Identification of Functional Elements of p18\(^{\text{INK4C}}\) Essential for Binding and Inhibition of Cyclin-dependent Kinase (CDK) 4 and CDK6\(^1\)

Seong J. Noh, Yan Li, Yue Xiong, and Kun-Liang Guan\(^2\)

Department of Biological Chemistry and the Institute of Gerontology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606 [S. J. N., K.-L. G.]; and Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599-7295 [Y. L., Y. X.]

ABSTRACT

Members of the INK4 family of cyclin-dependent kinase (CDK) inhibitors specifically bind and inhibit the G\(_1\)-specific CDK molecules CDK4 and CDK6. One of the INK4 molecules, p16, is also known as multiple tumor suppressor and has been found to be mutated or deleted in various tumors and cell lines. We have previously identified p18 as a member of the INK4 family. To determine the molecular basis for the inhibitory function of p18, we introduced 11 missense mutations of conserved residues that were identified in p16 of cancer cell lines into p18. The effects of these mutations on the ability of p18 to bind and inhibit CDK4 and CDK6 or to inhibit cell growth were determined. Our results indicate that the third ankyrin repeat and the NH\(_2\)-terminal portion of the fourth repeat constitute the essential element necessary for the ability of p18 to bind and inhibit CDK4 and CDK6. Apart from this core interaction element, p18 seems to use additional, distinct residues to differentially bind and inhibit CDK4 and CDK6, accounting for the known penchant of p18 to preferentially interact with CDK6.

INTRODUCTION

The molecular engines that propel mammalian cells through the first gap phase into S phase are: the G\(_1\)-specific CDKs\(^3\) CDK4 and CDK6, in complexes with their regulatory subunits, cyclin D1, D2, and D3; and CDK2, in complex with cyclin E (1). Of the numerous modes of regulation that impinge upon the cyclin D-associated CDK activity, two classes of CDK inhibitors exist. The first class of inhibitors, which consists of p21 (WAF1/Cip1), p27 (Kip1), and p57 (Kip2) is able to bind and inhibit all known cyclin-CDK complexes (2–6). On the other hand, the INK4 family, which consists of p15\(^{\text{INK4B}}\), p16\(^{\text{INK4A}}\), p18\(^{\text{INK4C}}\), and p19\(^{\text{INK4D}}\), specifically interacts with and inhibits the cyclin D-CDK4 and cyclin D-CDK6 complexes (7–12).

Although ample evidence exists that INK4 molecules share similar biochemical properties, namely, binding and inhibiting the activity of cyclin D-dependent CDK molecules (7, 8, 13), the exact physiological functions of each of the INK4 molecules are not well defined. It is postulated that p16 may function to inhibit CDK4/6 activity during S, G\(_2\), and M phases, at which time the kinase activity may be detrimental to the cell (7). In the macrophages and other hematopoietic cells, in which p16 is known to be absent (11, 14), p18 and p19 levels drop during G\(_1\) and accumulate as the G\(_1\)-S boundary is reached (11), possibly substituting for p16 function. Yet another role of INK in cell cycle is suggested by the induction of p15 expression upon transforming growth factor-\(\beta\) stimulation in human epithelial cell line (9) mediating transforming growth factor-\(\beta\)-induced G\(_1\) arrest. The varied functions of the INK4 molecules may result from the probable differential affinities between the various cyclin D-CDK holoenzymes and particular INK4 molecules (8) and also the differential tissue expression of the INK4 family members (9, 11, 14).

\(p16^{\text{INK4A}}\) is also known as multiple tumor suppressor because mutations in this gene were documented in a wide variety of tumors and cell lines (15). Deletions of 9p21, the chromosomal region that includes p16, were reported for lung and bladder carcinomas, acute lymphoblastic leukemias, melanomas, pancreatic adenocarcinomas, and gliomas. Most of the reported mutations were frameshift or nonsense mutations; however, a handful of missense mutations were also reported (15–21). In vivo characterization of some of these p16 missense mutants revealed that some of the mutations caused complete loss of CDK4 inhibitory activity, whereas others did not affect inhibition at all (13, 22–26). The majority of the loss of activity mutants of p16 identified in these studies, such as H83Y, R87P, P114L, and V126D, involved residues that are conserved between the INK4 family members. In contrast, mutations of nonconserved residues, such as I49T, N71S, A127S, and A148T, did not affect the inhibitory function of p16. The fact that the loss of function mutants of p16 involve conserved residues is significant, given that the INK4 family members share very low amino acid sequence identity (~30\%) and that these rare, conserved residues occur in clusters (Fig. 1). These clusters of conserved residues, therefore, might constitute critical structural or functional elements of the INK4 family.

As alluded to above, p18 may quite possibly serve a physiological function different from that of p16. Two lines of evidence further support this idea. (a) The frequency of deletion or mutations of p18 in cancer tissues is very low compared to that of p16 (27, 28). (b) In vivo communoprecipitation experiments demonstrated that p18 preferentially associates with CDK6, whereas p16 associates with both CDK4 and CDK6 (8). In light of these observations, we wished to determine the possible biochemical basis for the functional differences between p16 and p18. Because the clusters of conserved residues of p18 likely constitute elements critical for the inhibitor’s function, biochemical characterization of the mutants of these conserved residues was expected to garner insights into the molecular mechanism of the function of p18. By selectively mutating conserved residues of p18 that span the entire molecule, we hoped to attain a clearer identification of functional modules of the inhibitor.

To achieve this end, we produced 11 mutants of p18, each (except for one) with a mutation of a single conserved residue corresponding to that found in p16 of cancer tissues. Biochemical characterization of the eleven mutants revealed that the third ankyrin repeat of p18, along with the NH\(_2\)-terminal portion of the fourth ankyrin repeat, is critical for the biochemical activity of p18. Some mutants displayed differential binding affinities toward CDK4 and CDK6, suggesting a possible mode by which p18 discriminates between the two G\(_1\)-specific CDK molecules.

MATERIALS AND METHODS

Cell Culture. Both U-2OS and SaOS cells (human osteosarcoma cell lines) were maintained in DMEM supplemented with 10\% fetal bovine serum. Cells were cultured in a 37°C incubator with 5\% CO\(_2\).

**Mutagenesis and Purification of Mutant Proteins.** The full coding sequence of p18 (8) was cloned into pALTER mutagenesis vector (Promega), and the oligonucleotide-directed mutagenesis was achieved according to recom-
mended protocol (Promega). Mutants were confirmed by sequencing (United States Biochemical). Confirmed mutant p18 clones were then subcloned into pGEX-KG vector (29), and the resulting pGEX-KG-p18 (or mutant) plasmid was transformed into BL21 strain of Escherichia coli. These transformed cells were cultured and used for purification of the GST-tagged p18 protein using glutathione-agarose affinity resin as detailed by Guan and Dixon (29).

**In Vitro CDK6 Activity Assay.** The assays used to determine the kinase activity of CDK4 and CDK6 were identical except for the use of the appropriate kinase subunit. The activity assay will be described for CDK6. Recombinant CDK6 and cyclin D2 were expressed as NH2-terminal GST fusion proteins and purified by a method identical to that described above for GST-p18. For use as substrate, the COOH-terminal 137 amino acid residues of pRb was also expressed as GST-fusion protein and purified as described above. For each kinase reaction, 3 mCi of [γ-32P]ATP, 0.95 μM ATP, and 10 mM DTT were mixed with 0.2 μg of GST-CDK6 and 0.2 μg of GST-cyclin D2 in the presence of 1 mM ATP. This activation was allowed to proceed for 1 h at room temperature. After incubation, 5% of (v/v) glutathione agarose beads per kinase reaction were used to affinity purify the activated GST-CDK6/GST-cyclin D2 complex. The activated kinase complex was eluted in 10 μl of kinase buffer (50 mM HEPES (pH 7.0) containing 10 mM MgCl2, 5 mM MnCl2, 1 mM DTT, and 0.1 mM orthovanadate in 5 mM HEPES (pH 7.4), supplemented with 5 μg/ml leupeptin, 3.5 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM DTT) was mixed with 0.2 μg of GST-CDK6 and 0.2 μg of GST-cyclin D2 in the presence of 1 mM ATP. This activation was allowed to proceed for 1 h at room temperature. After incubation, 5% of (v/v) glutathione agarose beads per kinase reaction were used to affinity purify the activated GST-CDK6/GST-cyclin D2 complex. The activated kinase complex was eluted in 10 μl of kinase buffer (50 mM HEPES (pH 7.0) containing 10 mM MgCl2, 5 mM MnCl2, 1 mM DTT, and 0.1 mM ATP) containing 10 mM glutathione. The eluent containing the activated GST (or GST-p18 or mutant) concentration was defined to be 100% activity. The percentage of activity remaining at each inhibitor (p18 (or mutant)) concentration was set to IC50, IC50 s for the wild-type and mutant p18, mutations at the central cluster of residues in the third ankyrin repeat had the most significant effect on the activity of CDK6.

**Determination of Binding Constants.** The only mutant deviating from this trend was Q93W, which is the only

**Cyclin D2 Displacement Assay.** GST-cyclin D2 (10 ng) was incubated with 5 μl of in vitro translated CDK6 in the presence of varying concentrations of untagged, purified p18 (wild-type, A61T, or D76N). The mixture was incubated at 30°C for 30 min and then immunoprecipitated using anti-cyclin D1 antiserum that is known to cross-react with cyclin D2. The immunoprecipitated complex was extensively washed with TNE buffer (10 mM Tris (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1% NP-40, and 50 mM NaF) and solubilized with 40 μl of SDS-PAGE sample buffer. Half of this sample was resolved on SDS-polyacrylamide gel and analyzed by double-gated FACS analysis.

**Cell Cycle Analysis.** Full length coding sequences of p18, A61T, D76N, Q93W, and D100Y were subcloned into pcDNA3 vector (Invitrogen), behind the CMV promoter. pcMV-CD20 was a generous gift from Dr. E. Harlow (30). U-2OS cells grown to log phase were seeded at a density of 5 × 105 cells per 1 cm tissue culture dish 1 day prior to transfection. Plasmids encoding wild-type or mutant p18 was cotransfected into the cells with pcDNA3-CD20 using Lipofectamine (Life Technologies, Inc.). After a 48-h incubation at 37°C, cells were washed with PBS and detached using PBS containing 0.1% EDTA (v/v). Detached cells were then incubated with FITC-conjugated anti-CD20 antibody for immunofluorescence staining of transfected cells. Cells were then fixed and stained with propidium iodide for nuclear staining. Red fluorescence, as a measure of DNA content, and green fluorescence, as a measure of CD20 expression level, were analyzed by double-gated FACS analysis.

**RESULTS**

**In Vitro CDK6 Activity Assays.** To determine the effect of each mutation on the ability of p18 to inhibit CDK6, we performed an in vitro CDK6 activity assay in the presence of varying concentrations of the GST fusion protein with wild-type or mutant p18. Although mutations at G16, Q93, and A110 minimally affected the CDK6 inhibitory function of p18, mutations at the central cluster of residues in the third ankyrin repeat (D76, R79, L89, and D100) drastically reduced the ability of p18 ability to inhibit CDK6 (Figs. 1 and 2b). For example, D76N did not significantly inhibit CDK6 activity, even at 1000 ng, whereas 30 ng of wild type p18 almost completely inhibited CDK6 activity. For a more quantitative measure of the loss of inhibition, IC50s for the wild-type and mutant p18 were determined using PhosphorImager-derived quantification of the intensities of the phosphorylated Rb bands (Table 1). It is clear from comparison of the IC50s that mutations of residues D76, R79, L89, or D100 caused the most severe reduction (>50-fold) in the potency of p18 as CDK6 inhibitor. These results suggest that the residues in the third ankyrin repeat are essential for the ability of p18 to inhibit CDK6 activity.
mutations would differentially affect the ability of p18 to inhibit CDK4 or CDK6. The inhibition observed in all mutants is due to gross structural changes. Therefore, it is unlikely that the loss of inhibitory function of the mutant tested that involved a nonconserved residue (Q93 is glycine in both p16 and p19). It is also noteworthy that mutations of some of the residues that flank the third ankyrin repeat also caused substantial reduction in the ability of p18 ability to inhibit CDK6. A61T, P106L, and V118D were 11-, 16-, and 11-fold less potent than wild-type p18 as CDK6 inhibitor (Figs. 1 and 2; Table 1).

In Vitro CDK4 Activity Assays. It has been shown previously that p18 interacts more strongly with CDK6 than with CDK4 in vivo (8). Therefore, we were interested in determining whether the various mutations would differentially affect the ability of p18 to inhibit CDK4 and CDK6. An in vitro activity assay similar to that for CDK6 was performed using CDK4. Results (Fig. 2; Table 1) indicate that each of the mutants exhibited similar levels of inhibition toward both CDK4 and CDK6. This suggests that common structural elements of p18 are required for inhibition of both CDK4 and CDK6.

In Vitro Binding Assays. The loss of inhibitory function of the various mutants could be due to one or both of two factors. The mutated residue may be critically involved in the inhibition itself or the mutation may prevent proper binding of p18 to the CDK molecule. To distinguish between these two possibilities, the ability of each mutant to bind to CDK4 or CDK6 was tested by in vitro binding assay. The most striking result of the in vitro binding experiments was that two of the mutants, D76N and D100Y, did not bind to CDK6 at all (Fig. 3; Table 1). Mutation of other residues within the third ankyrin repeat (G81, L89, and Q93) did not affect CDK4 binding, in contrast to the results for CDK6. This suggests that p18 may use both common and distinct attributes of the third ankyrin repeat to specifically bind to CDK4 or CDK6. Still more interesting is the observation that D76N retained CDK4 binding ability, yet was completely inactive as an inhibitor. This observation suggests that aspartate 76 has a critical role in CDK inhibition.

Saturation Binding. Although activated holoenzyme CDK-cyclin D2 complex was used for assessing the ability of the mutants to inhibit the RB kinase activity of CDK4 or CDK6, the in vitro binding assays were performed in the absence of cyclin D2. Saturation binding experiments were performed to corroborate the in vitro binding data in the context of holoenzymes.

The concentration of cold, untagged p18 that caused the dissociation of 50% of the labeled wild-type p18 from the CDK6-cyclin D2-p18 complex, which is an estimate of the apparent $K_{D}$, was determined to be 55 nM. The apparent $K_{D}$ for A61T binding to the CDK6-cyclin D2 complex was 250 nM (Fig. 4). The same analysis was carried out using D76N but did not yield useful data because the labeled D76N did not bind to CDK6 or CDK6-cyclin D2 complex at a detectable level (data not shown). The lack of binding of labeled D76N to CDK6-cyclin D2 complex is completely consistent with the results of the in vitro binding experiment described above. The same experiments were attempted using GST-CDK4-GST-cyclin D2 complex. Whereas CDK6-D2 complex precipitated an easily detectable and quantifiable level of labeled p18 and A61T, the CDK4-D2 complex precipitated much less labeled p18 (data not shown). The amount of labeled p18 that was precipitated with CDK4 was so low that reliable PhosphorImager quantitation could not be obtained. Again, this observation is entirely consistent with the previously reported in vivo data that p18 preferentially forms a complex with CDK6.

Cyclin D2 Displacement Assay. Addition of free p16 to preformed CDK4-cyclin D1 complex or to a mixture of CDK4 and cyclin...
Table 1  Inhibitory and binding capacity of the wild-type p18 and mutants toward CDK4 or CDK6

<table>
<thead>
<tr>
<th>CDK4</th>
<th>CDK6</th>
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<tr>
<td>IC_{50} (nM)</td>
<td>Binding</td>
</tr>
<tr>
<td>p18</td>
<td>10</td>
</tr>
<tr>
<td>G15D</td>
<td>30</td>
</tr>
<tr>
<td>A61T</td>
<td>100</td>
</tr>
<tr>
<td>D76N</td>
<td>&gt;1000</td>
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<tr>
<td>R79P</td>
<td>&gt;1000</td>
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<tr>
<td>G81S</td>
<td>20</td>
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<tr>
<td>L89P</td>
<td>50</td>
</tr>
<tr>
<td>Q93W</td>
<td>50</td>
</tr>
<tr>
<td>D100Y</td>
<td>&gt;1000</td>
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<td>P106L</td>
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<td>A110S</td>
<td>20</td>
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<td>V118D</td>
<td>60</td>
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*IC_{50}s were derived from the quantification of intensity of phosphorylated Rb bands relative to concentration of inhibitor (Fig. 2). Scores for binding ability of the wild-type or mutant p18 are derived from the intensity of 35S-labeled CDK4 or CDK6 band (Fig. 3). +++, 90-100% of wild type; +, 50-89% of wild type; +, 10-49% of wild type; −, 0-9% of wild type. Identities of the inhibitors are shown in the far left column.

D1 was shown to disrupt CDK4-cyclin D1 complex formation, concomitant with the accumulation of CDK4-p16 complex (32, 33). To assess whether p18 also acts by displacing cyclin D from CDK-cyclin D complex, cyclin D2 displacement assay was performed (Fig. 5). Our data indicate that addition of wild-type p18 causes efficient dissociation of cyclin D2 from CDK6 (Fig. 5). D76N, which does not bind D-type CDK activity, the same vectors were transfected into SaOS cells, to assess whether p18 also acts by displacing cyclin D from CDK-cyclin D complex, cyclin D2 displacement assay was performed. The bands resulting complex was immunoprecipitated using anti-cyclin D1 antibody and resolved on SDS-PAGE for visualization and quantitation allowing a similar level of G1 arrest as the wild-type p18. The same argument holds for the G1 arrest observed in Q93W-transfected cells. D76N, on the other hand, cannot bind to CDK6 (although it can bind to CDK4) and does not inhibit either of the CDKs. Overexpression of D76N and also D100Y, as predicted from the in vitro data, does not cause cells to arrest at G1.

Growth Inhibition. It has been shown previously that overexpression of wild-type p18 inhibits cell growth and proliferation in an Rb-dependent manner (8). To further demonstrate that the growth suppression of U-2OS cells by p18 is due to its ability to inhibit D-type CDK kinase activity, the effects of the various mutations on the ability of p18 to inhibit cell growth were determined by using the colony formation assay (31). The plasmid constructs (pcDNA3) containing wild-type p18, antisense p18, A61T, D76N, Q93W, or D100Y were transfected individually into U-2OS cells. The transfected cells were grown in medium supplemented with G418 to select for resistant colonies. Number of colonies forming in this selective media for each transfection sample was counted and compared with the number of colonies forming in a transfection sample with vector alone, as described previously (31). The data indicate that overexpression of wild-type p18 and the mutants is, thus, specific and dependent on functional Rb.

The effects of the various mutants on cell cycle are consistent with the in vitro binding and inhibition data. A61T binds well to both CDK4 and CDK6 but is a less potent inhibitor of both CDK6 and CDK4 relative to wild-type p18 (~10-fold, Table 1). In the transfection system, A61T is overexpressed. The increased concentration of the mutant inhibitor most likely compensated for the loss of potency, allowing a similar level of G1 arrest as the wild-type p18. The same argument holds for the G1 arrest observed in Q93W-transfected cells. D76N, on the other hand, cannot bind to CDK6 (although it can bind to CDK4) and does not inhibit either of the CDKs. Overexpression of D76N and also D100Y, as predicted from the in vitro data, does not cause cells to arrest at G1.
pared to the number of colonies formed on plates of cells transfected with pcDNA3 itself (Table 3). Three independent experiments were performed. Transfection with vector alone produced an average of 37 neomycin-resistant colonies. In contrast, an average of only 4 neomycin-resistant colonies were formed on plates of wild-type p18-transfected cells, confirming that p18 overexpression inhibits the growth and proliferation of the transfected cells. This inhibition is specific for functional p18 expression because plates of cells transfected with antisense p18 formed an average of 39 colonies, similar to the result of transfection by vector alone. Transfection with A61T, which binds well to both CDK4 and CDK6 and retains some inhibitory capacity toward CDK4 and CDK6, caused a reduction in the number of colonies to 16. Although not quite as complete as that with the wild-type p18, A61T did significantly inhibit cell growth and proliferation. Similar results were obtained with Q93W, which binds much less tightly to the CDK molecules than does p18 but retains at least 10% of the inhibitory capacity toward both CDK4 and CDK6. Transfection with D100Y, on the other hand, resulted in similar number of resistant colonies as the vector control. This result further supports the idea that the growth suppression is probably due to the ability of p18 to curtail CDK4/CDK6 activities because D100Y neither binds nor inhibits either of the CDK molecules. Expression of D76N, the mutant that has

Fig. 6. FACS analysis profiles. Cells transfected with the expression vector containing wild-type p18 or mutant coding sequence (mutant as indicated on top of each panel) were analyzed by two parameter FACS analysis. Histograms represent the DNA content of CD20-positive U-2OS cells.
Table 2  FACS analysis summary

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>S</th>
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<tbody>
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<td>44</td>
<td>31</td>
</tr>
<tr>
<td>p18</td>
<td>47</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>A61T</td>
<td>51</td>
<td>29</td>
<td>36</td>
</tr>
<tr>
<td>D76N</td>
<td>29</td>
<td>36</td>
<td>35</td>
</tr>
<tr>
<td>Q93W</td>
<td>48</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>D100Y</td>
<td>16</td>
<td>33</td>
<td>51</td>
</tr>
</tbody>
</table>

Expression vector (pcDNA3), either empty or containing p18 or mutant, as designated in the first column, was transfected into U-2OS cells. Double-parameter FACS analysis was performed. The numbers in each column represent the percentages of transfection-positive cells (CD20-positive) residing in the indicated stage of cell cycle, as determined by DNA content.

DISCUSSION

Although the sequences of INK4 proteins are only ~30% identical, these molecules share the feature that they consist almost exclusively of tandem ankyrin repeats (34). The three-dimensional structures of p18 and p19 were recently solved by X-ray crystallography and nuclear magnetic resonance, respectively (35, 36). p18 and p19, as expected, appear to be similar in overall structure. In p18, each ankyrin repeat folds into an NH2-terminal -helix (35). P106 occurs at position 5 of ankyrin repeat to allow the sharp bend between the two -helices of repeats 1 and 3, respectively (35). Although mutation at this proline to leucine slightly impaired CDK6 binding and reduced CDK inhibition by at least 10-fold (Table 1; Fig. 2). The importance of proper alignment of the fourth ankyrin repeat with the third repeat is reemphasized by the data for D100Y. D100 occurs at position 32 of repeat 3. This aspartate residue is thought to be involved in a hydrogen-bonding interaction with the second residue of the loop that connects the third and fourth repeat (35). Mutation of this residue to tyrosine abolished all binding and inhibition of p18 to either CDK4 or CDK6. Stabilization of the tight turn linking the third and fourth ankyrin repeats, therefore, seems to be essential for the ability of p18 ability to function as CDK inhibitor. D76 and R79 are solvent exposed residues that occur on the helices of the third repeat that are strictly conserved among the INK4 proteins. It has been speculated that these solvent-exposed residues may be involved in interaction with CDK molecules (35). Mutations of D76 or R79 abolished the CDK inhibitory activity of p18 and also significantly impaired CDK6 and CDK4 binding (Table 1). It seems, therefore, that the solvent exposed, conserved residues of the helices in the third ankyrin repeat of p18 are critical for the ability of p18 to bind and inhibit the CDK4 and CDK6. Additionally, the ability of D76N to bind well to CDK4 but not CDK6 (Fig. 3) suggests that p18 may be using different portions of the solvent-exposed face of the -helices in the third ankyrin repeat to interact differentially with CDK4 and CDK6.

Saturation binding experiments, which involved binding of p18 to CDK6-cyclin D2 complex, yielded data that are consistent with the results of the in vitro binding experiments, in which p18 bound to free CDK6 subunit. Therefore, p18 most likely uses the same interactions to bind to the free and the complexed forms of CDK6. This conclusion is further supported by our observation that p18 competes with cyclin D2 for formation of a binary complex (Fig. 5) and that the ability of p18 mutants to displace cyclin D2 from CDK6 correlates with their CDK6 binding affinity (Figs. 3 and 4). These data suggest that p18 likely inhibits CDK6 by binding to the kinase subunit to the exclusion of cyclin D2, thus preventing activation of the kinase. Additionally, the saturation binding experiments also confirmed the preferential interaction of p18 with CDK6-cyclin D relative to the CDK4-cyclin D complex.

The in vitro binding and CDK inhibition data reported in this study agree with previously reported biochemical characterization of analogous p16 mutants. Yang et al. (22), for example, reported that G101W (corresponding to Q93W) exhibited reduced binding to CDK4 and was 50–100-fold less potent than wild-type p16 in CDK4 inhibition (22). Substitution of H83 (the residue adjacent to what corresponds to D76 in p18) with tyrosine also reduced binding and completely abolished the ability of p16 to inhibit CDK4 (22). P114L mutant, which corresponds to the P106L mutant of p18 (23), was at least 10-fold less potent than wild-type p16 in CDK4 inhibition (22). P114L mutant, which corresponds to the P106L mutant of p18 (23), was at least 10-fold less potent than wild-type p16 as CDK4 inhibitor in the hands of Koh et al. (23). Lukas et al. (13) found that P114L mutant did not bind to either CDK4 or CDK6. The in vivo and in vitro characterization of D84N, R87P, and H98P, which correspond to D76N, R79P, and H89P (residue just pre-
ceding L90P in p18, were also consistent with our data (23). These corroborating data support the idea that the INK4 family likely share structural features.

The effects of the mutations on the physiological function of p18, namely, to cause G1 arrest and stop cell growth, were tested using colony formation assay and FACS analysis of transiently transfected cells. By and large, the mutant p18 molecules affected cell cycle distribution and growth properties of U-2OS cells in manner consistent with their biochemical properties. Q93W, the inhibitory and binding capacities of which were very minimally affected, caused accumulation of U-2OS cells at G1 levels comparable to the wild-type p18. The CDK4/6-inhibitory activity of A61T but not its binding was adversely affected, and it caused the G1 accumulation of U-2OS cells at a level comparable to wild-type p18. Both A61T and Q93W also inhibited the formation of G418-resistant colonies in the growth assay, although not as effectively as the wild-type p18. As expected, D100Y, which neither binds nor inhibits the two CDK molecules, did not cause G1 arrest and did not inhibit the formation of G418-resistant colonies. It is interesting to note that D100Y transfection, in fact, caused a slight accumulation of cells in G2 and also a mild increase in the number of neomycin-resistant colonies. D100Y may, therefore, possibly be acting as a weak dominant interfering mutant. D76N, on the other hand, yielded some confounding results. Consistent with its inability to inhibit either of the CDK molecules in \textit{vitro}, D76N did not cause accumulation of the transfected U-2OS cells in G1 (Table 2). When its ability to inhibit cell proliferation was assessed by the colony formation assay, however, D76N was able to partially inhibit colony formation, at the same level as A61T and Q93W. Although D76N can no longer inhibit CDK4 or CDK6, the mutant still binds well to CDK4. This residual binding activity, in the setting of overexpression, may be enough to interfere with active CDK4-cyclin D complex formation, thereby inhibiting cell growth.

In summary, the biochemical properties of the p18 mutants, when analyzed in light of the recently reported three-dimensional structure of p18, suggest that the proper folding of the third ankyrin repeat and the proper alignment of the fourth repeat relative to the third repeat are essential for the ability of p18 to inhibit CDK activity. Mere binding of p18 to CDK, however, does not seem to require such extensive contacts, because the mutation of the second and fourth ankyrin repeat determinants do not affect the ability of p18 to bind to CDK4 or CDK6. The finding that mutation of one of the solvent exposed residues of the first helix in third ankyrin repeat abolished the ability of p18 ability to bind to CDK6 but did not affect CDK4 binding further suggested that p18 may be using slightly different binding elements of the third repeat to discriminate between the two CDK molecules.

ACKNOWLEDGMENTS

We thank Dr. E. Harlow for the pCMV-CD20 plasmid, Dr. E. R. Butch for critical reading of the manuscript, E. Tang for suggestions, and Dr. M. G. Spigarelli for invaluable help in the preparation of this manuscript.

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