogenization buffer using N\textsubscript{2} cavitation as described by Graber et al. (16). Cell lysates were centrifuged to remove debris.

Purification of His-p16 and GST Fusion Proteins from Escherichia coli Lysates—GST fusion proteins were purified using a glutathione-Sepharose column as described by Serizawa et al. (3, 17). His-p16 was prepared from E. coli lysates under denaturing conditions and purified using nickel-nitrotriacetic acid column (Qiagen) as described in Koh et al. (18). The purified His-p16 was dialyzed against buffer C (17) containing 50 mM KCl and centrifuged at 30,000 rpm for 30 min. The resulting supernatant was loaded onto DEAE-5PW (7.5 \times 75 mm) (TosoHaas) pre-equilibrated with buffer C containing 25 mM KCl. The column was washed using buffer C (17) containing 100 mM KCl and eluted at 1 ml/min using a 33 mM linear gradient from 100 to 500 mM KCl in buffer C (17). 1-ml fractions were collected.

Coprecipitation Experiments Using Recombinant GST Fusion Proteins and Glutathione-Sepharose Beads—Except as indicated in the figure legend, reaction mixtures were incubated at 27 °C for 30 min under conditions similar to those of the CTD kinase assay in the absence of ATP and N\textsubscript{6}-digested pDN-AdML. The reactions were subsequently incubated with glutathione-Sepharose beads at 4 °C for 60 min and were spun down in a centrifuge. The supernatant was removed. The precipitate was extensively washed using ElB (19) containing 1 mM DTT and 0.5 mg/ml BSA, and proteins in the precipitate were eluted using 1 mM glutathione. The precipitates and supernatants were subjected to SDS-PAGE, and proteins were detected on a gel.
Phosphorylation of RNA pol II CTD by TFIIH but not CDK2. a, phosphorylation of RNA pol II was carried out using RNA pol II (0.001 units) and TFIIH purified from calf thymus. GST-p16 (5 and 10 μg) and GST (5 and 10 μg) were added in the reactions of lanes 4, 5, 7, and 8, respectively. The arrowhead indicates the largest subunit of the RNA pol II phosphorylated by TFIIH. b, phosphorylation of GST-CTD was carried out using 10 μg of GST-CTD and calf thymus TFIIH. GST-p16 (5 and 10 μg) and GST (5 and 10 μg) were added in the reactions of lanes 2, 3, 4, and 5, respectively. The arrowhead indicates the CTD kinase inhibitory activity and phospho-

To determine whether p16INK4A associates with TFIIH, purified GST-p16 was incubated with a TFIIH preparation. GST-p16 was precipitated using glutathione-Sepharose beads, and the
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p16\textsuperscript{INK4A} might associate with the RNA pol II, a protein substract of the CTD kinase of TFIIH, was next examined. The purified GST-p16 was incubated with a fraction containing purified RNA pol II, and GST-p16 was precipitated using glutathione-Sepharose beads. The largest subunit of RNA pol II was detected in the precipitates on Western blots using anti-p16INK4A antibody. The supernatant (sup) and precipitates (ppt) were prepared and analyzed as described under “Experimental Procedures.” The arrowheads indicate p16\textsuperscript{INK4A} detected on Western blots using anti-p16\textsuperscript{INK4A} antibody.

To determine whether p16\textsuperscript{INK4A} associates with the CTD, the purified GST-CTD fraction was incubated with insect cell lysates.
containing His-p16 overexpressed by the baculovirus expression system. The His-p16 was precipitated using an anti-p16INK4A antibody and protein A-Sepharose beads (Fig. 5b). GST-CTD was detected in the precipitates on Western blots using a monoclonal antibody against RNA pol II CTD (lane 1). In control experiments, when an excess of a synthetic peptide that was used to generate the anti-p16INK4A antibody was incubated in the precipitation reactions (lane 2), the amount of GST-CTD detected in the precipitates on the Western blots was traceable but was much smaller than that in lane 1. These data suggest that GST-CTD associates and coprecipitates with His-p16.

To confirm that GST-CTD and His-p16 are coprecipitated together, glutathione-Sepharose beads were used to precipitate GST-CTD, when GST-CTD was incubated with purified His-p16 or the insect cell lysates containing His-p16 overexpressed and precipitated using glutathione-Sepharose beads (lane 1 and 7), the amount of His-p16 detected in the precipitates on Western blots using the anti-p16INK4A antibody was traceable but was much smaller than that in lanes 2 and 8, respectively. These data suggest that GST-CTD directly bound and coprecipitated with His-p16.

p16INK4A has been reported to bind and inhibit CDK4 and CDK6 and to regulate the cell cycle via the inhibition of these CDks (15, 18). CDK4 and CDK6 phosphorylate pRb, which forms a protein complex with RNA binding transcription factor E2F. The phosphorylation of pRb results in the release of E2F from the complex, which activates transcription by RNA pol II to express gene products that are required for the cell cycle (20). Thus, p16INK4A is thought to regulate the cell cycle through the governance of a signaling pathway involved in the CDks and RNA pol II transcription. RNA pol II CTD has been shown to be essential for cell viability and to regulate transcription in vivo (9–11). Phosphorylation of the RNA pol II CTD by TFIIH is thought to be important for transcriptional regulation in vivo and in vitro (1, 9, 21–24). A hypophosphorylated form of RNA pol II preferentially forms a preinitiation complex, and the CTD of RNA pol II engaged in transcriptional elongation is highly phosphorylated. Thus phosphorylation of the RNA pol II CTD has been proposed to be a regulatory event in transcription initiation. The present study shows that p16INK4A is capable of inhibiting phosphorylation of RNA pol II CTD by TFIIH, indicating an alternative possibility that p16INK4A may directly regulate transcription via phosphorylation of RNA pol II CTD in the cell cycle.

The RNA pol II holoenzyme has been reported to be a ribosome-sized protein complex containing factors involved in regulating transcription, cell cycle, and DNA repair (25–28). The precise subunit composition of the holoenzyme is still unclear. This work indicates that p16INK4A is a CTD-binding protein. Therefore, it is possible that p16INK4A may be a subunit of the holoenzyme. CDK5 and cyclin C appear to be subunits of the holoenzyme (28). Yeast homologues of these molecules are Srb10 and Srb11, and the CTD kinase by Srb10 was shown to be responsible for transcriptional activation (29). It is possible that p16INK4A might also inhibit the CTD phosphorylation by CDK5, and furthermore, that p16INK4A might inhibit transcriptional activation.

Mutations and deletions in the p16INK4A gene have been identified from many types of tumor cells (Ref. 18 and references therein). Mutants of p16INK4A derived from tumor cells have revealed defects in inhibiting CDK4 and in regulating cell cycle at the G1 phase (18). These p16INK4A mutants may also have defects in inhibiting the CTD kinase of TFIIH and in binding RNA pol II CTD and TFIIH, and these defects might be involved in tumorigenesis.

CDK7 and cyclin H have been shown to form a 175-kDa complex in vitro (19, 30). This 175-kDa complex activates the histone H1 kinase activity of CDK2 by phosphorylation of the threonine activation site. The kinase capable of activating a CDK is called CDK-activating kinase, CAK. Most of the CAK activity in cell lysates fractionates in a 175-kDa complex (30). TFIIH has been reported to contain in vitro CAK activity and to be a complex of about 700 kDa (3). However, the function of the 175-kDa CAK complex and TFIIH in the cell cycle is not well understood. This work reveals that p16INK4A binds the recombiant CDK7 and the purified TFIIH and that although p16INK4A inhibits CTD kinase of TFIIH, it does not inhibit CDK2 phosphorylation by TFIIH. These results suggest that CAK complexes, including TFIIH and the 175-kDa complex, may play a role in the cell cycle via a signaling pathway regulated by p16INK4A. However, p16INK4A may not be involved in a pathway to activate CDK2 by CAK.

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