Progression through the eukaryotic cell cycle is driven by the activity of cyclin-dependent kinases. The cyclin D-dependent kinase Cdk4 promotes progression through the G1 phase of the cell cycle and is deregulated in many human tumors. The tumor suppressor protein \( p16^{INK4A} \) (p16) forms a complex with Cdk4 and inhibits kinase activity. Here we report that p16 is phosphorylated, and the phosphorylated form of p16 is preferentially associated with Cdk4 in normal human fibroblasts. We mapped phosphorylation sites on exogenously overexpressed p16 to serines 7, 8, 140, and 152 and found that endogenous p16 associated with Cdk4 is phosphorylated at serine 152. All mapped phosphorylation sites lie outside of the conserved kinase-binding domain of p16 but in regions of the protein affected by mutations in familial and sporadic cancer. Our results suggest a novel regulation of p16 activity.

Progression through the cell cycle is driven by the activity of cyclin-dependent kinases (Cdks)\(^1\) (1). An active Cdk holoenzyme consists of a catalytic kinase subunit associated with a regulatory cyclin partner (1). Cdk4 promotes progression through the G1 phase of the cell cycle in response to extracellular growth signals (2). The D-type cyclins promote Cdk4 activity; however, cellular cyclin D and Cdk4 associate only in response to additional growth factor-dependent signaling events (3, 4). Such events include the recruitment of assembly factors such that an inactive p16 complex (3, 4). Thus, the formation of an active cyclin D-Cdk4 complex is a highly regulated event (3).

p16 is a specific inhibitor of the D-type Cdks (5). p16 association with Cdk4 results in dissociation of the D-cyclin and other assembly factors such that an inactive p16-Cdk4 complex contains no other proteins (6). In contrast to the assembly of an active Cdk4 complex, current models presume that the assembly of an inactive p16-Cdk4 complex is regulated only by the abundance of the two proteins (3). Post-translational regulation of either p16 or a p16-Cdk4 complex has not been described. Furthermore, past studies have determined that p16 is not phosphorylated (7).

Here we report that p16 is phosphorylated and map the phosphorylation sites to regions of the protein outside of the kinase-binding domain. We show that phosphorylation site mutants of p16 associate with Cdk4 as wild type p16 but that the phosphorylated form of p16 is selectively associated with Cdk4 in normal human fibroblasts. Our results suggest novel post-translational regulation of p16 activity.

**Materials and Methods**

Cell Lines, Plasmids, and Transfections—WI-38 and U2OS cells were obtained from the American Type Culture Collection and maintained in DMEM + 10% fetal bovine serum (FBS, Invitrogen). WI-38 cells were used at passage 3–5. U2OS cells were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Wild type p16 and all Cdk4 plasmids have been described elsewhere (8). p16 phosphorylation site mutants were constructed using the QuikChange kit (Stratagene).

**Metabolic Labeling**—For \(^{32}\)P labeling, cells were washed twice in phosphate-buffered saline and incubated for 20 min in methionine/cysteine-free DMEM (Invitrogen) supplemented with 10% dialyzed FBS (Hyclone). The medium was aspirated and fresh medium was added containing 0.2\(\mu\)Ci/ml Easy Tag Express in vitro labeling mix (PerkinElmer Life Sciences). The cells were labeled for 3 h before lysis and immunoprecipitation. For \(^{32}\)Porthophosphate labeling, cells were washed twice in phosphate-free DMEM and incubated for 30 min in phosphate-free DMEM supplemented with 10% dialyzed FBS. The medium was aspirated and replaced with medium containing 1\(\mu\)Ci/ml \(^{32}\)Porthophosphate (PerkinElmer Life Sciences). The cells were incubated for 4 h prior to lysis and immunoprecipitation.

**Immunoprecipitation and Western Blotting**—Cells were lysed in ELB (50 mM HEPES, pH 7.0, 0.1% Nonidet P-40, 250 mM NaCl, 5 mM EDTA, 20 mM sodium fluoride, 2 mM sodium orthovanadate, and 1 mM dithiothreitol) containing one complete miniprotease inhibitor tablet/10 ml (Roche Molecular Biochemicals)). The cells were incubated for 10 min on ice and clarified by centrifugation at 20,000 \(\times g\) for 10 min. The supernatant was transferred to a clean tube and immunoprecipitated with the indicated antibody: p16 (JC-4; Ref. 9), Cdk4 (Santa Cruz Biotechnology H-22), Myc tag 9E10 clone, laboratory stock), or a combination of normal mouse serum and rabbit serum (Jackson Immunoresearch Laboratories, Inc.). Antibody complexes were captured with protein G-Sepharose (Amersham Biosciences). All immunoprecipitations were washed three times in ELB prior to further manipulation. For phosphatase treatment of the Cdk4 immunoprecipitate (IP) (Fig. 1A), the Cdk4 IP was washed twice in supplied phosphatase buffer prior to incubation with λ-phosphatase (New England Biolabs) for 30 min at 30 °C. For blots, IPs were separated by SDS-PAGE and transferred to Immobilon P (Millipore). The membranes were probed as directed using a combination of JC-2 and JC-8 to detect p16 (9). Cdk4 was detected with Santa Cruz Biotechnology H-22.

**Two-dimensional Gel Electrophoresis**—IPs were washed twice in water to remove salts. Bound proteins were eluted by vortexing gently for 20 min in 2-D buffer (160 \(\mu\)l total volume, 2-D buffer contained 7 M urea, 2 \(\times\) thiourea, 8 M dithiothreitol, 4% Chaps, and 0.2% Biolyte, pH 3–8 (Bio-Rad)). 125 \(\mu\)l of the reaction mixture was loaded onto a pH 5–8 2-D gel, stained with Coomassie blue, and destained with 50% methanol and 10% acetic acid. The remaining volume of sample in 2-D buffer was separated by one-dimensional SDS-PAGE.

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\(^{2}\)The abbreviations used are: Cdk, cyclin-dependent kinase; p16, \( p16^{INK4A} \); p21\(^{CIP1} \); p27\(^{KIP1} \); IP, immunoprecipitate; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography.
Phosphorylation of p16 Correlates with Cdk4 Association

RESULTS

Normal human fibroblasts (WI-38 cells) were labeled with \(^{35}\text{S}\)-protein labeling mix, and either p16 or Cdk4 was immunoprecipitated. A single protein of approximate molecular mass 16 kDa was present in each IP but not in control reactions (Fig. 1A, top). The identity of this protein was confirmed as p16 by Western blot (Fig. 1B). When separated by isoelectric focusing, two major populations of p16 were present in these cells. Of the two populations, a single species was associated with Cdk4 (Fig. 1A, compare IP: p16 to IP: cdk4). Upon alignment, the species appeared to be the more acidic form of p16. To test whether the form of p16 associated with Cdk4 was phosphorylated, the Cdk4 IP was treated with \(\lambda\)-phosphatase for 30 min prior to separation. Treatment with phosphatase caused the appearance of a second, less acidic form of p16. These results suggested that a phosphorylated form of p16 is associated with Cdk4 in WI-38 cells.

To confirm phosphorylation of p16, WI-38 cells were labeled with \(^{32}\text{P}\)orthophosphate. p16 recovered from these lysates had incorporated \(^{32}\text{P}\) (Fig. 1A, lower panel), demonstrating that p16 is a phosphoprotein. Moreover, p16 recovered in the Cdk4 IP was greatly enriched for phospho-p16. This was not due to increased recovery of p16 since the abundance of p16 in the Cdk4 IP was lower by Western blot. The reason for incomplete recovery of p16 in the p16 IP is unclear, but it is possible that phosphorylation results in decreased availability of the epitope (11). Together with the isoelectric focusing results from Fig. 1A, these data demonstrate that a phosphorylated form of p16 is preferentially associated with Cdk4 in cultured normal human fibroblasts.

p16 is expressed at low levels in early passage WI-38 cells. We thus mapped phosphorylation sites on exogenous p16 to obtain sufficient protein for analysis. Myc-tagged p16 was overexpressed in p16-null U2OS cells, a human osteosarcoma cell line. The cells were labeled with \(^{32}\text{P}\)orthophosphate, and exogenous p16 was isolated via the Myc tag. We found that endogenous p16 was exclusively serine-phosphorylated (Fig. 2A) just like endogenous p16 (data not shown). The labeled protein was digested with trypsin following separation by SDS-PAGE. The peptide digests were then separated by HPLC. An acetonitrile gradient was used to elute proteins from a C8 column so that larger, more hydrophobic peptides tend to elute later in the run. Scintillation counting of each fraction following the
separation demonstrated the presence of three distinct phosphopeptides (Fig. 2B, top panel).

Fractions containing each labeled peptide were pooled, concentrated, and subjected to Edman degradation. Scintillation counting of the amino acid released following each round of degradation allowed identification of the phosphorylation sites in overexpressed p16. Each of the three labeled peptides contained different phosphorylation sites. These sites were mapped as serines 7 and 8, serine 140, and serine 152 (for mapping details and data, see Supplemental Figs. 1 and 2). We believe that both serine 7 and serine 8 can be phosphorylated because mutating either residue to alanine did not completely abrogate phosphorylation of this peptide. However, a p16 mutant lacking the eight N-terminal amino acids did not yield a corresponding N-terminal phosphopeptide. Similarly, mutation of serine 152 to alanine abolished the presence of the serine 152 peak. The peak corresponding to serine 140 maps to a partial digestion product. The appearance of this peak was variable, probably due to the efficiency of digestion. We believe the completely digested peptide containing phosphorylated serine 140 does not bind the column. However, serine 140 is the only amino acid in p16 that is capable of being phosphorylated and maps correctly to a potential tryptic peptide following Edman degradation.

We then asked which of these sites were phosphorylated on endogenous p16. p16 isolated from 32P-labeled WI-38 cells was digested and assayed for phosphorylation as above. We detected a single reproducible peak following labeling and digestion of endogenous p16 with trypsin (Fig. 2B, middle panel). This peak eluted in the fractions corresponding to phosphorylated serine 152 on exogenous p16 (Fig. 2B, upper panel) and eluted very close to an artificially synthesized phosphopeptide similar to the observed tryptic peptide (not shown). This peak was also present, even enriched, in p16 isolated by co-immunoprecipitation with Cdk4. We cannot rule out serine 140 phosphorylation in these IP's since the site maps to a partial tryptic digestion product. Similarly we could not rule out that other residues are phosphorylated at low levels, below the detection limit in our assay due to the low signal. However, by two-dimensional gel electrophoresis, we detect only two major species of p16 in WI-38 cells.

Of particular interest was the location of the phosphorylation sites in human p16 (Fig. 3A). These sites lie outside of the conserved kinase-binding region of p16 (12). The four ankyrin repeats that form the core structural unit of p16 are highly conserved between species; however, the regions surrounding the mapped phosphorylation sites are not conserved. The lack of conservation would suggest either that p16 phosphorylation is not important or that human p16 has acquired characteristics not shared between species. The correlation between p16 phosphorylation and Cdk4 association suggests the latter. In fact, at least two reports suggest functional differences between mouse and human p16 (13, 14).

To determine whether the phosphorylation sites on p16 are required for Cdk4 association, we mutated each of the sites to alanine, treating the adjacent serines 7 and 8 as a single site (p16 S7A/S8A). We expressed these mutants in p16-null U2OS cells at varying levels and compared association with Cdk4 (Fig. 3B). In the experiment shown, the cells were transiently transfected with plasmids expressing untagged p16 constructs driven by a retroviral long terminal repeat. In our experiments, these vectors yield lower expression than the more commonly used cytomegalovirus promoter (data not shown). To ensure transfection of equal cell numbers, the amount of total DNA was held at a constant 2 μg, consisting of increasing amounts of p16 DNA together with the appropriate amount of empty vector. Even when expressed at low levels, each p16 mutant was present in a Cdk4 IP at levels comparable to wild type p16. Moreover, a Myc-tagged p16 construct consisting of only amino acids 9–139 of the protein and lacking all phosphorylation sites still associates with Cdk4 and inhibits cell cycle progression, although the construct appears less stable than wild type p16 (data not shown). These results indicate that phosphorylation of p16 does not result in increased association with Cdk4.

**DISCUSSION**

We have shown that p16 is phosphorylated and that the phosphorylated form of p16 is preferentially associated with Cdk4 in normal human fibroblasts. We have mapped phosphorylation sites on exogenous p16 to regions outside of the kinase-binding portion of the protein. At least one of these sites, serine 152, is phosphorylated on endogenous p16 in WI-38 cells. We have further shown that p16 phosphorylated at this site is associated with Cdk4 in WI-38 cells.

Several models could explain the preferential association of phosphorylated p16 with Cdk4. The first possibility is that phosphorylation of p16 promotes association with Cdk4. The fact that phosphorylation site mutants retain their ability to associate with Cdk4 argues against this model, but these results are derived from overexpression studies in an immortalized cell line and may poorly represent the case in normal human fibroblasts. Studies of p16 mutants in normal fibroblasts are complicated by the presence of wild type p16 and difficulties in regulating delivery of a transgene. The second possibility is that phosphorylation of p16 is a consequence of association with Cdk4. We are currently investigating this...
hypothesis, and while possibly true, the relationship does not appear simple.

Perhaps the most intriguing question is the significance of p16 phosphorylation. Overexpression of p16 does not alter the abundance or the subcellular localization of endogenous Cdk4 (data not shown). One possibility is that phosphorylation of p16 results in stabilization of an inactive p16-Cdk4 complex. p16 has three closely related INK4 family members, p15, p18, and p19 (15). Each of these family members has affinity for Cdk4 equal to that of p16 (6, 7); however, p15, p18, and p19 do not associate stably with Cdk4 in cells in which p21 is expressed (6). Furthermore, it has been noted that p15 forms complexes with Cdk4 only if it has access to the kinase prior to p27 and that p15 cannot disrupt preformed p27-containing complexes (16). A regulatory mechanism that actively assembles or stabilizes a phospho-p16-Cdk4 complex could explain why p16 appears to be the selective Cdk4 inhibitor in cells expressing p21 and p27.

The phosphorylation sites mapped in human p16 are poor substrates for known kinases, including Cdns, which require an (S/T)P motif (25). We believe that Cdk4 does not phosphorylate p16 because 1) the mapped sites are poor Cdk4 substrates, 2) levels of p16 phosphorylation remain high even in the presence of a kinase-dead form of Cdk4, and 3) Cdk4 does not phosphorylate p16 in vitro (data not shown). It will be of interest to identify the kinases responsible for p16 phosphorylation and determine the role these kinases might play in cell cycle regulation.

The phosphorylation sites mapped in human p16 could explain mutational events associated with cancer progression. Alteration of the gene encoding p16 is among the most common mutations in human cancer, and many cases of familial melanoma are associated with inactivation of this gene (17). The majority of mutations abrogate the ability of p16 to associate with its cognate kinase and simultaneously disrupt the coding region of an unrelated tumor suppressor, p14ARF (17). Of note, however, are mutations that affect specifically p16 and do not alter the affinity of p16 for Cdk4 (17). The effects of these mutations remain unclear despite the fact that at least one such alteration is associated with familial melanoma (17). This mutation is a duplication of the 24-base pair region comprising the initial eight amino acids of p16 including the serines 7/8 potential phosphorylation sites (18, 19). Deletion of the same eight amino acids has also been noted as a tumor-associated mutation (20, 21). The proteins encoded by the mutant p16 cDNAs behave like wild type p16 in previously described assays for function (17).

Similar data have been reported concerning mutations at the C terminus of p16 (17). One mutation affects splicing of p16 and results in an exchange of the C-terminal four amino acids DIPD for the amino acids GED (17, 22, 23). A separate tumor-derived mutation eliminates the p16 stop codon and extends the C terminus by 13 amino acids (24). Since the proteins encoded by these cDNAs are not defective in Cdk4 association, it has been difficult to explain why these mutations would be associated with cancer (17). All of the above listed mutations affect regions of p16 harboring the mapped phosphorylation sites.

While the consequences of p16 phosphorylation remain unclear, two observations support a role for p16 phosphorylation in protein function. First, phosphorylated p16 is selectively associated with Cdk4 in normal human fibroblasts. Second, phosphorylated regions of the protein are found mutated in cancer, and these mutations do not affect known functions of the protein. That such tumor-derived p16 mutants behave normally in known assays for function suggests that novel assays must be developed to discern behavior differences for p16 phosphorylation site mutants. Elucidation of the mechanistic nature by which p16 phosphorylation affects function will be of paramount interest in understanding how p16 regulates Cdk4 activity and acts as a tumor suppressor.

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