Mechanistic insights into avian reovirus p17-modulated suppression of cell cycle CDK–cyclin complexes and enhancement of p53 and cyclin H interaction

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The avian reovirus p17 protein is a nucleocytoplasmic shuttling protein. Although we have demonstrated that p17 causes cell growth retardation via activation of p53, the precise mechanisms remain unclear. This is the first report that avian reovirus p17 possesses broad inhibitory effects on cell cycle CDKs, cyclins, CDK–cyclin complexes, and CDK-activating kinase activity in various mammalian, avian, and cancer cell lines. Suppression of CDK activity by p17 occurs by direct binding to CDKs, cyclins, and CDK–cyclin complexes; transcriptional down-regulation of CDKs; cytoplasmic retention of CDKs and cyclins; and inhibition of CDK-activating kinase activity by promoting p53–cyclin H interaction. p17 binds to CDK–cyclin except for CDK1–cyclin B1 and CDK7–cyclin H complexes. We have determined that the negatively charged \(^{151}\)LAVXDVDA(E/D)DGADPN\(^{165}\) motif in cyclin B1 interacts with a positively charged region of CDK1. p17 mimics the cyclin B1 sequence to compete for CDK1 binding. The PSTAIRE motif is not required for interaction of CDK1–cyclin B1, but it is required for other CDK–cyclin complexes. p17 interacts with cyclins by its cyclin-binding motif, \(^{12\,}RXL\(^{127}\). Sequence and mutagenic analyses of p17 indicated that a \(^{140}\)WXFD\(^{143}\) motif and residues Asp-113 and Lys-122 in p17 are critical for CDK2 and CDK6 binding, leading to their sequestration in the cytoplasm. Exogenous expression of p17 significantly enhanced virus replication, whereas p17 mutants with low binding ability to cell cycle CDKs had no effect on virus yield, suggesting that p17 inhibits cell growth and the cell cycle, benefitting virus replication. An in vivo tumorigenesis assay also showed a significant reduction in tumor size.

Cyclin-dependent kinases (CDKs)\(^2\) are catalytic subunits of a family of serine/threonine protein kinases that govern progression through the cell cycle (1). CDK activity requires binding of cyclin regulatory subunits. There are three interphase CDKs (CDK2, CDK4, and CDK6), a mitotic CDK1, and 10 cyclins that belong to four different classes (1). CDK activity is regulated by two families of inhibitors: INK4 and CIP/KIP (2, 3). The CIP/KIP family binds to and inhibits a broader range of kinases than does the INK4 family and exhibits a preference for CDK–cyclin complexes (2–4). INK4 proteins are composed of repeated 32-amino acid ankyrin motifs and specifically inhibit the catalytic subunits of CDK4 and CDK6 (4), whereas CIP/KIP preferentially inhibits CDK2 and CDK1.

Avian reoviruses (ARVs) cause considerable economic losses in the poultry industry (5, 6). ARV diseases include viral arthritis, chronic respiratory diseases, and malabsorption syndrome (5, 6). The ARV p17 protein is a nucleocytoplasmic shuttling protein (7) that performs specific duties in the nucleus and the cytoplasm and regulates signaling pathways, autophagy, and the cell cycle and interacts with several cellular proteins (8–13). The ARV p17 protein acts as a positive regulator by stabilizing PTEN by phosphorylation of its cytoplasmic form and by elevating Rak–PTEN interaction to prevent it from E3 ligase NEDD4-1 targeting (11). p17 suppresses CDK1 function by suppressing both Plk1 and CDC25C to cause inhibitory phosphorylation of CDK1 (12). More recently, we found that the p17 and αA proteins of ARV act cooperatively to down-regulate Akt by suppressing both mTORC2 and CDK2 and up-regulating proteasome PSMB6 (13). Although we have shown that p17 inhibits CDK1 and CDK2 activities (12, 13) and causes cell growth retardation via activation of p53 (8), the precise mechanisms by which p17 modulates suppression of cell cycle CDKs, cyclins, and CDK–cyclin complex formation and triggers p53 to inhibit the cell cycle remain largely unknown. In this study, we have undertaken a comprehensive investigation to explore

\(^*\)The abbreviations used are: CDK, cyclin-dependent kinase; ARV, avian reovirus; CAK, CDK-activating kinase; IP, immunoprecipitation; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GST, glutathione S-transferase; MOI, multiplicity of infection; p-, phosphorylated; PMSF, phenylmethylsulfonyl fluoride; hpi, hours postinfection; MEM, minimum essential medium.

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This article contains Tables S1–S4 and Figs. S1–S11.
Suppression of CDK–cyclin complexes by ARV p17 protein

how the nucleocytoplasmic shuttling protein p17 performs specific duties in the nucleus and the cytoplasm to suppress the formation of active CDK–cyclin complexes. Findings from the current study provide mechanistic insights into p17-modulated inhibition of the formation of active CDK–cyclin complexes in the nucleus by transcriptional down-regulation of CDKs, cytoplasmic retention of CDKs and cyclins, and enhancement of p53 and cyclin H interaction to suppress CDK7 activity. Importantly, our findings suggest that the PSTAIRE motif of CDK1 is not required for interaction with cyclin B1, but the PSTAIRE motif is critical for other cell cycle CDK–cyclin complexes (14).

Results

p17 competes with cyclin B1 for CDK1 binding

A previous study suggested that a cyclin box in cyclin B1 is involved in CDK1 binding (15). However, to date, actual interaction sites were not determined. We found a region, 241LAIPSFTAITGADP538, in p17 that mimics the sequence 151LAVXDVD(E/D)DGADPN165 in the cyclin box of cyclin B1 (Fig. 1A). The LA and GADP sequences in this region are conserved in p17 and cyclin B1 (Fig. 1A). To examine whether the LA and GADP sequences in p17 are important for competing with cyclin B1 for CDK1 binding, site-directed mutagenesis was used to create three p17 mutants. p17 co-immunoprecipitated with CDK1 (Fig. 1B and Fig. S1B), but not with cyclin B1 (Fig. 1C and Fig. S1C). Mutation of Gly-34 minimally affected CDK1 binding of p17, but p17Δ25A had no effect (Fig. 1D and Fig. S1D). Mutation of Asp-36 caused a significant loss of binding (Fig. 1D and Fig. S1C). To rule out the possibility of overexpression artifacts, GST pulldown assays were carried out. To perform GST pulldown assays, fusion proteins were expressed, purified, and analyzed (Fig. S1D and Table S1). GST pulldown assays revealed that p17 directly interacts with CDK1, but the p17Δ36A mutant has weak association (Fig. 1E), suggesting that Asp-36 is essential for p17 and CDK1 interaction. Furthermore, to investigate whether other acidic residues in p17 are involved in CDK1 binding, site-directed mutagenesis was used to create three additional p17 mutants. We discovered that mutations of other conserved acidic residues Glu-84 and Asp-86 in p17 also caused a dramatic loss of binding, but mutation at Lys-122 and Asp-143 had no effect (Fig. 1E). Thus, we have identified several conserved acidic residues in p17 that form a negatively charged pocket required for CDK1 binding (Fig. S1E). Importantly, several conserved basic residues in CDK1 are predicted by PyMOL software analysis to form a positively charged pocket in the N lobe of CDK1 (Fig. 1F and Fig. S1F). To test whether this positively charged pocket in CDK1 interacts with the negatively charged pocket in p17, site-directed mutagenesis was used to create three CDK1 mutants (K6/9A, K34A/R36A, and R75A) (Fig. S1D). GST pulldown assays revealed that mutations of these basic residues caused a significant loss of p17 interaction, as did simultaneous mutation of both p17 and CDK1 (Fig. 1F and J). All CDK1 and p17 mutants showed weak interaction (35% or less; Fig. 1J).

Identification of CDK1 and cyclin B1 interaction sites

To further confirm whether p17 mimics the GADP motif in cyclin B1 for competing with CDK1 binding, we conducted similar experiments with a site-specific cyclin B1D163A mutant to examine the conserved residue in the GADP motif of cyclin B1 (Fig. 1A). To perform GST pulldown assays, fusion proteins were expressed, purified, and analyzed (Fig. S2A and Table S1). This mutant has impaired ability to bind CDK1 compared with the WT cyclin B1 (Fig. S2B), suggesting that Asp-163 is required for CDK1 binding. To investigate whether other acidic residues in the negatively charged 151LAVXDVD(E/D)DGADPN165 motif in cyclin B1 are involved in CDK1 binding, site-directed mutagenesis was used to create three cyclin B1 mutants (D155A, D157A, and E159A/D160A). Mutations of these conserved acidic residues also affected cyclin B1 interaction with CDK1 (Fig. S2B). Furthermore, simultaneous mutation of both CDK1 and cyclin B1 severely impaired their interaction (down to 8–50%; Fig. 2A and Fig. S2C). In vitro kinase assays revealed that CDK1 and cyclin B1 mutants dramatically reduce phosphorylation of vimentin (Fig. 2B). Collectively, these observations highlight the importance of the negatively charged 151LAVXDVD(E/D)DGADPN165 motif in cyclin B1 for interaction with the positively charged pocket in CDK1. The corresponding interaction sites in p17, CDK1, and cyclin B1 are summarized in Table 1.

p17 inhibits CDK1 kinase activity by direct binding to CDK1, leading to cytoplasmic retention of CDK1

Having demonstrated that p17 competes with cyclin B1 for CDK1 binding, we next determined whether p17 inhibits CDK1 kinase activity. An in vitro kinase assay (12) detected decreased levels of phosphorylated vimentin (Ser-56) by p17 in a dose-dependent manner, whereas controls had no effect (Fig. 3, A and B). The $K_i$ value for inhibition of CDK1–cyclin B1 or CDK1–cyclin A2 complexes is about 105 nm (Fig. 3, A and B). Increasing concentrations of cyclin B1 in the CDK1–cyclin B1 complex led to increased kinase activity, whereas decreased kinase activity was correlated with increasing concentrations of p17 (Fig. 3A). After preincubation of p17 with CDK1, the $K_i$ value was 60 nm, whereas no change was detected in cyclin B1 (Fig. 3B). The inhibitory effect of CDK1 by preincubation with p17 was higher compared with the simultaneous addition of p17, CDK1, and cyclin B1, indicating that p17 competes with cyclin B1 for CDK1 binding (Fig. 3A).

The failure of cells to enter mitosis mediated by p17 results from inhibition of the kinase activity of the CDK1–cyclin B1 complex and inhibitory phosphorylation of CDK1 (12). Immunofluorescence staining revealed a marked increase in CDK1 level in the cytoplasm in ARV-infected and p17-transfected Vero cells (an African green monkey kidney cell line) (Fig. 3D). However, the failure of the p17Δ36A mutant to sequester CDK1 in the cytoplasm correlated with lower binding ability of CDK1 (Fig. 1E). These results were further supported by Western blotting (Fig. 3E), which revealed that the CDK1 level in the cytoplasm increased 3-fold in p17-transfected Vero cells. p17 uses a novel strategy in which it competes with cyclin B1 for CDK1 binding and sequesters CDK1 in the cytoplasm, preventing the formation of active CDK1–cyclin B1 complex in the nucleus.
p17 binds to the CDK1–cyclin A2 complex but not the CDK1–cyclin B1 complex

To further support the finding that p17 competes with cyclin B1 for CDK1 binding, we carried out complex formation assays. The formation of the CDK1–cyclin B1 complex was detected by Western blotting. We found that p17 is unable to bind the preassembled CDK1–cyclin B1 complex, further confirming our conclusion. Taken together, this supports the idea that p17 suppresses CDK–cyclin complexes by ARV p17 protein.
our results revealed that p17 binds CDK1, but not cyclin B1 or the CDK1–cyclin B1 complex. The formation of CDK1–cyclin A2, CDK1K34A/R36A–cyclin 2, and cyclin A2–CDK1K34A/R36A complexes was detected by Western blotting (Fig. 4, B and D). The CDK1K34A/R36A mutant has reduced ability to interact with cyclin B1 (Fig. 2A). In contrast, mutations at Lys-34 and Arg-36 in CDK1 did not affect CDK1–cyclin A2 complex formation, suggesting that CDK1 interacts differently with specific cyclin B1 and cyclin A2 residues. Furthermore, p17 exhibits binding specificity for the preassembled CDK1–cyclin A2 complex, and the $K_i$ value for inhibition of CDK1–cyclin A2 was about 125 nM (Fig. 4E). Based on our findings, we propose models for interaction of p17 with CDK1–cyclin B1 and CDK1–cyclin A2 complexes (Fig. 4F). The first model explains how p17 inhibits formation of the CDK1–cyclin B1 complex. The second model indicates that inhibition of CDK–cyclin A2 kinase activity occurs through direct binding of p17 to CDK1, cyclin A2, or the complexes.

The PSTAIRE region of CDK1 is not involved in CDK1 and cyclin B1 interaction but is required for other CDK–cyclin complexes

It has been demonstrated that Ile-49/Arg-50 in the PRTAIRE domain of CDK2 directly interacts with cyclin A (14). Furthermore, several reports predicted that CDK1 is activated by residue Ile-49 in CDK1 interacting with residue Tyr-258 of cyclin B1 (16–18). However, due to technical difficulties, such as poor...
expression and the insolubility of recombinant cyclin B1, these groups used a human cyclin B1_{AN165} mutant for crystal structure determination and prediction of CDK1–cyclin B1 interaction (17, 18). Surprisingly, our data revealed that mutations I49A and R50A in CDK1 retained strong binding ability with cyclin B1 or cyclin B1_{Y258A}, and CDK1 did not lose its kinase activity (Fig. 5A and Fig. S2A). Our findings contrast with previous conclusions that the PSTAIRE motif is required for interaction with CDK1–cyclin B1. Notably, mutations in CDK2 or CDK6 profoundly reduced their ability to bind partner cyclins and inhibited their kinase activity (Fig. 5, B and C). A similar trend was also found in CDK1–cyclin A2 (Fig. 5, B and C). Although the positively charged pocket in CDK1 lies very close to the PSTAIRE region, we show that the pocket is distinct from the PSTAIRE region (Fig. 5D). Taken together, our data confirm that the PSTAIRE region of CDK1 is not directly involved in the interaction with cyclin B1, but the PSTAIRE region of CDK2 and CDK6 is crucial for interaction with partner cyclins.

**p17 directly binds to CDK2, cyclins (A2 and E1), and the complexes of CDK2–cyclin A2 and CDK2–cyclin E1**

By alignment of p17, p21_{CIP}, and p27_{KIP} sequences (19, 20), we identified a conserved 140_{WFXD}143 motif (Fig. 6A). We found that p17 bears resemblance to cyclin-binding sequences (125_{RXL}127 motif) in the CIP/KIP family (21). Both 140_{WFXD}143 and 125_{RXL}127 motifs were conserved in p17 across different strains (Fig. S1E) (22, 23). Reciprocal co-immunoprecipitation assays revealed that p17 and the p17_{27–146} mutant interact with CDK2, cyclin A2, and cyclin E1 but not cyclin A1 (Fig. S3, A–C). Fusion proteins were expressed, purified, and analyzed (Fig. S3D and Table S1). GST pulldown assays revealed that the 140_{WFXD}143 and 125_{RXL}127 motifs of p17 are critical for CDK2 and cyclin binding (Fig. S3, E–G). Mutagenesis analysis suggested that CDK2_{K98}, cyclin A2_{D283}, and cyclin E1_{E154} are required for p17 interaction, whereas cyclin A2_{E220} and cyclin E1_{D218} are not involved (Fig. S3, F and G). The binding abilities of p17 and mutants are shown in Fig. 6B. Mutations at Asp-36 and Lys-122 in p17 (critical for CDK1 or CDK6 interaction) have no effect on the p17–CDK2 interaction (Fig. S3H). These residues of CDK2 and cyclins are conserved across different species (Fig. S3I). All p17, CDK2, cyclin A2, and cyclin E mutants showed weak interaction (40% or less; Fig. 6B). Taken together, our results demonstrate that p17 interacts directly with CDK2, cyclin A2, and cyclin E1.

With increasing concentrations of p17, a dose-dependent decrease in levels of phosphorylated Rb (Ser-249) was observed (Fig. 6C). The K_i value for inhibition of CDK2–cyclin A2 or CDK2–cyclin E1 by p17 was 40 and 80 nm, respectively (Fig. 6, C and D). Mutations at Arg-125, Trp-140, Phe-142, and Asp-143 in p17 attenuated binding to cyclins (A2 and E1) or CDK2 (Fig. 6, C and D). The addition of BSA to the mixture had no effect on Rb phosphorylation. Preincubation of p17 with either CDK2 or cyclins (A2 and E1) resulted in K_i values of 60 and 125 nm, respectively (Fig. S4, A and B). The inhibitory effect on CDK2 was moderately reduced compared with the simultaneous addition of p17, CDK2, and cyclins, implying that p17 interferes with formation of CDK2–cyclin A2 and CDK2–cyclin E1 complexes, which reduces its effect on the inhibition of Rb kinase activity of CDK2.

Similar to CDK1 sequestration by p17, immunofluorescence analysis revealed that the majority of CDK2, cyclin A2, and cyclin E1 is associated with p17 in the cytoplasm (Fig. S4, C, E, and G) in p17-transfected and ARV-infected Vero cells. In mock controls and p17 mutant (D143A and R125A)-transfected cells, most of the accumulation was in the nucleus. Western blotting confirmed that CDK2 and its partner cyclins are sequestered in the cytoplasm (Fig. S4, D, F, and H).

We also examined whether p17 binds to the CDK2–cyclin A2 and CDK2–cyclin E1 complexes. The formation of CDK2–cyclin A2 (24) and CDK2–cyclin E1 complexes was examined by Western blotting (Fig. S5A). We found that p17 binds pre-
assembled CDK2–cyclin A2 or CDK2–cyclin E1 complexes. In contrast to the CDK1–cyclin B1 complex, p17-modulated inhibition of the CDK2–cyclin A2 and CDK2–cyclin E1 complex kinase activity occurs through direct binding to CDK2, partner cyclins, or complexes. The $K_i$ value for binding of the CDK2–cyclin A2 complex by p17 was 200 nM, which showed stronger
inhibition of Rb kinase activity compared with that of the CDK2–cyclin E1 complex (Fig. S5, C and E). In addition, the CDK inhibitor p21 is a substrate of cyclin A–CDK2, and phosphorylated p21 is a potent CDK inhibitor (25). However, phosphorylation of p17 by CDKs was not detected.

**p17 directly binds to individual CDKs (CDK4 and -6), cyclin D1, and the CDK6–cyclin D1 complex**

To assess whether p17 interacts with CDK6 and cyclin D1, co-immunoprecipitation (co-IP) assays were performed. Co-IP assays revealed that p17 and p17 mutants interact with CDK4/6 and cyclin D1 (Fig. S6, A–D), but not cyclin D2 and D3 (Fig. S6D). Deletion of the C terminus of p17 in p17 (1–118) appeared to disrupt CDK4/6- and cyclin D1-binding activity (Fig. S6, A–D). By alignment of p17, p16\textsuperscript{INK4a}, p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d} sequences (26), we identified several conserved charged residues in p17 (Fig. 7A). To define whether these conserved residues are involved in CDK4/6 binding, a series of p17, CDK6, and cyclin D1 constructs were made, purified, and analyzed (Fig. S6F). Mutations at Asp-113 and Lys-122 in p17 impaired CDK6 binding, whereas mutations at Asp-36 and Asp-143, which are involved in CDK1 and CDK2 interaction, did not affect the binding ability of p17 (Fig. 7B and Fig. S6, G and H), suggesting that residues Asp-113 and Lys-122 are critical for p17–CDK6 interaction. Mutagenesis analysis revealed that Glu-18/Glu-21 in CDK6 and Glu-66/Glu-70 in cyclin D1 are conserved and required for interaction with p17 (Fig. S6, E, G, and H). p17 has higher affinity for the N lobe of cyclin D1, whereas only some detectable affinity for the N lobe of CDK6 was detected (Fig. S6, G and H). Our results reveal that p17 may function similar to p16\textsuperscript{INK4a} to inhibit the CDK4–cyclin D1 complex by distorting the kinase catalytic cleft and interfering with ATP binding (27).

Decreased levels of the p-Rb(Ser-780) by p17 were observed in a dose-dependent manner (Fig. 7C). The $K_c$ value for inhibition of CDK6–cyclin D1 by p17 was 125 nm (Fig. 7C). After preincubation of p17 with CDK6 or cyclin D1 for 30 min, the $K_c$ values were 125 and 250 nm, respectively. The results imply that p17 may interfere with complex formation of CDK6–cyclin D1, thereby reducing Rb kinase activity of CDK6 (Fig. S6f). In contrast to p21, which not only promotes the formation of the CDK6–cyclin D1 complex but also promotes increased kinase activity (28), p17 exhibits weak binding ability to the preassembled CDK6–cyclin D1 complex (less than 50%; Fig. 7, D and E), and the $K_c$ value for inhibition of CDK6–cyclin D1 by p17 was only 500 nm (Fig. 7F). Collectively, our findings suggest that p17 interacts specifically with various cell cycle CDKs, cyclins, and CDK–cyclin complexes (Table 1). Our findings suggest that p17 has a higher inhibitory effect on CDK1 and CDK2 than on CDK6 (Table 1).

Similar to p17-mediated cytoplasmic retention of other CDKs and cyclins (cyclins A2 and E1), we found that CDK6 and cyclin D1 were also sequestered by p17 in the cytoplasm (Fig. S6, J and K) as compared with the mock control. Immuno blot analyses revealed that the cytoplasmic retention of CDK6 and cyclin D1 was increased as compared with the mock control (Fig. 7, G and H). In mock controls and p17 mutant–transfected cells, most of the proteins accumulated in the nucleus (Fig. 7, G and H). However, the ratio of cytoplasmic retention of CDK6 and cyclin D1 is much lower than that of other cell cycle CDKs or cyclins. Collectively, our findings reveal that ARV p17 has a higher binding affinity for CDK1 and CDK2 than for CDK6 (Figs. 4 C and D) and 7E and Fig. S5 B and D) and stronger inhibition of CDK1 and CDK2 kinase activity than of CDK6 (Table 1). p17 appears to use a novel mechanism in which it sequesters different cell cycle CDKs, cyclins, and/or their complexes in the cytoplasm, preventing the accumulation of CDK–cyclin complexes in the nucleus and retarding the host cell cycle.

**p17 diminishes CAK activity through enhancing p53–cyclin H interaction**

An earlier study suggested direct involvement of p53 in triggering growth arrest by its interaction with the CAK complex without the need of cyclin-dependent kinase inhibitors (29). We have shown that p17 activates and protects p53 via multiple mechanisms (11, 13, 30). Therefore, we next wanted to examine whether p17 enhances p53–cyclin H interaction to inhibit CDK7 kinase activity. Reciprocal co-immunoprecipitation assays revealed that p17 interacts neither with CDK7 nor cyclin H, but increases p53–cyclin H interaction (more than 2-fold) (Fig. 8A and Fig. 8G), which dissociates the CDK7–cyclin H complex and suppresses CDK2 phosphorylation at Thr-160 (Fig. 8B). The effect of p17 on CDK7–cyclin H could be reversed in p53 knockdown cells (Fig. 8B), suggesting that p17 diminishes CAK activity in a p53-dependent manner. In addition to the two interaction models of p17 with CDK–cyclin complexes, as shown in Fig. 4F, a third model of p17-modulated suppression of CAK activity via activation of p53 is possible (Fig. 8C).

**p17 structure and interaction with CDKs, cyclins, and CDK–cyclin complexes**

p17 structure was predicted by homology modeling. The modeling confidence estimated by Phyre2 is 74%, suggesting reliability of the target and template protein sequences. During the 250-ns MD simulation, the potential energy of the system converged after 25 ns (Fig. S8A), indicating that the system had equilibrated sufficiently. By monitoring the root mean square deviation on calcium atoms, we found that the overall fold pattern became stable after 170 ns of simulation (Fig. S8B), sug-

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**Figure 3.** p17 inhibits CDK1 kinase activity by direct binding to CDK1, leading to cytoplasmic retention of CDK1. A, and B, in vitro kinase assays were carried out to determine the inhibitory effect of CDK1–cyclin B1 and CDK1–cyclin A2 by p17. The kinase assay measured vimentin phosphorylation at Ser-56 as analyzed by Western blotting. The p17/\textsuperscript{D16A} mutant and BSA were used as negative controls. Experiments were done in duplicate. C, the inhibitory effect of p17 on CDK1 kinase activity was assessed via preincubation of p17 with cyclin B1 or cyclin D1 for 30 min, followed by an in vitro kinase assay. Vimentin phosphorylation at Ser-56 was analyzed by Western blotting. D, a pcDNA3.1-GFP-p17 plasmid was used to assess the distribution of CDK1 in the cytoplasm and the nucleus of Vero cells under the fluorescence microscope. Mutation at D36A was used as a negative control. Scale bar, 10 μm. E, distribution of CDK1 in the cytoplasm and the nucleus was detected by Western blotting. All data shown represent the mean ± S.D. (error bars) calculated from three independent experiments. Protein levels were normalized to those for histone H2A or β-tubulin, markers for the cytoplasm and the nucleus, respectively. Signals in all Western blots were quantified with ImageJ software.
suggesting that our protein model is reliable and stable. We observed that a reduction of exposed hydrophobic area on the protein surface and a decreased number of residues in unstructured regions (Fig. S8, C and D) imply the progress of protein folding. Because the hydrophobicity and the secondary structure content become stable after 200 ns (Fig. S8, C–E), we presumed that protein folding was near completion and stopped the simulation at 250 ns. The z-score value estimated by ProSA is within the acceptable region (Fig. S8F). The Ramachandran statistics showed the percentage of residues in the disallowed...
regions is only 0.8% (Fig. S8G). Taken together, our results validate our model structure of p17 (Fig. S9A). The residues important for p17–CDK, p17–cyclin, or CDK–cyclin interaction are highlighted as spheres (Fig. S9B).

p17 down-regulates several cell cycle CDKs in cancer cell lines

Co-immunoprecipitation assays revealed that p17 interacts with CDKs and cyclins of cancer cell lines (Fig. 9A). Decreased levels of CDKs 2, 4, and 6 in HeLa cells (a human cervical cancer cell line) and SW620 cells (a human colorectal adenocarcinoma cell line) were detected in p17-transfected cells (Fig. 9B). The data are consistent with our previous findings with Vero and DF-1 cells (11, 13). Notably, the level of CDK1 was not altered, whereas increased inhibitory phosphorylation of CDK1 of HeLa and SW620 cancer cells was detected in p17-transfected cells (Fig. 9C). The results are consistent with our previous findings with Vero and DF-1 cells (12). Collectively, the previous and current studies suggest that p17-modulated inhibitions of CDK1 function by direct binding to CDK1 to suppress the formation of CDK1–cyclin B1 complex and by suppression of both Plk1 and CDC25C to cause inhibitory phosphorylation of CDK1 (12). Furthermore, decreased amounts of the phosphorylated forms of vimentin and Rb were detected (Fig. 9C), indicating inhibition of the respective CDK kinase activities. Our results reveal that p17-mediated down-regulation of CDKs is not through the...
ubiquitin-proteasome pathway but via transcriptional down-regulation (Fig. 9, D and E).

Exogenous expression of p17 enhances virus replication in ARV-infected cells

The effects of p17 and p17 mutants on the cell cycle are consistent with inhibition of cell proliferation and binding capacity of p17 toward cell cycle CDKs, cyclins, or CDK–cyclin complexes. Notably, exogenous expression of p17 significantly elevated virus titer more than 1.5 log in ARV-infected cells with various multiplicities of infection (MOIs) (Fig. 10A) but only caused a slight increase in p17 mutant–transfected cells (Fig. 10B), suggesting that p17 induces cell cycle retardation, benefiting virus replication.
p17 inhibits cell growth and the cell cycle in cancer cell lines

We compared the viable cell number and cell proliferation rate of p17- and p17 mutant-expressing cells (Vero, DF-1, HeLa, SW620, and A459) with those of vector-transfected or mock-transfected cells. The viable cell number was significantly reduced by about 2.5–4.5-fold in p17-expressing cells.
Our results reveal that p17 inhibits cell growth independent of host cell type. The current study has found significant growth inhibition of p17 on avian, mammalian, and cancer cell lines. We further investigated whether p17 could inhibit cell cycle progression of these cell lines. A diagram showing that cells are synchronized at G0/G1 transition using serum deprivation by maintenance of cells in Dulbecco's modified Eagle's medium (DMEM) either supplemented with fetal bovine serum (FBS) or not is shown in Fig. S10A. The expression levels of p17, p17 mutants, and the empty pcDNA3.1 control for every condition are shown in Fig. S10B. The percentages of cells accumulating in each phase of the cell cycle at different time points are shown in Fig. S10C. The p17-expressing cell lines grew at a slower rate than control cells (Fig. 11B), whereas p17 mutants that weakly interact with different CDKs or cyclins caused retardation of the cell cycle, although it was still slightly faster compared with p17-transfected cells (Fig. 11B). An in vivo tumorigenesis assay was carried out to evaluate effects of p17 on the growth of A549 cancer cells (a lung cancer cell line), which showed a significant reduction in tumor size (Fig. 11C).

**Discussion**

This study provides novel insights into p17-modulated suppression of cyclins, CDK activity, complex formation, and the cell cycle in mammalian, avian, and cancer cell lines. We demonstrate that nucleocytoplasmic shuttling protein p17 performs specific duties in the nucleus and the cytoplasm to modulate the p53 pathway and the cell cycle. In addition to activation of p21 (CIP/KIP family) (8, 11), p17 has broader inhibitory effects on cell cycle CDK kinases in several cell lines compared with the NK4 and CIP/KIP families, suggesting that the ARV p17 protein represents a new class of CDK inhibitor.

In this work, we propose three putative interaction models to explain the mechanisms by which p17 modulates cell cycle CDK–cyclin complex kinase activities. We found that in addition to CDK1–cyclin B1 and CDK7–cyclin H complexes, p17 binds directly to other cell cycle CDK–cyclin complexes. CDK1 is the only essential cell cycle CDK in human cells and is required for successful completion of M phase. The novel discovery that p17 mimics the cyclin B1 motif led to the identification of a conserved LAVDVNAV/E/DGYMDP motif in cyclin B1 that forms a negatively charged pocket to interact with a positively charged pocket in CDK1. p17 uses a novel strategy to compete with cyclin B1 for CDK1 binding, leading to cytoplasmic retention of CDK1, which prevents the formation of active CDK1–cyclin B1 complex in the nucleus, resulting in G2/M cell cycle retardation. We also found that other CDKs...
CDK2 and CDK6) and cyclins (A2, E1, and D3) could be sequestered by p17 in the cytoplasm, suggesting that p17 uses a novel strategy to sequester these molecules in the cytoplasm, preventing the formation of complexes and inhibiting CDK activity.

This is the first report to suggest that the PSTAIRE region of CDK1 is not involved in CDK1–cyclin B1 interaction but is required for formation of other complexes. Due to technical difficulties, such as poor expression and the insolubility of recombinant cyclin B1, other research groups used a human cyclin B1<sub>N165</sub> mutant for crystal structure determination and prediction of CDK1–cyclin B1 interaction (17, 18). Surprisingly, our data revealed that mutations I49A and R50A in CDK1 retained strong binding ability with cyclin B1 or cyclin B1<sub>Y258A</sub>, and CDK1 did not lose its kinase activity, suggesting that the PSTAIRE motif of CDK1 is not directly involved in the interaction with cyclin B1. Mutations in CDK2 or CDK6 profoundly reduced their ability to bind partner cyclins and inhibited their kinase activity, suggesting that the PSTAIRE motif of CDK1 is required for cyclin A2, and likewise the PSTAIRE motif of

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### Figure 9. The effects of p17 on CDKs in cancer cell lines.

**A**. Co-immunoprecipitation assays were performed in p17-transfected HeLa and SW620 cells and in mock controls using antibodies against p17, CDK, and cyclins. **B**. In reciprocal co-immunoprecipitation experiments, the interaction of p17 and CDKs was further examined in p17-transfected HeLa cells by Western blotting (WB) of p17 immunoprecipitates. **C**. The levels of CDKs, p-Rb (Ser-249 and Ser-780), and p-vimentin (Ser-56) were examined in p17-transfected HeLa and SW620 cells. **D**. MG132 (2.5 μM) was used to treat mock, ARV-infected, and p17-transfected cells. The protein levels of CDK2 and CDK4 were examined by Western blotting. **E**. To examine whether CDK transcription was down-regulated by p17, mRNA levels of CDKs in pCDNA3.1-p17–transfected HeLa cells were examined at the indicated time points. HeLa cells were transfected with pCDNA3.1-p17, and transfected cells were collected at 0, 12, or 24 h, followed by semiquantitative RT-PCR for analysis of CDK genes. After electrophoretic analysis, PCR products were stained with ethidium bromide. Data in the graph represent the mean ± S.D. (error bars) calculated from three independent experiments. Signals for all blots were quantified using ImageJ software.
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CDK2 and CDK6 is required for interaction with partner cyclins.

The growth suppressor p53 is an important key element which governs cell cycle progression in response to cellular stresses, including DNA damage. We have previously shown that p17 can prevent MDM2-mediated p53 ubiquitination and degradation (13, 30, 31). ARV infection and p17 transfection induce p53 phosphorylation at multiple sites (30), impairing the ability of MDM2 to target p53 (13). Previous reports suggested that p17 enters the nucleus, leading to suppression of nucleoporin p53 and p53 nuclear accumulation, consequently activating p53, PTEN, and p21 and down-regulating the PI3K/Akt/mTOR and REK signaling pathways, all of which result in translation shutoff, induction of autophagy, and retardation of cell growth (9–11, 13). However, the mechanism underlying p17-modulated retardation of the cell cycle in a p53-dependent manner remains largely unknown. Here, we show that suppression of CAK activity by p17 involves enhanced p53–cyclin H interaction, which disassociates the CDK7–cyclin H complex. Earlier reports suggested that phosphorylation of CDKs at Thr161/160/172 by CAK is essential for stabilization of CDK–cyclin complexes and increases the flexibility of the T-loop of CDK2 (14, 32, 33). This study further elucidates the mechanism for how p17 causes p53-dependent cell cycle arrest in several cell lines.

In the present study, we found that p17 bears resemblance to the cyclin-binding motif 125RXL127 in the CIP/KIP family (21). Notably, we discovered that p17 interacts with several cyclins in the cytoplasm through an RXL motif, leading to cytoplasmic retention of cyclins and prevents the formation of active CDK–cyclin complexes in the nucleus. Sequence and mutagenic analyses of the ARV p17 protein indicated that a 146W XF D145 motif and residues Asp-113 and Lys-122 conserved in p17 across different strains (22, 23) are critical for CDK2 and CDK6 binding. Mutations at these motifs or residues in p17 impaired CDK2 and CDK6 binding. Thus, the third interaction model appears to best explain how p17 suppresses formation of CDK1–cyclin A2, CDK2–cyclin A2, CDK2–cyclin E1, and CDK6–cyclin D1 complexes by directly binding to CDK, cyclin, or CDK–cyclin complexes.

Modulation of cell growth is a common feature of infection of animal viruses and also contributes to the progress of pathogenesis. A number of viral proteins induce cell cycle arrest and apoptosis (34–36). The ARV p17 and σC proteins induce cell cycle retardation and apoptosis, respectively (37, 38). The current study has found significant growth inhibition of and cell cycle arrest by p17 in avian, mammalian, and cancer cell lines, benefiting virus replication. Our findings further support previous studies suggesting that depletion of CDKs in ARV-infected cells reduces virus replication (11–13). Importantly, ARV replication can be dramatically enhanced by exogenous expression of p17 in ARV-infected cells. Thus, ARVs have evolved strategies that alter the physiology of host cells during infection to enhance viral replication. Based on our findings, the effect of p17 on growth of viruses may accelerate virus propagation, thereby benefiting virus isolation or vaccine production in cell culture.

Animal-derived viruses that do not circulate extensively in the human population represent a potential source of oncolytic viruses that can circumvent preexisting immunity. Mammalian reoviruses are now being studied in the clinic and have been demonstrated to have efficacy in conjunction with chemotherapy against head and neck cancers (38). The present and earlier studies suggest that ARVs possess oncolytic characteristics and can potentially be utilized for treatment of human hepatocellular carcinoma and other malignancies (39). ARVs have several unique characteristics that are different from mammalian reoviruses. They can induce syncytia, thereby facilitating virus spread and distribution within a tumor. Additionally, p17 can induce autophagy and activate protein kinase RNA-activated signaling (10), thereby activating the innate immune system, which may induce the immune response against tumors. Our work shows that p17 exhibits significant growth inhibition and cell cycle retardation in several cancer cell lines and significantly reduces tumor size in vivo. ARV is not associated with human diseases, and preexisting immunity would not hamper its clinical application. Understanding of p17-modulated growth inhibition and cell cycle arrest in cancer cell lines will provide important information for further clinical applications. Targeting p53 or CDKs using ARV p17 may provide a complementary platform for oncolytic virotherapy.

Experimental procedures

Virus and cells

The S1133 strain of ARV used in this study is our laboratory stock. African green monkey kidney (Vero) cells, immortalized chicken embryo fibroblasts (DF-1), and human colorectal ade-
necarcinoma (SW620) cells were maintained in DMEM and supplemented with 10% FBS and 10 mM HEPES (pH 7.2). Human cervical cancer (HeLa) and lung cancer (A549) cell lines were maintained in RPMI1640 supplemented with 10% FBS and 10 mM HEPES (pH 7.2). Cells were seeded 1 day before each experiment in 6-well culture dishes with 5 × 10^5 cells and grown at 37 °C in a 5% CO₂ humidified incubator. Cell lines HeLa and DF-1 were purchased from the Food Industry

Figure 11. p17 results in cell growth inhibition and cell cycle retardation in various mammalian, avian, and cancer cell lines. A, the growth curve of various cell lines transfected with pcDNA3.1, p17-pcDNA3.1, or p17 mutant genes. The data represent the average of triplicate plates with S.D. (error bars) indicated. The x axis represents the post-transfection time of incubation. The viable cells were counted with a hemocytometer in the presence of trypan blue and are represented on the y axis. B, p17 causes cell cycle retardation in various mammalian, avian, and cancer cell lines. Cells were transfected for 18 h with constructs after serum deprivation for 54 h. As shown in Fig. S10C, the percentages of cells accumulating in each phase of the cell cycle at different time points were analyzed. B was derived from the results of Fig. S10C. The x axis represents the time period of incubation post-transfection, whereas cell cycle number is represented on the y axis. C, 1 × 10^7 A549 lung cancer cells were injected subcutaneously into BALB/c nude (nu/nu) mice and monitored for tumorigenesis in vivo. A549 cancer cells in nude (nu/nu) mice were allowed to grow for 17 days, followed by gene gun transformation with pcDNA3.1-p17 and pcDNA3.1 plasmids, respectively. n = 3 for each group.

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Research and Development Institute (Hsinchu, Taiwan). SW620 and A549 cell lines were from Professors Chang and Chen (National Chung Hsing University), respectively.

Primers and vectors

The expected GST or His-tagged fusion protein molecular weights are summarized in Table S1. Primers with restriction enzyme sites and vectors are summarized in Table S2.

Reagents and antibodies

Proteasome inhibitor MG132 was from Calbiochem. mAb against p17 protein is our laboratory stock. Mouse anti-GST, mouse anti-CDK1, rabbit anti-p-vimentin (Ser-56), rabbit anti-p-CDK2 (Thr-160), mouse anti-CDK7, mouse anti-p53, rabbit anti-histone H2A, rabbit anti-β-tubulin, and rabbit anti-p-Rb (Ser-780) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-β-actin antibody was from Millipore (Billerica, MA). Mouse anti-CDK2, mouse anti-CDK4, mouse anti-CDK6, mouse anti-cyclin A1, mouse anti-cyclin A2, mouse anti-cyclin D1, rabbit anti-cyclin D2, mouse anti-cyclin D3, mouse anti-cyclin E, and rabbit anti-cyclin H antibodies were from Santa Cruz Biotechnology, Inc. Mouse anti-cyclin B1 was purchased from BD Biosciences. Mouse anti-FLAG mAb was purchased from Sigma-Aldrich. Rabbit anti-p-RB (Ser-249) antibody was purchased from LifeSpan Biosciences (Seattle, WA). Mouse anti-His mAb was purchased from Abcam (Cambridge, UK). Anti-mouse IgG (H+L), anti-rabbit IgG (H+L) antibodies, and rhodamine-labeled affinity-purified antibody to mouse IgG (H+L) were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). The catalogue numbers of the antibodies used in this study are shown in Table S3.

Determination of the location of the positively charged pocket region and PSTAIRE motif in CDK1

A colored ribbon diagram of CDK1 protein structure was generated by PyMOL software analysis (49).

Generation of p17, CDK, and cyclin constructs and mutants

The pcDNA3.1-FLAG-p17, pcDNA3.1-FLAG-p17(1–118), and pcDNA3.1-FLAG-p17(27–146) constructs were generated previously (11). p17 mutants (p17D25A, p17G34A, p17D36A, p17D113A, p17K122A, p17R125A, and p17D143A), CDK mutants (CDK1K34A/R36A, CDK2K89A, and CDK6E18/21A), and cyclin mutants (cyclin A2E230A, cyclin A2D283A, cyclin D1E166/70A, cyclin E1E154A, and cyclin E1D121A) were generated using a QuikChange II site-directed mutagenesis kit (Agilent). Substitution of nucleotides was confirmed by DNA sequencing.

Sequence analysis and GenBank™ accession numbers for p17, CDK, cyclins, and CDK inhibitors

The GenBank™ accession numbers for p17, CDKs, cyclins, and CDK inhibitors are shown in Table S4.

Protein expression and purification

The pET32a-p17 plasmid used in this study was described previously (11). For construction of a variety of genes (CDK1, CDK2, CDK6, cyclin B1, cyclin D1, and vimentin) into the pGEX4T-1 expression vector (GE Healthcare), full-length cDNAs were generated by RT-PCR of total RNA from Vero cells. RNA template (2 μg of total RNA) prepared from cells was mixed with oligo(dT) primer (50 ng; Genomics Inc., New Taipei City, Taiwan), and incubated at 72 °C for 10 min. RT was carried out at 42 °C for 90 min in 25 μl containing 5 μl of RT buffer, 5 μl of dNTPs (10 mm), RNA template (2 μg), 0.5 μl of Moloney murine leukemia virus reverse transcriptase (200 units), and diethyl pyrocarbonate–treated water to a final volume of 25 μl. Reactions were incubated at 42 °C for 90 min followed by PCR. PCR was performed with 50 ng of plasmid DNA, 2 μl of 2.5 mm dNTP (MD Bio Inc.), 10 μM forward and reverse primers, 0.5 μl of Pfu DNA polymerase (2.5 units) (MD Bio), 5 μl of 10× Pfu buffer and adjusted with nuclease-free water to a final volume of 50 μl. PCR cycling conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 30 s, followed by a final 10-min extension at 72 °C. Both plasmid DNA and the amplified full-length gene products were digested with the respective restriction enzymes. Inserts and vectors were isolated by gel purification, and ligation was carried out at 16 °C overnight. Unfortunately, after RT-PCR, we could not amplify cyclin A2 and cyclin E1 cDNA products from Vero cells. Thus, human cyclin A2 (MHS6278-202857779) and cyclin E1 (MHSM278-202830068) cDNA clones were purchased from Thermo Fisher Scientific. PCR was performed with 2 μl of cDNA (50 ng), 2 μl of dNTP (2.5 mm), 1 μl of primer pairs, 0.5 μl of Pfu DNA polymerase (2.5 units), 5 μl of 10× Pfu buffer and adjusted with nuclease-free water to a final volume of 25 μl. PCR amplification was performed under the following conditions: 95 °C for 5 min and 35 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 90 s, followed by a final 10-min extension at 72 °C. The purified PCR products of cyclin A2 and cyclin E1 were digested with EcoRI and Xhol, followed by ligation into the corresponding restriction sites of the pGEX4T-1 vector. All recombinant plasmids were transformed into Escherichia coli BL21(DE3). Transformed E. coli cells were grown in Luria-Bertani broth with 100 μg/ml ampicillin at 37 °C to an optical density of 0.6 and then induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside for 5 h at 28 °C.

For the His-tagged p17 fusion proteins, cells were harvested by centrifugation, followed by resuspension in pET system lysis buffer (20 mM Tris–HCl, pH 8.0, 300 mM NaCl, 0.2 mM PMSF, 10% glycerol, 5 mM imidazole) and sonicated. The cell suspension was centrifuged at 12,000 × g for 20 min at 4 °C. The
supernatant was applied to a nickel column. After washing beads with 150 ml of washing buffer, the TrxA-His–tagged p17 fusion protein was eluted from the affinity column with elution buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.2 mM PMSF, 10% glycerol, 200 mM imidazole). To obtain soluble forms of GST-tagged fusion proteins, cells were harvested by centrifugation, followed by resuspension in lysis buffer (1 × PBS, 0.2 mM PMSF, 1% Triton X-100, 0.5% sodium lauroyl sarcosinate). After sonication, the cell suspension was centrifuged at 12,000 × g for 20 min at 4 °C. The supernatant was changed to 1 × PBS with Amicon Ultra 0.5-mli 10K filters (Millipore) by adding the same volume of 1 × PBS and centrifuging at least five times. The supernatant was applied to a GSH-Sepharose 4B column (GE Healthcare). After washing beads with 1 × PBS washing buffer, the GST fusion proteins were eluted from the column with elution buffer (1 × PBS, 10 mM reduced GSH). Finally, all purified fusion proteins were changed to PBS buffer using Amicon Ultra 0.5-mi 10K filters. Samples were stored at −80 °C for further experiments.

**Mass spectrometry analysis of p17 phosphorylation by CDK1**

To determine whether p17 is phosphorylated by CDK1, 2 μM purified His-p17 was mixed with purified GST-CDK1 (10 nM) and GST-cyclin B1 (10 nM) in kinase buffer (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, pH 7.1, 25 mM MgCl₂, 0.1 mM Na₃VO₄, and 0.5 mM DTT) that contained 40 μM Na3VO4 and 0.5 mM DTT) for 20 min at 4 °C. The supernatant was removed by centrifugation, followed by resuspension in lysis buffer (1 × PBS, 10 mM reduced GSH). Finally, all purified fusion proteins were changed to PBS buffer using Amicon Ultra 0.5-ml 10K filters after centrifugation, followed by resuspension in lysis buffer (1 × PBS, 10 mM reduced GSH) by adding the same volume of 1 × PBS and centrifuging at least five times. The supernatant was applied to a GSH-Sepharose 4B column (GE Healthcare). After washing beads with 1 × PBS washing buffer, the GST fusion proteins were eluted from the column with elution buffer (1 × PBS, 10 mM reduced GSH). Finally, all purified fusion proteins were changed to PBS buffer using Amicon Ultra 0.5-mi 10K filters. Samples were stored at −80 °C for further experiments.

**Transcriptome analysis of p17 phosphorylation by CDK1**

To determine whether p17 is phosphorylated by CDK1, 2 μM purified His-p17 was mixed with purified GST-CDK1 (10 nM) and GST-cyclin B1 (10 nM) in kinase buffer (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, pH 7.1, 25 mM MgCl₂, 0.1 mM Na₃VO₄, and 0.5 mM DTT) that contained 40 μM ATP in a final volume of 25 μl at 37 °C for 30 min. The mixture was resolved by SDS-PAGE and visualized by Coomassie Blue. A band of the molecular mass of TrxA-p17 (35 kDa) in the gel was excised and washed twice with 50% acetone. In-gel trypsin-digested proteins were identified by LC/MS/MS sequencing (National Chung Hsing University).

**Cell lysate preparation and Western blot analysis**

ARV-infected or p17-transfected cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with complete protease mixture inhibitor. Total proteins from each lysate were quantified using a Bio-Rad protein assay and electrophoresed in 10 or 12% polyacrylamide gels at 70 V through the stacking gel and at 100 V through the resolving gel and then transferred to a polyvinylidene difluoride membrane (GE Healthcare). Expression of individual proteins was examined using the respective antibodies, followed by secondary antibody conjugated with horseradish peroxidase. After incubation with enhanced chemiluminescence (ECL Plus) (Amersham Biosciences) according to the manufacturer’s instructions, the polyvinylidene difluoride membranes were exposed to high-performance chemiluminescence films.

The relative intensity of target proteins was calculated using ImageJ (National Institutes of Health). Signals in all Western blots were quantified with ImageJ software.

**Reciprocal co-IP assays**

The procedure used has been described previously (13). Reciprocal co-IP assays were performed using a Catch and Release Reversible Immunoprecipitation System (Millipore). Briefly, 6-well plates were seeded with 5 × 10⁵ Vero or cancer cells, and the cells were cultured in DMEM containing 10% FBS overnight. Vero cells were infected with ARV at an MOI of 10 and collected 24 h postinfection. Vero or cancer cells were transfected with pCDNA3.1-p17 plasmid and collected 24 h post-transfection. Cells were washed twice with 1 × PBS and scraped in 200 μl of CHAPS lysis buffer (40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycophosphate, 50 mM NaF, and 0.3% CHAPS). 1000 μg of cellular proteins were incubated with 4 μg of the respective antibodies at 4 °C overnight. Mouse or rabbit IgG was used as negative control. The immunoprecipitated proteins were separated by SDS-PAGE followed by a Western blotting assay with the respective antibodies. Because the molecular masses of the light chain (25 kDa) or the heavy chain (50 kDa) of anti-mouse IgG (H+L) or anti-rabbit IgG (H+L) are close to that of the target proteins, to avoid interference by the heavy chains and light chains of IgG, we cut the regions for the heavy and light chains from the membranes after transfer of proteins from the gel.

**GST pulldown assays**

The procedure for GST pulldown has been described previously (12). Briefly, 1 μg of purified GST or GST fusion proteins was coupled to GSH-Sepharose 4B beads (GE Healthcare) and incubated at 4 °C overnight, followed by washing with 1 × PBS. GST beads–GST fusion proteins were then incubated at 4 °C overnight with 100 ng of purified TrxA-His–p17 or mutant proteins in binding buffer (20 mM Tris-HCl, 25 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, and 10 μg/ml mixture protease inhibitor). The protein-bound GSH beads were washed five times with binding buffer and eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced GSH). Elution fractions were denatured and examined by Western blot analysis with the corresponding antibodies. A total of 30% input of TrxA-His fusion protein represents the internal control.

**In vitro complex formation assays and binding assays of p17 to preassembled CDK–cyclin complexes**

An overall flow chart shows steps in the formation of CDK–cyclin complexes (Fig. 4A). To test the formation of these complexes, 100 μl of Pierce™ protein G–agarose beads (Thermo Fisher Scientific) were incubated with 10 μl of 100 ng/μl antibody capture affinity ligand (Millipore), 2 μl of GST mAb, and 1 μg of purified GST–CDK (GST–CDK1, GST–CDK1K34A/R36A, GST–CDK2, or GST–CDK6) or GST–cyclin (GST–cyclin B1, GST–cyclin A2, GST–cyclin A2D283A, GST–cyclin D1, or GST–cyclin E1) in binding buffer (1% Nonidet P-40, 0.25% deoxycholic acid, 15 mM imidazole, and 10 μg/ml mixture protease
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Inhibits the kinase activities of these complexes, the CDK1–cyclin B1 or CDK1–cyclin A2 complex, which to those described above. To assess whether p17 could bind to p17. The procedures used for in vitro kinase assay. To examine the binding ability of p17 to the pre-assembled CDK–cyclin complexes, 1 μg of purified p17 was added to the preassembled CDK–cyclin complex for 30 min at 37 °C. Beads were washed three times with cold binding buffer and eluted with elution buffer. Fractions were examined by Western blotting with the respective antibodies. The inhibitory effect of the preassembled CDK–cyclin kinase activity examined by an in vitro kinase assay. To examine the binding ability of p17 to the pre-assembled CDK–cyclin complexes, 1 μg of purified p17 was added to the preassembled CDK–cyclin complex for 30 min at 37 °C. Beads were washed three times with cold binding buffer and eluted with elution buffer. Fractions were examined by Western blotting with the respective antibodies. The inhibitory effect of the preassembled CDK–cyclin kinase activity examined by an in vitro kinase assay.

In vitro kinase assays

In vitro kinase assays using purified fusion proteins were performed as reported previously (12, 40, 41). To assess the effect of kinase activity inhibition by p17 on the CDK1–cyclin B1 complexes, vimentin (2 μM) was used as a substrate and incubated with purified cyclin B1 or cyclin A2 (10 nm) and CDK1 (10 nm) at 37 °C for 30 min in cold kinase buffer (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, pH 7.1, 25 mM MgCl2, 0.1 mM Na2VO4, and 0.5 mM DTT) that contained 40 μM ATP in a final volume of 25 μL. Simultaneously, different concentrations of purified His-p17 protein were added to the mixture of GST-vimentin, GST-CDK1, and GST-cyclin B1. To investigate whether p17 competes with cyclin B1 for CDK1 binding, increasing concentrations of purified GST-cyclin B1 were added to the mixture of GST-vimentin, GST-CDK1, and GST-cyclin B1. The procedure used for in vitro kinase assays are similar to those described above. To assess whether p17 could bind to the CDK1–cyclin B1 or CDK1–cyclin A2 complex, which inhibits the kinase activities of these complexes, in vitro kinase assays were performed. Purified His-p17 and GST-vimentin proteins were added after preincubation of CDK and cyclin for 30 min. To further investigate whether p17 affects the formation of CDK–cyclin complexes, leading to inhibition of kinase activity, purified His-p17 and CDK or cyclin were preincubated at 37 °C for 30 min followed by the addition of substrates, CDK, or cyclin at 37 °C for 30 min, followed by in vitro kinase assays.

Immunofluorescence staining

To study whether p17 sequesters CDKs or cyclins in the cytoplasm, monolayer Vero cells grown in 6-well plates were infected with ARV at an MOI of 10 or transfected with pcDNA3.1-GFP, pcDNA3.1-GFP-p17, pcDNA3.1-GFP-p17D36A, pcDNA3.1-GFP-p17D143A, pcDNA3.1-GFP-p17K125A, and pcDNA3.1-GFP-p17K122A, respectively. After 24 h of transfection or infection, the cells were washed twice with 1× PBS followed by incubation for 10 min in 4% paraformaldehyde (Alfa Aesar) at room temperature. The cells were blocked with SUPERBLOCK® T20 solution (Thermo Scientific) (Bellefonte, PA) at room temperature for 1 h with gentle shaking. After triple washes with 1× PBS, cells were incubated with CDK or cyclin monoclonal antibodies (500-fold dilution in PBS), followed by additional washing with 1× PBS. Rhodamine-conjugated anti-mouse IgG (500-fold dilution in PBS) was added to the culture wells and incubated at 4 °C in the dark overnight. After several rinses with 1× PBS, cell nuclei were stained with 10 μM 4′,6-diamidino-2-phenylindole for 10 min in the dark, followed by observation with a BX51 fluorescence microscope (Olympus). The images were processed using Viewerfinder Lite software (Pixera Corp.).

Isolation of cytosol and nuclear fractions

The procedures used in this study were described previously (11). To further investigate the subcellular distribution of p17, CDKs, and cyclins in the nucleus or the cytoplasm in ARV-infected or p17-transfected cells, fractions of the cytosol and nucleus were isolated using a CNM compartmental protein extraction kit according to the manufacturer’s protocol (Bio-Chain Institute Inc., Newark, CA). Briefly, cells were seeded into 6-cm cell culture dishes. At about 75% confluence, cells were either transfected with pcDNA3.1-p17 or infected with ARV at an MOI of 10. All cultures were harvested at 24 h post-transfection or postinfection. Cells were harvested after washing with ice-cold PBS twice and pelleted. They were resuspended in 150 μL of ice-cold buffer C, and the mixture was rotated at 4 °C for 20 min. A 26.5-gauge needle was attached to a syringe, and the needle tip was removed by bending the needle several times, leaving only the needle base on the syringe. The cell mixture was passed through the needle base 50–90 times to disrupt the cell membrane and release the nuclei from the cells. After centrifugation at 15,000 × g at 4 °C for 20 min, the cytoplasmic proteins in the supernatant were collected. The pellet was resuspended in 500 μL of ice-cold buffer W, and the mixture was rotated at 4 °C for 5 min. The supernatant was centrifuged at 15,000 × g for 20 min at 4 °C, and the pellet was resuspended in 75 μL of ice-cold buffer N, followed by rotation at 4 °C for 20 min. After centrifugation at 15,000 × g at 4 °C for 20 min, the nuclear proteins in the supernatant were collected.
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Semiquantitative RT-PCR

To investigate whether p17 transfection affects CDK transcript levels, HeLa cells were transfected with pcDNA3.1-p17 and collected at the indicated time points. Total RNA was isolated from the transfected cells using TRIzol reagent and subjected to semiquantitative RT-PCR according to previously published methods (11). The glyceraldehyde-3-phosphate dehydrogenase gene was used as an internal control for normalization.

Ethics statement and mouse xenograft assay

The animal use protocol was approved by the institutional animal care and use committee at the National Chung Hsing University (approval 104-073), and the animal studies were performed in compliance with these guidelines. The tumorigenesis assay was as described previously (42). 1 × 10^7 A549 cancer cells mixed with sterile 1 × PBS (1:1) were injected subcutaneously into female BALB/c nude (nu/nu) mice (n = 3 for each group) and monitored for tumorigenesis in vivo. Female BALB/c nude (nu/nu) mice were purchased from National Laboratory Animal Center (Taiwan). A549 cancer cells in nude (nu/nu) mice were allowed to grow for 17 days, followed by two injections with pcDNA3.1-p17 or pcDNA3.1 vector (negative control), respectively, at days 17 and 21 via a gene gun. Each injection was applied to the propidium iodide histogram plot. The percentage of cells in each phase of the cell cycle were analyzed by BD FACSDiva™ software.

Virus titration

To determine the role of p17 in ARV replication, 4-well cell culture plates were transfected with pcDNA3.1-p17 for 6 h and infected with different MOI of ARV. The supernatants of each well were harvested at 24 hpi for viral titration. To determine the role of p17 mutant genes in ARV replication, 4-well cell culture plates were transfected with pcDNA3.1-p17 or its mutants for 6 h and infected with ARV at an MOI of 0.1. The supernatants of each well were harvested at 24 hpi for titration to determine virus titer as described previously (11). Briefly, ARV-infected cell supernatants were collected. Virus titer was determined by an agar overlay plaque assay carried out in triplicate. Cells in 6-cm cell culture dishes were incubated for 1 h with diluted virus in 100 μl of serum-free minimal essential medium (MEM). The cells were then washed twice with MEM to remove unabsorbed viruses and overlaid with 2 ml of 1% agarose in MEM containing 2% FBS and antibiotics. Plaques were examined after an incubation period of 2 days at 37 °C by staining with neutral red for 3 h.

Homology modeling, MD, and protein–protein docking

The structure of p17 was modeled using the Phyre2 server (43). Phyre2 chose the β-propeller domain of prolyl endopeptidase (Protein Data Bank entry 3IUN) (44) as the template and built the initial model of p17. To improve model quality, the resulting model was subjected to molecular dynamics simulation using GROMACS version 4.6.7 (45) with an AMBER99SB-ILDN force field. The initial structure was soaked in an orthorhombic water box, and the net charge was neutralized by the addition of sodium or chloride ions (at 150 mM salt). Long-range electrostatics was controlled using the particle mesh Ewald method. Possible bad contacts from the particle mesh Ewald method. Possible bad contacts from the initial structure were removed by the steepest descent energy minimization method until energy convergence reached 1000 kJ/(mol·nm). The system was then subjected to equilibration at 300 K and normal constant pressure (1 bar) for 100 ps under the conditions of position restraints for heavy atoms and LINCS constraints. The equilibrated structure was used to perform the production run. The virtual site hydrogen was used to remove angle vibrations involving hydrogens and to speed up the calculation. The time step of the simulation was set to 5 fs, and the coordinates were saved for analysis every 100 ps. The stereo-chemical qualities of the final model were validated by ProSA (46) and PROCHECK (47). Protein–protein docking simulations were conducted using the HADDOCK server (48), and final structures were visualized and analyzed with PyMOL (49).
**Statistical analysis**

All data were evaluated for statistical significance using Student’s *t* test. Data are expressed as mean ± S.D. of at least three independent experiments. In all tests, *p* < 0.05 was considered statistically significant.

**Author contributions**—H.-C. C. and W.-R. H. performed most of the experiments. H.-C. C., W.-R. H., P.-I. C., J.-H. L., T.-L. L., B. L. N., and H.-J. L. analyzed the data. H.-J. L. conceived and designed the experiments, wrote the paper, and supervised the project. H.-J. L. and B. L. N. revised and edited the manuscript.

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