Critical Role for the 3_10 Helix Region of p57\textsuperscript{Kip2} in Cyclin-dependent Kinase 2 Inhibition and Growth Suppression*

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Although crystal structural analysis of cyclin A/cyclin-dependent kinase 2 (Cdk2)/p27 (Russo, A. A., Jeffrey, P. D., Pattern, A. K., Massague, J., and Pavletich, N. P. (1996) Nature 382, 325–331) has suggested that the 3_10 helix region in Cdk inhibitors of the Cip/Kip family may be involved in the inhibition of cyclin/Cdk activities, there is no biochemical evidence supporting this hypothesis. In the present study, we demonstrated that cyclin and Cdk binding domains of p57 were necessary but were not sufficient in themselves for the inhibition of cyclin A/Cdk2 and cyclin E/Cdk2, and that the 3_10 helix region of this protein is indispensable for the inhibition of these complexes. In contrast, the 3_10 helix regions of p21 and p27 were not required, and cyclin- and Cdk-binding domains alone were sufficient for the inhibition of all cyclin/Cdk complexes examined. Site-directed mutagenesis identified phenylalanine 79 and tyrosine 80 within the 3_10 helix region of p57 as crucial residues for kinase inhibition, supporting the structural evidence that the 3_10 helix binds deep inside the catalytic cleft of Cdk2, mimicking ATP. Mutations within the 3_10 helix region of the p57 molecule completely abolished the ability to arrest the cell cycle at G\textsubscript{1} in vivo. These results indicate that this region is specifically utilized by p57 in selectively inhibiting cyclin A or E/Cdk2 activities. Thus the 3_10 helix motif may confer a specific regulatory mechanism by which p57 differentially regulates Cdk2 and Cdk4 activities.

Cyclin-dependent kinases (Cdks)\textsuperscript{1} are key regulators of cell cycle progression in eukaryotic cells (1). The activities of Cdks are regulated through a number of mechanisms, including phosphorylation of a conserved threonine residue by Cdk-activating kinase (CAK, cyclin H/Cdk 7) (2). In addition, individual Cdks are temporally activated by their cognate cyclins, whose expression is induced in certain situations (3). The well-characterized cyclins are classified into four types, cyclin D, E, A, and B, which are required in the G\textsubscript{1}, G\textsubscript{1}/S, S, and G\textsubscript{2}/M phases of the cell cycle, respectively.

Another mechanism of regulation has emerged with the discovery of the Cdk inhibitors (cyclin-dependent kinase inhibitors, CKIs) (4, 5). Two distinct families of CKIs have been described in mammalian cells. The Cip/Kip family includes the structurally related proteins, p21, p27, and p57, all of which inhibit a variety of Cdk activities (6–13). CKIs of this family share significant homology in their N-terminal region, suggesting that they inhibit cyclin/Cdk activity in a similar manner. The INK4 family, including p15, p16, p18, and p19, is not structurally related to the p21 family, but shares characteristic 4-fold ankyrin repeats and specifically inhibits cyclin D/Cdk4 and cyclin D/Cdk6 kinases (14–18).

Recent reports revealed that several regions within CKI molecules of the Cip/Kip family, including a cyclin binding site(s), a Cdk binding region, and a 3_10 helix, are involved in the inhibition of cyclin/Cdk activities. On the basis of the crystal structure of cyclin A/Cdk2/p27, the 3_10 helix region has been proposed to insert itself within the catalytic cleft of Cdk2, mimicking ATP (19). Although this region is well conserved among CKI molecules of the Cip/Kip family, the actual contribution of the 3_10 helix region to Cdk inhibition remains to be determined.

To address this important question, we generated mutants in the 3_10 helix region of CKIs of the Cip/Kip family and tested their abilities to inhibit cyclin/Cdk activities. Using these mutants, we demonstrate here that the 3_10 helix is specifically utilized by p57, but not by p21 or p27, for the inhibition of cyclin A/Cdk2 and cyclin E/Cdk2, and is crucial for its growth suppressing function in vivo.

MATERIALS AND METHODS

Plasmids and Baculoviruses—Baculoviruses expressing cyclins A, D1, and E were kindly provided by Dr. D. Morgan. pGEX-wild-type p57 was generated by PCR with a 5\textsuperscript{'}-primer (GAAT-CCC-CCG-GCG-GTC-GTT-GAG-GAA-GTC-CCC) and a 3\textsuperscript{'}-primer (TCC-GAA-TTC-CTC-TGC-GGC-CCC-TGC-GTC-CCC-CGC-CCC-GCG) using PCR-CMV-mouse p57 (courtesy of Dr. S. Elledge) as a template. p57 mutants Δ18–23 and W50G were generated by PCR with the common 5\textsuperscript{'}-primer (G-TG-GA-GCT-CAT-CC-TCC-CTG-GAT-GAG-GCC) and a 3\textsuperscript{'}-primer described in our previous report (20) and a set of inner primers as follows: CGT-AGC-AGC-CCC-GGC-GCG-CCC-GAA-GGC-GTG-CGA-CAC-CCG (for Δ18–23) and CAG-GAC-TTC-AGC-GGC-GTC-TTC-GGC-TTC-TCT-GGC (for W50G). C-terminally truncated mutants of p57 (Δ75–335, Δ85–335, Δ105–335, and Δ165–335) were also generated by PCR with the pGEX 5\textsuperscript{'}-primer and specific 3\textsuperscript{'}-primers: GGA-TTC-CTC-GAG-CTA-AGC-GTA-ATC-TGG-GTA-GTC-GCC-GTC-GGC (for Δ75–335), GTC-GAA-TTC-ATG-ATC-GTA-CTG-GTA-GTC-GATT-GCT-CAC-CTC (for Δ85–335), GTC-GAA-TTC-CTA-AGC-GTA-ATC-TGG-GTA-GTC-GCC-GTC-GGC (for Δ105–335), and GTC-GAA-TTC-CTA-AGC-GTA-ATC-TGG-GTA-GTC-GCC-GTC-GGC (for Δ165–335).

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The abbreviations used are: Cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; PCR, polymerase chain reaction; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; CMV, cytomegalovirus; PMSF, phenylmethylsulfonyl fluoride.

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105–335, and 165–335 were also generated by PCR with the pGEX 5\textsuperscript{'}-primer and specific 3\textsuperscript{'}-primers: GGA-TTC-CTC-GAG-CTA-AGC-GTA-ATC-TGG-GTA-GTC-GCC-GTC-GGC (for Δ75–335), GTC-GAA-TTC-ATG-ATC-GTA-CTG-GTA-GTC-GCC-GTC-GGC (for Δ85–335), GTC-GAA-TTC-CTA-AGC-GTA-ATC-TGG-GTA-GTC-GCC-GTC-GGC (for Δ105–335), and GTC-GAA-TTC-CTA-AGC-GTA-ATC-TGG-GTA-GTC-GCC-GTC-GGC (for Δ165–335).
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GTA-GGA-GGT-GCC-GAC-CAG-GTT-CAC-CTC (for \(\Delta 165\)–335), respectively.

The construction of a C-terminally truncated p21 mutant (\(\Delta 162\)–164) was described in our previous report (20). pGEX-p27 wild type was generated by PCR with a 5\prime primer (GGA-TCC-TCT-CTG-GTA-ATC-TGC-G-TAC-GTA-GTC-GCC-GAC-CAG-GTT-CAC-CTC) and a 3\prime primer (GAA-TTC-GCT-GTG-GCA-TCA-GGA-CTG-GTC-GGC-GAA). Two 5\prime primers were used to insert between the GST domain and p21 or p57, the same seven-amino acid linker as that used previously for the construction of GST-p21 (20). This linker increased the ability of CKIs of the GST-Cip/Kip family to inhibit cyclin/Cdk activities. A C-terminally truncated p2\textsubscript{7} (SA3–198) was generated by PCR with the common 5\prime primer and a 3\prime primer: GAA-TTC-GCT-GTG-GCA-TCA-GGA-CTG-GTC-GGC-GAA.

A eukaryotic expression construct (pcDNA3-p57) was generated by removing a HindIII/BamHI fragment from pCMV-p57 and ligating the fragment into the pcDNA3 vector. pcDNA3-p57/HA was generated by PCR using pcDNA3-p57 as a template and a 5\prime primer (AG-AAG-GCA-ATG-GGT-GAG-ATT-CAG) and a 3\prime primer (GAG-TTC-CGC-GAC-CAG-GTT-CAC-CTC) and a 3\prime primer: GAA-TTC-GCT-GTG-GCA-TCA-GGA-CTG-GTC-GGC-GAA. pcDNA3-p57 mutants were generated using the same set of inner primers described above and the INVS\prime primer and a 3\prime primer: AGG-ACC-GTA-GGC-GTA-ATC-TGC-G-TAC-GTA-GTC-GCC-GAC-CAG-GTT-CAC-CTC.

Production of GST Fusion Proteins—Overnight cultures of Escherichia coli transformed with plasmids encoding the GST fusion proteins were diluted 10-fold with fresh medium and were further cultured for 2 h at 37 \textdegree C. Recombinant proteins were then induced with 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside for 2 h at 37 \textdegree C. Cells were harvested and lysed by sonication in NETN150 buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and a mixture of protease inhibitors: 20 \mu g/ml soybean trypsin inhibitor, 2 \mu g/ml aprotinin, and 100 \mu g/ml phenylmethylsulfonyl fluoride (PMSF)). Recombinant proteins were adsorbed to glutathione-Sepharose beads (Pharmacia Biotech Inc.) and were washed with NETN100 (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and then with 50 mM HEPES, pH 8.0. Purified proteins were eluted in 50 mM HEPES, pH 8.0, containing 5 mM reduced glutathione (Sigma) and were dialyzed against 50 mM HEPES, pH 8.0. Purified proteins were quantitated by densitometric analysis of the bands after SDS-PAGE using bovine serum albumin as a standard.

In Vitro Binding Assay—Spodoptera frugiperda (SF9) cells infected with recombinant baculovirus vectors encoding cyclin or Cdk for 48 h were harvested in EBC buffer containing 50 mM Tris, pH 8.0, 120 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 2.5 mM dithiothreitol, 0.5% Nonidet P-40, and mixtures of protease and phosphatase inhibitors (1 mM NaF, 10 \mu M \(\beta\)-glycerophosphate, 0.1 mM Na\textsubscript{3}VO\textsubscript{4}). 5\mu l of lysates were diluted with 0.3 ml of EBC buffer and were mixed with 0.1 \mu g of GST or GST-fused p57 proteins. After 1 h of incubation at 4 \textdegree C, the complexes were adsorbed to glutathione beads and were washed three times with EBC buffer. Bound proteins were then analyzed by SDS-PAGE followed by Western blotting.

Kinase Assay—SF9 cell lysates containing cyclin/Cdk complexes were mixed with purified, soluble GST or GST-p57 fusion proteins in 50 mM HEPES, pH 8.0, and were incubated for 1 h at 4 \textdegree C. Kinase activity was determined at 30 \textdegree C for 10–30 min in a 30-\mu l reaction mixture containing 50 mM HEPES, pH 8.0, 10 mM MgCl\textsubscript{2}, 2.5 mM EGTA, 1 mM dithiothreitol, 10 \mu M \(\beta\)-glycerophosphate, 1 mM NaF, 0.1 mM Na\textsubscript{3}VO\textsubscript{4}, 0.1 \mu M PMSF, 10 \mu M ATP, and 185 kBq of [\(\gamma\text{-32P}\)]ATP (222 TBq/mM; NEC Life Science Products). Either 0.2 \mu g of soluble GST-p57 or 1 \mu g of histone H1 (Boehringer Mannheim) was used as a substrate. The reaction products were separated on SDS-PAGE, and phosphorylated proteins were detected by autoradiography and quantitated using a Fuji imaging analyzer BAS-1500.

For the measurement of kinase activity of active cyclin A/Cdk2/p57 complexes, 1 \mu l of cyclin A/Cdk2 complexes expressed in SF9 was mixed with increasing amounts of GST-p57 fusion proteins in IP kinase buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, and 10% glycerol, 0.1% NP-40, 2 \mu g/ml aprotinin, 0.1 \mu M PMSF, 1 mM NaF, 0.1 mM Na\textsubscript{3}VO\textsubscript{4}, and 10 \mu M \(\beta\)-glycero-phosphate) and incubated for 1 h at 4 \textdegree C. The resultant cyclin A/Cdk2/p57 complexes were adsorbed to glutathione-Sepharose beads and washed three times with IP kinase buffer. Beads were then used for the kinase assay as described above.

Transfection and Fluorescence-activated Cell Sorter Analysis—MDA041 cells (1 \times 10\textsuperscript{6} cells/10-cm dish) were cotransfected with 5 \mu g of pcDNA3 CS and 10 \mu g of pcDNA3 vector-expressing wild-type p57HA or its mutants, using the calcium precipitation method. The total amount of DNA was adjusted to 25 \mu g/10-cm dish by adding the pcDNA3 empty vector. Forty-eight hours after the transfection, cells were immunostained with anti-CDS antibody, counterstained with propidium iodide, and analyzed by flow cytometry to determine the DNA content of CDS\textsuperscript{+} (transfected) and CDS\textsuperscript{−} (not transfected) cells using a fluorescence-activated cell sorter Calibur (Becton-Dickinson).

Western Blot Analysis—MDA041 cells were cotransfected with pCMV-\(\beta\)-galactosidase and 10 \mu g of pDNAS vectors expressing wild-type p57HA or its mutants. Forty-eight hours after the transfection, cells were lysed in EBC buffer, and \(\beta\)-galactosidase activities in the lysates were determined to normalize the transfection efficiency. The lysates that possessed equivalent \(\beta\)-galactosidase activity were separated by SDS-PAGE, and the exogenously expressed p57HA proteins were detected by anti-HA antibody at 1:1000 (12CA5, Boehringer Mannheim). The precipitated complexes were washed four times in EBC buffer, separated by SDS-PAGE, and p57HA proteins were detected using anti-HA antibody.

RESULTS

Cyclin and Cdk Binding Domains in p57—It has recently been reported that CKIs of the Cip/Kip family have contiguous cyclin- and Cdk-binding domains in their N-terminal regions (19, 20, 22–25). In the case of p21, the sites responsible for cyclin and Cdk binding are within the regions of amino acids 17–24 (CB1/146–164 (CB2), and 49–71 (KB), respectively (23). Although CB1 and KB appear to be well conserved among members of the Cip/Kip family (Fig. 1), it remains to be determined whether p57 binds cyclins or Cdns in the same manner as does p21. To address this question, we introduced a deletion or a mutation in either one of the two domains and examined their binding properties.

As shown in Fig. 2, wild-type p57 effectively bound all cyclins tested in a dose-dependent manner, whereas \(\Delta 18\)–23 was completely defective in cyclin binding even at the highest amount.
In terms of Cdk binding, wild-type p57 bound to Cdk2, but not to Cdk4, in a dose-dependent manner. Substitution of glycine for tryptophan at amino acid 50 in the p57 molecule completely abolished Cdk2 binding, while W50G retained the ability to bind all cyclins (Fig. 2). These results indicate that domains critical for cyclin and Cdk binding are well conserved between p21 and p57. Although two sites, CB1 and CB2, have been reported to be involved in p21 binding to cyclins, our data suggest that in case of p57, the N-terminal site, which corresponds to the CB1 motif of p21, plays a major role in cyclin binding.

Both Cyclin and Cdk Binding Domains of p57 Are Indispensable for the Inhibition of Cyclin/Cdk Activities—To clarify the role of the cyclin and/or Cdk binding domains of p57 in the inhibition of cyclin/Cdk activities, we generated GST-fused wild-type p57 and its mutants defective in either cyclin (Δ18–23) or Cdk (W50G) binding, and tested their abilities to inhibit various cyclin/Cdk activities. As shown in Fig. 3, wild-type p57 effectively inhibited cyclin A/Cdk2, cyclin E/Cdk2, and cyclin D1/Cdk4, whereas both Δ18–23 and W50G were defective in kinase inhibitory activities. Thus, both cyclin and Cdk binding domains of p57 seem to be required for the inhibition of cyclin A/Cdk2, cyclin E/Cdk2, and cyclin D1/Cdk4 activities.

Cyclin and Cdk Binding Domains of p57 Are Not Sufficient for the Inhibition of Cyclin A/Cdk2 and Cyclin E/Cdk2—We next asked whether cyclin and Cdk binding domains of p57 are sufficient for the inhibition of cyclin/Cdk activities. To address this question, we generated C-terminally truncated mutants of p57 (Δ72–164; p57N) as well as mutants of p21 (Δ72–164; p21N) and p27 (Δ83–198; p27N), and tested their abilities to inhibit cyclin/Cdk activities. All of the C-terminally truncated mutants were as effective as the respective wild-type molecules in inhibiting cyclin D1/Cdk4 (Fig. 4) and cyclin D1/Cdk6 (data not shown). Interestingly, whereas both p21N and p27N inhibited cyclin A/Cdk2 activity to the same extent as wild-type p21 and p27, respectively, p57N completely and specifically lost the ability to inhibit cyclin A/Cdk2 (Fig. 4). Similar results were obtained with cyclin E/Cdk2 (data not shown). Based on the collective findings, it is suggested that although cyclin and Cdk binding domains are sufficient for the inhibition of Cdk2 and Cdk4 by p21 and p27, these two domains are necessary but not sufficient in themselves for the inhibition of cyclin A/Cdk2 or cyclin E/Cdk2 by p57, raising the possibility that an additional region(s) may be required.

The 310 Helix Region Is Indispensable for the Inhibition of Cyclin A/Cdk2 and Cyclin E/Cdk2 by p57—In an attempt to identify the region(s) within the p57 molecule that is required for the inhibition of cyclin A/Cdk2, we generated p57 mutants serially truncated from the C terminus, and tested their abilities to inhibit cyclin A/Cdk2 activity. As shown in Fig. 5A, Δ85–335 inhibited cyclin A/Cdk2 activity to the same extent as wild-type p57, whereas Δ75–335 (p57N) did not, suggesting that the region between amino acids 75 and 84 is important for the inhibitory function.

Recently, elucidation of the crystal structure of the cyclin
A/Cdk2/p27 complex has suggested that the $3_{10}$ helix of p27 (amino acids 85–90) binds to the N-terminal lobe of Cdk2 and is inserted deep within the catalytic cleft, mimicking ATP (19). This raises the possibility that this insertion may represent an alternative mechanism by which CKIs of the Cip/Kip family inhibit cyclin/Cdk activities. To delineate the functional role of the $3_{10}$ helix region of p57, we introduced mutations that were assumed to disrupt the ATP-like structure, and tested the mutant molecules for their kinase inhibitory activities. As shown in Fig. 5B, a mutant of p57 (F79A/Y80A), but not corresponding mutants of p21 or p27, showed a complete loss in the ability to inhibit cyclin A/Cdk2. Similar data were obtained with cyclin E/Cdk2 (data not shown). Thus, the $3_{10}$ helix region of p57 seems indispensable for the inhibition of cyclin A/Cdk2 and cyclin E/Cdk2.

To rule out the possibility that the complete loss of the F79A/Y80A mutant in the ability to inhibit cyclin A or E/Cdk2 activities was due to a defective binding to the kinase complexes, we determined the binding constants to cyclin A or E/Cdk2 complexes. Increasing amounts of GST-fused wild-type p57 and mutant proteins were incubated with cyclin A or E/Cdk2 expressed in insect cells, and the complexes were precipitated using glutathione-Sepharose beads. After separation on SDS-PAGE, p57-bound cyclin A or E were detected by Western blotting using anti-cyclin A or E antibodies and quantitated with a densitometer. $K_d$ values were determined by Scatchard analysis.

| p57 protein | Cyclin A/Cdk2 $K_d$ (nM) | Cyclin E/Cdk2 $K_d$ (nM) |
|------------|--------------------------|--------------------------|
| Wild-type  | 6.0                      | 3.5                      |
| $\Delta18$–$23$ | 53.3                     | 20.8                     |
| W50G       | 51.5                     | 15.2                     |
| p57N (1–74) | 18.6                     | 9.9                      |
| F79A/Y80A  | 17.9                     | 10.3                     |

Increasing amounts of GST-fused wild-type p57 and mutant proteins were incubated with cyclin A or E/Cdk2 complexes expressed in Sf9 cells. After precipitation with glutathione-Sepharose beads, the complexes were separated by SDS-PAGE, and the amounts of cyclin A or E in the complexes were detected by Western blotting using anti-cyclin A or E antibody and quantitated with a densitometer. $K_d$ values were determined by Scatchard analysis.

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Fig. 5. The $3_{10}$ helix region is required for the inhibition of cyclin A/Cdk2 by p57, but not by p21 or p27. A, increasing amounts of wild-type p57 (wt) and its mutants serially truncated from the C terminus were incubated with cyclin A/Cdk2 expressed in Sf9 cells, and kinase activity was determined using histone H1 (HH1) as a substrate. B, wild-type p21, p27, and p57 (wt) and the respective $3_{10}$ helix region mutants (L76A/Y77A, F79A/Y80A/Y89A, and F79A/Y89A) were examined for their abilities to inhibit cyclin A/Cdk2 kinase activity using histone H1 (HH1) as a substrate.
Inhibition in Vivo—To determine whether the $3_{10}$ helix region of p57 is important for biological function in vivo, we analyzed growth inhibitory activities of wild-type p57 and its mutants. MDAH041 cells were cotransfected with a marker vector, pcDNA3-CD8, and either pcDNA3-wild-type p57 or its mutants ($\Delta18$–$23$, W50G, or F79A/Y80A). 48 h after the transfection, cells were simultaneously analyzed for CD8 expression, as a marker for transfected cells, and DNA content by two-color flow cytometry. As shown in Fig. 7A, cells transfected with the CD8 expression vector alone showed a DNA content distribution typical of asynchronously growing cells, whether CD8$^+$ (transfected) or CD8$^-$ (untransfected), suggesting that the transfec- tion procedure per se had no effect on cell growth. It is also worth noting that in all transfections, CD8$^+$ cells had a distribution pattern identical with that of the control cells (Fig. 7A, upper panel), which allowed us to examine the specific effects of transfected genes on cell cycle regulation.

As shown in Fig. 7A, cells expressing wild-type p57 showed an increased proportion in the G$_1$ phase (79.3% in wild-type/CD8$^+$ versus 51.2% in wild-type/CD8$^-$ cells). $\Delta18$–$23$ and W50G, which lack an inhibitory capacity toward all the cyclin/Cdk combinations (Fig. 3), were defective in arresting cells at the G$_1$ phase. Interestingly, cells expressing the p57 mutant with substitutions in the $3_{10}$ helix region (F79A/Y80A) completely lost the ability to cause G$_1$ arrest (Fig. 7A). The expression levels of these mutants were comparable to that of wild-type p57 (Fig. 7B), suggesting that the decreased ability of certain mutants to inhibit DNA synthesis was not due to changes in their expression levels or in the stability of the proteins. This notion was further supported by the results showing that, when overexpressed, all of the p57 mutants bound to Cdk2 complexes to the same extent as the wild-type protein in vivo (Fig. 7C). Taken together with the fact that F79A/Y80A retained kinase inhibitory activity toward cyclin D1/Cdk4 and cyclin D1/Cdk6 (data not shown), it is plausible that the inhibition of cyclin A/Cdk2 and cyclin E/Cdk2 activities is crucial for the growth-suppressing function of p57.

DISCUSSION

We have demonstrated in the present study that the interaction of the $3_{10}$ helix region with Cdk2 forms the basis of a molecular mechanism by which p57, but not p21 or p27, inhibits the kinase activity. From the crystal structure of cyclin A/Cdk2/p27, it has been inferred that the binding of p27 to cyclin and Cdk causes significant conformational changes in the Cdk2 molecule, thereby inhibiting its kinase activity (19). Structural analysis also revealed that the $3_{10}$ helix region of p27 is inserted within the catalytic cleft of Cdk2, mimicking ATP (19). Although the presence of a conserved $3_{10}$ helix region among CKIs of the Cip/Kip family raises the possibility that all members of this family inhibit cyclin/Cdk activity in the same manner, our results indicate that the $3_{10}$ helix motif is specifically utilized by p57, and not by p21 or p27, in selectively inhibiting Cdk2 activity.

Extensive structure and function studies on the p21 molecule have suggested that both cyclin and Cdk binding is not only necessary but sufficient for the inhibition of cyclin/Cdk activities (20, 23, 24). Interestingly, it has been proposed that p21 contains two copies of the cyclin-binding motif, CB1 (amino acids 17–24) and CB2 (amino acids 146–164) (23). Consistent with this concept are our findings that deletion mutants of p21, $\Delta17$–$22$ and $\Delta146$–$164$, which lack either one of the two cyclin binding regions, still retained the ability to interact with cyclins A and E, whereas a double mutant ($\Delta17$–$22$/146–164) completely lost the ability to bind cyclin A, E, or D1. In contrast, deletion mutants of p27 ($\Delta28$–$33$) and p57 ($\Delta18$–$23$) failed to interact with cyclins (Fig. 2 and data not shown), suggesting that p27 and p57 contain only one cyclin binding domain. Although the functional domain for Cdk binding seems
well conserved among CKIs of the Cip/Kip family, only p27, and not p21 or p57, effectively bind Cdk4 (Fig. 2), suggesting that minor differences may exist between members of this family in terms of their binding affinity for specific Cdks. It has been reported that both cyclin and Cdk binding activities of p21 are indispensable for the inhibition of a wide variety of cyclin/Cdk complexes (22–25), and the present results indicate that this is also the case with p57 (Fig. 3).

While the N-terminal fragments of p21 and p27 (p21N and p27N) containing cyclin and Cdk binding domains were sufficient for the inhibition of all cyclin/Cdk complexes tested, p57N specifically lost the ability to inhibit cyclin A/Cdk2 (Fig. 4) and cyclin E/Cdk2 (data not shown). Moreover, additional 10 amino acids (residues 75–84) encompassing the 310 helix region were required for the effective inhibition of cyclin A/Cdk2 (Fig. 5) as well as cyclin E/Cdk2 (data not shown). To further substantiate these findings, we tested the effect of mutations within the 310 helix region of p57 (F79A/Y80A) on cyclin A/Cdk2 (Fig. 4) and cyclin E/Cdk2 (data not shown). It has been suggested, on the basis of crystal structural analysis of cyclin A/Cdk2/p27, that the position of the tyrosine side chain (corresponding to Tyr-80 of p57) in the catalytic cleft, and its points of contact with Cdk2 are similar to those of the purine base of ATP in the binary complex (19). Although mutations within 310 helix regions of p21 (L76A/Y77A) and p27 (F87A/Y88A/Y89A) did not affect the inhibitory activity toward cyclin A/Cdk2 and cyclin E/Cdk2, the F79A/Y80A mutant of p57 completely lost the ability to inhibit these kinase activities (Fig. 5B). The minor difference between the F79A/Y80A mutant and wild-type p57 protein in the binding affinity to cyclin A or E/Cdk2 complexes (Table I) does not account for the complete loss of the kinase inhibitory function. Taken together, these results indicate that the 310 helix motif is specifically utilized by p57, and not by p21 and p27, in inhibiting cyclin A/Cdk2 and cyclin E/Cdk2.

Another interesting aspect of CKIs of the Cip/Kip family is their ability to form active complexes with cyclin/Cdk (26–28). In this study, we demonstrated the interaction of p57 at lower concentrations with catalytically active cyclin A/Cdk2 complexes, but also found that the kinase activity was quenched as more wild-type p57 was added (Fig. 6). These results are consistent with the reported bimodal association of p21 with cyclin/Cdk complexes (26–28). Interestingly, p57 mutants, Δ18–23, W50G, and F79A/Y80A, formed kinase-active complexes with cyclin A/Cdk2 without any evidence of inhibition, even at

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**Fig. 7.** The 310 helix region of p57 is required for growth inhibitory activity in vivo. A, MDAH041 cells were cotransfected with 5 μg of pcDNA3-CD8 and 10 μg of pcDNA3-p57HA vectors expressing wild-type p57 (Wt) or its mutants (Δ18–23, W50G, and F79A/Y80A). 48 h after the transfection, cells were immunostained with anti-CD8 antibody, and CD8− (untransfected, upper panel) and CD8+ (transfected, lower panel) cells were independently analyzed for DNA content, as described under “Materials and Methods.” B, wild-type p57HA and the mutant proteins expressed in MDAH041 cells were detected by anti-HA antibody (12CA5) at a dilution of 1:1000. Control indicates control transfections with pcDNA3 vector alone. C, complex formation of wild-type p57HA and the mutant proteins with Cdk2 in MDAH041 cells. MDAH041 cells were cotransfected with pCMV-β-galactosidase and pcDNA3 vectors expressing wild-type p57HA or the indicated mutants, and the transfection efficiencies were normalized as described under “Materials and Methods.” The lysates expressing wild-type p57HA or the mutants were immunoprecipitated with anti-Cdk2 antibody, and the complexes were analyzed by Western blotting using anti-HA antibody. Control indicates control transfections with pcDNA3 vector alone.
higher amounts (Fig. 6). Thus, multiple cyclin A/Cdk2-interacting modules within the p57 molecule seem to be required to fully inhibit the kinase activity.

An important question arises as to whether the inhibitory activity directed toward Cdk2 through the $\beta_{10}$ helix motif plays any significant role in the growth-suppressing function of p57 in vivo. The F79A/Y80A mutant, which is specifically impaired with respect to Cdk2 inhibitory function, provides a good tool for addressing this point. Transfection experiments revealed that not only A18–23 and W50G, which lack inhibitory activity toward both Cdk4 and Cdk2, but also the F79A/Y80A mutant failed to cause G1 arrest (Fig. 7A). In contrast, the corresponding mutations within the $\beta_{10}$ helix regions of p21 (L76A/Y77A) and p27 (F87A/Y88A/Y89A) did not affect the cell cycle distribution of both p57 and p27 (Fig. 2A and B) indicating that the Cdk2-inhibiting activity of p57 is essential for its growth-suppressing function in vivo. Taken together with the recent report suggesting that, in Mv1Lu cells, p27 acts as a cyclin A/Cdk2 inhibitor and not as a cyclin D-Cdk4/6 inhibitor (29), it is tempting to hypothesize that cyclin A and cyclin E/Cdk2 are molecular targets of the cell cycle regulatory activity of both p27 and p57 in vivo.

In conclusion, we have provided biochemical evidence that the $\beta_{10}$ helix region of p57 is specifically required for the inhibition of cyclin A and E/Cdk2, but not cyclin D/Cdk4, and is crucial for its growth-suppressing function. Specific and functional interactions between the $\beta_{10}$ helix region and cyclin A and E/Cdk2 complexes may form the basis of a molecular mechanism by which p57 selectively regulates these kinase activities. Since this inhibition via the $\beta_{10}$ helix motif appears to be specific for p57 and not for other members of the Cip/Kip family, p21 and p27 may utilize an as yet unidentified mechanism that differentially regulates Cdk2 and Cdk4 functions.

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