Transcriptional Repression of the D-Type Cyclin-dependent Kinase Inhibitor p16 by the Retinoblastoma Susceptibility Gene Product pRb

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Abstract

Progression of the eukaryotic cell division cycle is regulated by a series of structurally related serine/threonine protein kinases known as cyclin-dependent kinases (CDKs). The D-type cyclin-dependent kinases, CDK4 and CDK6, have been strongly implicated in the control of G1 progression and the phosphorylation of the retinoblastoma protein, pRb. The formation of complexes and enzymatic activity of cyclin D-CDK4 and cyclin D-CDK6 kinases is negatively regulated by p16INK4 (MTS1/CDK4i/CDKN2) via its specific interaction with CDK4 and CDK6 catalytic subunits. Here we report that the p16 mRNA accumulates to a high level in cells lacking pRb function and transcription of p16 is repressed by pRb. Our results provide evidence supporting a feedback regulatory loop involving pRb, p16, and cyclin-dependent kinases.

Introduction

The primary control of the eukaryotic cell cycle is provided by a family of serine/threonine protein kinase complexes consisting of a catalytic subunit, a CDK3; and a regulatory subunit, a cyclin (1). CDK subunits by themselves are inactive and binding to a cyclin protein is required for their activity as well as their regulation. CDKs are also regulated intrinsically by activating and inhibitory phosphorylations (reviewed in Refs. 1 and 2). In addition to cyclin activation and subunit phosphorylation, it has become clear recently that CDK activity is also regulated (predominantly negatively) by a number of small proteins that physically associate with cyclins, CDKs, or their complexes. In mammalian cells, the number of small cell cycle-regulatory proteins identified by virtue of their ability to physically interact with cyclin or CDK proteins is rapidly increasing. Although these small CDK-interacting proteins were identified only very recently, their biochemical and biological properties are being characterized very quickly. Most importantly, a striking connection between these small CDK-interacting proteins were identified only very recently, their biochemical and biological properties are being characterized very quickly. Most importantly, a striking connection between the tumor suppression function of p53 and cell cycle control has been discovered.

The first evidence that a CDK inhibitor may play an important role in tumor growth suppression came from the studies of the p21 CDK inhibitor. p21 (also variously known as WAF1, CIPI, SDII, CAP20, and CDKN1), first discovered in normal human fibroblast cells as a component of cyclin D-CDK quaternary complexes that also contain proliferating cell nuclear antigen (3), was subsequently identified as a regulatory subunit by itself and as a component of cyclin-CDK quaternary complexes that also contain cyclin D-dependent kinases (CDKs) (reviewed in Refs. 1 and 2). In addition to cyclin activation and subunit phosphorylation, it has become clear recently that CDK activity is also regulated (predominantly negatively) by a number of small proteins that physically associate with cyclins, CDKs, or their complexes. In mammalian cells, the number of small cell cycle-regulatory proteins identified by virtue of their ability to physically interact with cyclin or CDK proteins is rapidly increasing. Although these small CDK-interacting proteins were identified only very recently, their biochemical and biological properties are being characterized very quickly. Most importantly, a striking connection between these small CDK-interacting proteins were identified only very recently, their biochemical and biological properties are being characterized very quickly. Most importantly, a striking connection between the tumor suppression function of the Retinoblastoma Susceptibility Gene Product pRb, p16, and cyclin-dependent kinases.

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: CDK, cyclin-dependent kinase; CMV, cytomegalovirus.
Genomic Clones and Cell Transfection. A 500-base-pair cDNA fragment corresponding to the coding region of p16 was isolated by PCR using oligonucleotide primers derived from p16 cDNA (10) and used as a probe to screen a human placenta genomic library cloned in AFlX II (Stratagene, La Jolla, CA). A 1.1-kilobase genomic fragment corresponding to the 5' upstream region of p16 cDNA was determined by DNA sequencing and subcloned into a promoterless luciferase expression plasmid, pGL2-Basic (Promega, Madison, WI). The resultant plasmid, p16-Luc, or control pGL2-Basic and pSV40-Luc DNA were transfected with lipid reagents (Lipofectamine Reagent; Gibco-BRL, Gaithersburg, MD) into 4 × 10^5 cells cultured on 6-well dishes (40–60% confluence) that were plated 1 day prior to transfection. In cotransfection experiments shown in Fig. 3, B and C, 1 μg of p16-Luc DNA was cotransfected with an increasing amount of CMV-RB complemented by carrier pGL2-Basic DNA to provide equal amounts of a total of 2 μg transfected DNA in each transfection. Twenty-eight h after transfection, cells were lysed with 250 μl Triton lysis buffer (1% Triton X-100/15 mM MgSO_4/4 mM ethylenedinitrilo[2]tetraacetic acid/1 mM DTT/25 mM glycyglycine, pH 7.8) and gently shaken in a cold room for 15 min. Cell lysates were collected into a 1.5-ml tube and clarified by centrifugation for 5 min at 14,000 rpm on a microcentrifuge in the cold room. Twenty-five μl of clarified cell lysate was mixed with 231 μl of 50 mM glycyglycine (pH 7.8) and a 44-μl reaction mixture to give rise to a final volume of 300 μl that contains 15 mM MgSO_4, 6 mM ATP, and 0.67 μg/ml BSA. Luciferase activities were measured by integrating light emission over 50 s on a Berthold luminometer (Lumat LB 9501; Wallac, Inc., Gaithersburg, MD) with injection of 100 μl luciferin (ICN, Irvine, CA) to each reaction.

Results and Discussion

p16 was initially identified as a CDK4-associated protein in cells transformed by a variety of DNA tumor viruses including SV40, papilloma, and adenoviruses (5). It is present in the CDK4 complexes at a high level in transformed cells as determined by immunoprecipitation, but in normal untransformed cells the level of CDK4-associated p16 is extremely low or undetectable. A common property shared by these DNA tumor viruses is their ability to bind to and consequently inactivate the function of two tumor suppressors, pRb and p53, suggesting the possibility that pRb and/or p53 may regulate the expression of p16. To test this hypothesis, we first analyzed the steady state level of p16 mRNA.

Total RNA was prepared from a number of tumor-derived cell lines the pRb and/or p53 status of which has been previously characterized. Two such examples are shown in Fig. 1. Saos-2 and U-2 OS cell lines were both derived from human osteosarcomas. Northern analysis revealed a high level of p16 mRNA in Saos-2 cells, but p16 mRNA was undetectable in U-2 OS cells (Fig. 1B). Failure to detect the p16 mRNA in U-2 OS cells is not due to homozygous deletion of the p16 locus as is observed in many cultured tumor cell lines (11, 12), since we have verified the presence of the p16 gene in both cell lines by PCR (data not shown). Whether the p16 gene in U-2OS (and Saos-2) cells contains point mutation(s) in the coding region or promoter sequences has not been determined. The high level of p16 mRNA in Saos-2 cells is correlated with a high level of p16 protein and its association with CDK4 and CDK6, as shown in Fig. 1C. In addition to p16, we also noticed that both CDK4 and CDK6 appear to associate with several additional cellular proteins such as p13, p14, and p15 that do not correspond to any of the recently identified small CKD inhibitors as judged by their mobility. The specificity and identity of these potential CDK4 and CDK6 interacting proteins are currently un-
TRANSCRIPTIONAL REPRESSION OF p16 BY pRb

Fig. 2. Increased expression of p16 mRNA and association of p16 protein with CDK4 and CDK6 in cells lacking pRb function. A, Northern analysis of p16. The procedures on cell culture, RNA preparation, and Northern blotting were the same as described previously (20). Total RNA was prepared from normal human diploid fibroblasts IMR-90 cells and IMR-90 cells expressing type 16 papilloma viral oncoproteins E6, E7, and E6 together with E7 (19), and from a different strain of normal human fibroblasts (HSF43) and its SV40 large T antigen-transformed derivative [CT10 (5)]. Twenty µg of each of the RNA samples were resolved on a 1% agarose gel. Similar amounts of RNA were loaded for each sample as determined by hybridization with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Resolved RNA samples were transferred to a nitrocellulose filter and the blot was hybridized with a probe derived from full length human p16 cDNA. As a comparison, the same blot was also hybridized with a probe derived from full length human p21 cDNA, the expression of which is drastically reduced as the result of inactivation of p53 function by the E6 oncoprotein of type 16 papillomavirus (6, 9, 23). B, analysis of CDK4, CDK6, and p16 immunocomplexes. [35S]Methionine-labeled cell lysates were prepared from parental IMR-90 cells and IMR-90 cells infected with retroviral vectors containing the type 16 HPV E6 (IMR-90/E6), E7 (IMR-90/E7), or E6 plus E7 (IMR-90/E6+E7) oncogene. Labeled cell lysates were immunoprecipitated with antiserum specific to CDK4 (5), CDK6 (21), and p16 (10) with or without prior incubation with a competing antigen peptide as indicated at the top of each lane and resolved by SDS-PAGE. Left ordinate, positions of p16, p21 and GAPDH mRNA. The steady state level of p16 mRNA accumulation was determined on a PhosphorImager (Molecular Dynamics, ImageQuant software version 3.3). Right ordinate, relative position of proteins with established identities. Cyc D1, cyclin D1.

They further demonstrate that expression of either E6 or E7 oncoprotein leads to an increased level of p16 mRNA, with E7 being a more potent stimulus indicating that both p53 and pRb may negatively regulate the expression of p16 mRNA. Subsequent experiments therefore were focused on the regulation of the level of p16 mRNA by pRb. As a comparison, the same blot was also hybridized with a probe derived from human p21 CDK inhibitor cDNA. While expression of 16E7 protein had little effect, expression of 16E6 (and 16E6 together with 16E7) dramatically reduced the level of p21 mRNA (Fig. 2A) that is also correlated closely with a reduced level of p21 protein in these cells (data not shown). These results are entirely consistent with the previous finding that the expression of p21 mRNA is transcriptionally activated by p53 (6, 9).

We next investigated whether transcriptional activation of p16 contributed to the higher level of p16 mRNA seen in virally transformed or Rb-deficient cells. Genomic fragments containing p16 sequences were isolated from a human placenta genomic library and a 1.1-kilobase genomic fragment corresponding to the 5' upstream region of p16 cDNA was subcloned into a promoterless luciferase expression plasmid (pGL2-Basic). The resultant plasmid, p16-Luc, gave rise to considerable luciferase activity as compared to the parental promoterless luciferase construct pGL2-Basic when it was transiently expressed in several cell lines including Saos-2 and U-2 OS (Fig. 3A). To allow comparison of luciferase activity in cells transfected with p16-Luc, an SV40-Luc plasmid was transiently expressed in parallel as a control for normalizing the transfection efficiency between the different cells. In five independent experiments, we have reproducibly observed an average of 10-fold higher luciferase activity in Saos-2 cells than in U-2 OS cells (Fig. 3A), indicating that the higher level of p16 mRNA seen in Saos-2 cells was most likely the result of transcriptional activation of the p16 promoter.

* Unpublished observations.
Finally, to determine whether pRb can directly repress transcription through the p16 promoter, the p16-Luc reporter plasmid was co-transfected into Saos-2 cells with wild-type pRb cDNA under the control of the strong immediate-early promoter of CMV [pCMV-Rb (25)]. Ectopic expression of pRb in these Rb-deficient cells reproducibly resulted in an average 40% reduction in luciferase activity from the p16 promoter (Fig. 3B), but had no detectable effect on the SV40 promoter (data not shown). Similar results were also obtained in separate experiments using mouse NIH 3T3 cells. Cotransfection of the p16-Luc plasmid with an increasing amount of pCMV-pRb repeatedly led to a progressive reduction of luciferase activity up to 40% of the full activity from the p16 promoter (Fig. 3C). These results provide direct evidence for the repression of the p16 promoter by pRb. We have been consistently obtaining a maximal 40% reduction of luciferase activity in cotransfection with pCMV-pRb in different cell lines, suggesting that an additional factor(s) that is not controlled by pRb may also regulate the expression of p16. A number of cellular transcriptional factors such as E2F-1 have been identified as the functional targets of pRb (reviewed in Refs. 17 and 26). We have determined more than 3 kilobases of nucleotide upstream sequences of the p16 gene including the 1.6-kilobase region used in the transfection assay and found no apparent E2F binding site (26) or conventional TATA sequences. Whether the p16 gene contains a weak E2F binding site or pRb represses p16 transcription by a mechanism independent of E2F factors are currently under investigation.

The function of pRb is known to be down-regulated by cell cycle-dependent phosphorylation and both D-type cyclins and their associated kinases (primarily CDK4 and CDK6) have been strongly implicated as physiological pRb kinases (see two most recent reviews in Refs. 17 and 18). Biochemical analyses have indicated that p16 acts as an inhibitor of CDK4 (and likely CDK6 as well (10)). Our results demonstrate that transcription of p16 is repressed by pRb, providing evidence for a regulatory feedback loop involving pRb, CDKs, p16, and an as yet unidentified transcription factor (Fig. 4). In this model, phosphorylation of pRb by cyclin D-CDK4 or cyclin D-CDK6 results in inhibition of the cyclin D-CDK4 and cyclin D-CDK6 kinase activities. Ultimately, phosphorylation of pRb would decrease, as would the level of p16 expression. This model is consistent with, and provides a plausible explanation for, the absence of cyclin D proteins and cyclin D-CDK complexes in cells lacking pRb (27, 28). A high level of p16 in cells lacking pRb function may lead...
to dissociation of cyclin D from CDK complexes and subsequent degradation. In addition, an increased level of p16 mRNA in cells lacking p53 function [IMR-90 cells expressing 16E6 (Fig. 2A)] suggests that the expression of p16 may also be regulated negatively by p53. Our results also provide a potential pathway by which the expression of p16 is regulated by p53. An increased level of p21 activated by p53 inhibits the activity of various CDKs, preventing the phosphorylation of pRB and consequently leading to the repression of the p16 promoter. This study, together with the discovery of the transcriptional regulation of the p21 CDK by p53, dramatically exemplifies how the recently identified small CDK inhibitors link tumor suppressor gene function to cell cycle control.

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