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p12^{DOC-1} Is a Novel Cyclin-Dependent Kinase 2-Associated Protein

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Regulated cyclin-dependent kinase (CDK) levels and activities are critical for the proper progression of the cell division cycle. p12^{DOC-1} is a growth suppressor isolated from normal keratinocytes. We report that p12^{DOC-1} associates with CDK2. More specifically, p12^{DOC-1} associates with the monomeric nonphosphorylated form of CDK2 (p33CDK2). Ectopic expression of p12^{DOC-1} resulted in decreased cellular CDK2 and reduced CDK2-associated kinase activities and was accompanied by a shift in the cell cycle positions of p12^{DOC-1} transfectants ($\uparrow$ G_1 and $\downarrow$ S). The p12^{DOC-1}-mediated decrease of CDK2 was prevented if the p12^{DOC-1} transfectants were grown in the presence of the proteosome inhibitor *clasto*-lactacystin β-lactone, suggesting that p12^{DOC-1} may target CDK2 for proteolysis. A CDK2 binding mutant was created and was found to revert p12^{DOC-1}-mediated, CDK2-associated cell cycle phenotypes. These data support p12^{DOC-1} as a specific CDK2-associated protein that negatively regulates CDK2 activities by sequestering the monomeric pool of CDK2 and/or targets CDK2 for proteolysis, reducing the active pool of CDK2.

Cell cycle inhibitors of the p16^{INK4a} and p21$^{WAF1/CIP1/CAP20}$ families exert their effects by negatively regulating cyclin and cyclin-dependent kinase (CDK) complex formation and kinase activities (10, 14). While the p16^{INK4a} family is specific for CDK4 and CDK6, and the p21$^{WAF1/CIP1/CAP20}$ family of CDK inhibitors is universal for CDKs, there is no known specific inhibitor for CDK2. CDK2, when complexed with cyclins E and A, is implicated in G_1/S transition, DNA replication, and progression through the DNA synthesis phase (6, 7, 9).

p12^{DOC-1} is a growth suppressor identified and isolated from normal keratinocytes (12). It is a highly conserved cellular gene. Our laboratory (12, 13) and others (4, 5) have cloned p12^{DOC-1} cDNA from human, mouse, and hamster. The full-length human and mouse p12^{DOC-1} cDNAs are 1.6 kb and 1.2 kb, respectively. Human p12^{DOC-1} has one additional amino acid at residue 19, which corresponds to an alanine, and differs from the mouse and hamster p12^{DOC-1} at only two other amino acid residues (Ala → Thr at residue 8 and Gly → Ser at residue 100). Human and rodent p12^{DOC-1} polypeptides have 97% identity, and the mouse and hamster p12^{DOC-1} protein sequences are identical. Human p12^{DOC-1} is a 115-amino-acid peptide with a molecular mass of 12.4 kDa (pI, 9.62).

Ectopic expression of p12^{DOC-1} in keratinocytes is associated with increased doubling time, suggestive of a growth suppressor function (11). These observations prompted us to examine if p12^{DOC-1} interacts with regulatory proteins in the cell division cycle. We report that p12^{DOC-1} associates with CDK2. Data are presented to support the role of p12^{DOC-1} as a specifically CDK2-associated protein, which, when overexpressed, negatively regulates CDK2-associated kinase activities and cell cycle phenotypes.

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MATERIALS AND METHODS

Cell culture and transfections. Transfection of human 293 cells was performed using Lipofectamine Plus (Life Technologies, Inc., Rockville, Md.) according to the manufacturer's protocol. Cells were harvested 12 to 48 h posttransfection for respective experiments. Transfections were done in 60- or 100-mm dishes in triplicate. Each experiment was repeated at least three times.

Site-directed mutagenesis. Mutations of the amino acid sequence from positions 103 to 111 of p12^{DOC-1} were introduced using the QuickChange site-directed mutagenesis system (Stratagene, La Jolla, Calif.). Sequences were confirmed by automated DNA sequencing.

In vitro, cellular, and endogenous p12^{DOC-1} and CDK2 association assays. For p12^{DOC-1} in vitro binding experiments, glutathione *S*-transferase (GST)–p12^{DOC-1} fusion protein was prepared from *Escherichia coli*. Cellular lysates from HeLa, 293, and A431 cells were prepared in ice-cold 0.5% NP-40 lysis buffer. GST or GST-p12^{DOC-1} (1 μg) was incubated with cell lysates (200 μg) for 2 h at 4°C in 500 μl of lysis buffer. Beads were washed four times with lysis buffer, loaded onto a sodium dodecyl sulfate (SDS)–10 or 12% polyacrylamide gel and blotted onto polyvinylidene difluoride (PVDF) membranes. The anti-CDK2 antibody (C18520 Clone 55; Transduction Laboratories, San Diego, Calif.) was used for immunodetection.

For p12^{DOC-1} and CDK2 cellular binding assays, 293 cells were transfected with pFLAG-DOC-1-wt (or pFLAG-DOC-1-A3) (cloned into pFLAG-CMV-2; Eastman Kodak Co., New Haven, Conn.) and wild-type CDK2 using Lipofectamine Plus (Life Technologies). Forty-eight hours posttransfection, cells were lysed in 0.5% NP-40 lysis buffer. The cell lysate (300 μg) was incubated with anti-FLAG (1 μg) (M5; Sigma Chemicals, St. Louis, Mo.) and anti-CDK2 antibodies (1 μg) (C18520 Clone 55; Transduction Laboratories, Lexington, Ky.) for 1 h at 4°C and then incubated with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, Calif.) for 2 h at 4°C. Immunoprecipitated complexes were loaded onto 12 or 15% polyacrylamide gel and blotted onto polyvinylidene difluoride membranes. Anti-p12^{DOC-1} (Ab3) and anti-CDK2 antibodies (C18520 Clone 55; Transduction Laboratories) were used for immunodetection.

For protein preparations from human lung tissue, snap-frozen tissues were rapidly homogenized in 5 volumes of 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris [pH 7.4], 1 mM sodium vanadate, 25 μg of aprotinin/ml, and 25 μg of leupeptin/ml). Tissues were obtained from the Massachusetts General Hospital Tumor Bank.

Gel filtration chromatography. Normal human lung homogenates or HaCaT cell lysate (0.5 ml; 2.5 mg of total protein) was applied to a Protein PAK300sw gel filtration high-pressure liquid chromatography column (Waters). Elution was

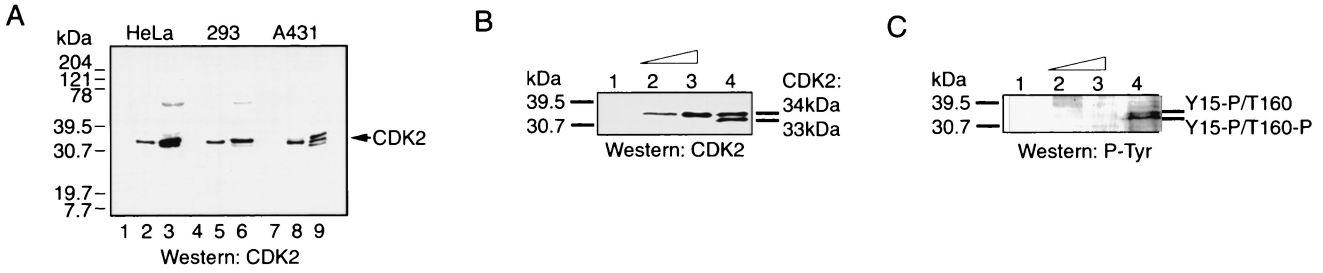

FIG. 1. GST-p12^{DOC-1} associates with CDK2 in cell lysates. (A) CDK2 immunoblot showing cellular CDK2 from HeLa, 293, and A431 cells associating with the GST-p12^{DOC-1} fusion protein. Lanes 1, 4, and 7, GST control; lanes 2, 5, and 8, GST-p12^{DOC-1}; lanes 3, 6, and 9, input lysate at 0.1×. (B) CDK2 immunoblot showing that GST-p12^{DOC-1} associates with the 34-kDa forms of CDK2. Lane 1, GST control; lanes 2 and 3, GST-p12^{DOC-1} (1 and 2 μg, respectively); lane 4, immunoprecipitation of CDK2. (C) Phosphotyrosine immunoblot showing that the p34CDK2 that associates with GST-p12^{DOC-1} does not contain phosphotyrosine residues (4G10; Upstate Biotechnology). The samples for panels B and C were run on long SDS-PAGE gels to resolve the 33- and 34-kDa CDK2 bands.

achieved with an isocratic flow of 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 50 mM NaF, and 2 mM EDTA at a flow rate of 0.45 ml/min. Fractions of 0.45 ml were collected and subjected to Western blotting analyses for p12^{DOC-1} and CDK2. The column was calibrated with protein standards of known molecular weights (catalase, aldolase, bovine serum albumin, ovalbumin, and chymotrypsin).

CDK2 kinase assays. For kinase assays, cell lysates were prepared from 293 cells transfected for 48 h with pFLAG, pFLAG-DOC-1-wt, or pFLAG-DOC-1-A3. Immunoprecipitations were performed with antibodies specific to CDK2 (M2; Santa Cruz Biotechnology), cyclin A (BF683; Santa Cruz Biotechnology) and cyclin E (M20; Santa Cruz Biotechnology) and protein A/G agarose beads. The immune complexes were washed four times with kinase buffer (50 mM Tris [pH 7.4], 0.1 mM EDTA, 1 mM dithiothreitol) and resuspended in a final volume of 10 μl of kinase buffer. The kinase reactions included 500 ng of histone H1 or pRBc (the carboxyl-terminal fragment of pRB), 5 μM ATP, 10 mM $MgCl_2$, and 10 μCi of [γ-^{32}P]ATP and were incubated for 15 min at 37°C. At the end of the reaction, 10 μl of 2× SDS sample buffer was added, and proteins were loaded onto SDS–10% polyacrylamide gels. The gels were stained with Coomassie blue prior to autoradiography.

RESULTS

p12^{DOC-1} associates with CDK2. We examined if the p12^{DOC-1}-mediated growth suppression is due to its association with cyclins and CDKs. In vitro binding experiments were performed using a GST-p12^{DOC-1} fusion protein and human cellular lysates, followed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. The bound complexes were examined for the presence of cyclins and CDKs. Of the candidates surveyed (CDC2, CDK2, CDK4, CDK6, cyclin A, cyclin B, cyclin D1, cyclin D3, and cyclin E), GST-p12^{DOC-1} was found to associate with CDK2 (Fig. 1).

Figure 1A shows that the GST-p12^{DOC-1} fusion protein can associate with CDK2 in different human cell lysates. Similar experiments with CDC2, CDK4, and CDK6 showed that although the cellular levels of these proteins are similar, there is no association, indicating that the association of p12^{DOC-1} to CDK2 is specific (data not shown). Neither cyclin E nor cyclin A was detected in the bound GST-p12^{DOC-1}–CDK2 complexes, suggesting that p12^{DOC-1} either associates with the monomeric form of CDK2 or has displaced the cognate cyclin (data not shown). CDK2 electrophoretically migrates as a doublet (HeLa and 293) or triplet (A431) on SDS-PAGE gels (Fig. 1B). Gu et al. have demonstrated that phosphorylation affects the electrophoretic mobility of CDK2 (6). The 34-kDa CDK2 band (p34CDK2) represents the monomeric nonphosphorylated and Tyr15-phosphorylated (by WEE1) forms of CDK2. The 33-kDa band (p33CDK2) represents forms of CDK2 that have been phosphorylated by CAK at amino acid residue Thr160. Figure 1B shows that p12^{DOC-1} associates predominantly with p34CDK2. To ascertain whether p12^{DOC-1} associates with the WEE1-Tyr15-phosphorylated or the monomeric nonphosphorylated form of p34CDK2, GST-p12^{DOC-1}–CDK2-bound complexes were immunoblotted for the presence of phosphotyrosine. While phosphorylated tyrosine was detected in both the immunoprecipitated 33- and 34-kDa CDK2 bands (Fig. 1C, lane 4), the p34CDK2 associated with GST-p12^{DOC-1} did not contain detectable phosphorylated tyrosine (Fig. 1C, lanes 2 and 3), suggesting that p12^{DOC-1} associates with the monomeric nonphosphorylated form of CDK2.

To examine if p12^{DOC-1} and CDK2 can interact in cells, a FLAG-tagged DOC-1 vector (pFLAG-DOC-1-wt) was cotransfected with pCDK2 into 293 cells. p12^{DOC-1} and CDK2 coprecipitated from the 293 cells (Fig. 2A and B, lanes 1 to 4). The immunoprecipitated p12^{DOC-1}–CDK2 complexes were also examined for the presence of cyclins A and E, but they were not detected (Fig. 2C and D). To determine if a similar interaction can be detected for endogenously expressed p12^{DOC-1} and CDK2, a number of human tissues and cell types were examined for endogenous p12^{DOC-1} expression. p12^{DOC-1} was not detectable in tumor or immortalized cell lines (35 were examined). Immunoblotting lysates from a panel of normal human tissues revealed that p12^{DOC-1} is detectable in tissue from the spleen, kidney, placenta, and lung (Fig. 3A). Since lung tissue expresses the highest endogenous level of p12^{DOC-1}, lysate preparations from two normal lungs were used to examine endogenous interactions of these proteins. Figure 3B and C showed that endogenous p12^{DOC-1} does interact with CDK2 in the lysate preparations from both lung tissue samples. Immunohistochemical staining of normal lung sections revealed that the majority of lung parenchymal cells coexpress p12^{DOC-1} and CDK2 (data not shown). To examine whether the CDK2 that coprecipitated with p12^{DOC-1} from the normal lung tissue is p34CDK2 and/or p33CDK2, the immunoblot shown in Fig. 3C was reprobed by the antiphosphotyrosine antibody 4G10. Figure 3F reveals that while immunoprecipitation of total CDK2 from the lung lysates contained tyrosine-phosphorylated forms (lanes 3 and 7), the CDK2 associated with p12^{DOC-1} was apparently not tyrosine phosphorylated (lanes 2 and 6), suggesting that p12^{DOC-1} predominantly associates with p33CDK2, the monomeric nonphosphorylated form. While the lack of detectable phosphotyrosine in the CDK2 associated with p12^{DOC-1} may reflect a smaller amount of CDK2 coimmunoprecipitated with p12^{DOC-1} (lanes 2 and 6), the data do support that endogenous p12^{DOC1} associates predominantly with the non-tyrosine-phosphorylated form of CDK2.

To provide additional evidence that endogenous p12^{DOC-1} associates with CDK2, normal lung lysates were subjected to gel filtration chromatography to determine if these proteins

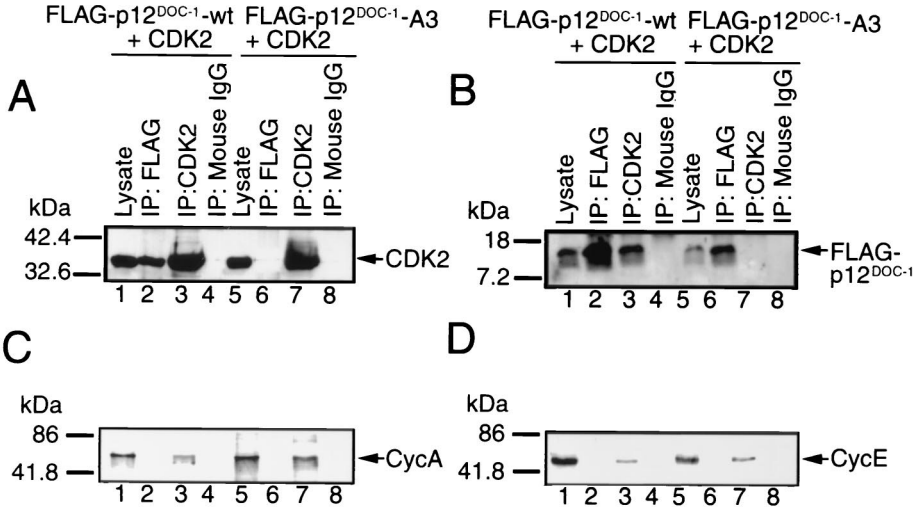

FIG. 2. Association of $p12^{DOC-1}$ and CDK2 in cells. (A and B) CDK2 and $p12^{DOC-1}$ immunoblots showing the coprecipitation of $p12^{DOC-1}$ with CDK2 in 293 cells cotransfected with pCDK2 and pFLAG-DOC-1-wt or pFLAG-DOC-1-A3. Lanes 1 and 5, lysate (30 μg); lanes 2 and 6, immunoprecipitation using anti-FLAG monoclonal antibody (M5; Sigma Chemicals); lanes 3 and 7, CDK2 immunoprecipitation using anti-CDK2 monoclonal antibody (C18520 Clone 55; Transduction Laboratories); lanes 4 and 8, negative control using nonimmune mouse immunoglobulin G for immunoprecipitation. (C and D) Reprobing of the same membranes shown in panels A and B for cyclins A and E, respectively.

comigrate. Normal human lung homogenates were applied to a protein PAK300sw gel filtration high-pressure liquid chromatography column. Eluted fractions were immunoblotted for $p12^{DOC-1}$ and CDK2. Figure 3D shows the elution profiles of $p12^{DOC-1}$ and CDK2 from normal lung 2 (with the same preparation used for panels B and C), showing that the two proteins coelute from fractions 21, 22, and 23, corresponding to the apparent molecular masses of 56, 46, and 37 kDa, respectively, as calibrated with protein standards of known molecular weights (catalase, aldolase, bovine serum albumin, ovalbumin, and chymotrypsin). We have recently found that human keratinocyte HaCaT cells express detectable levels of endogenous $p12^{DOC-1}$ and CDK2. Gel filtration chromatography was similarly performed using these cell lysates. Figure 3E shows the

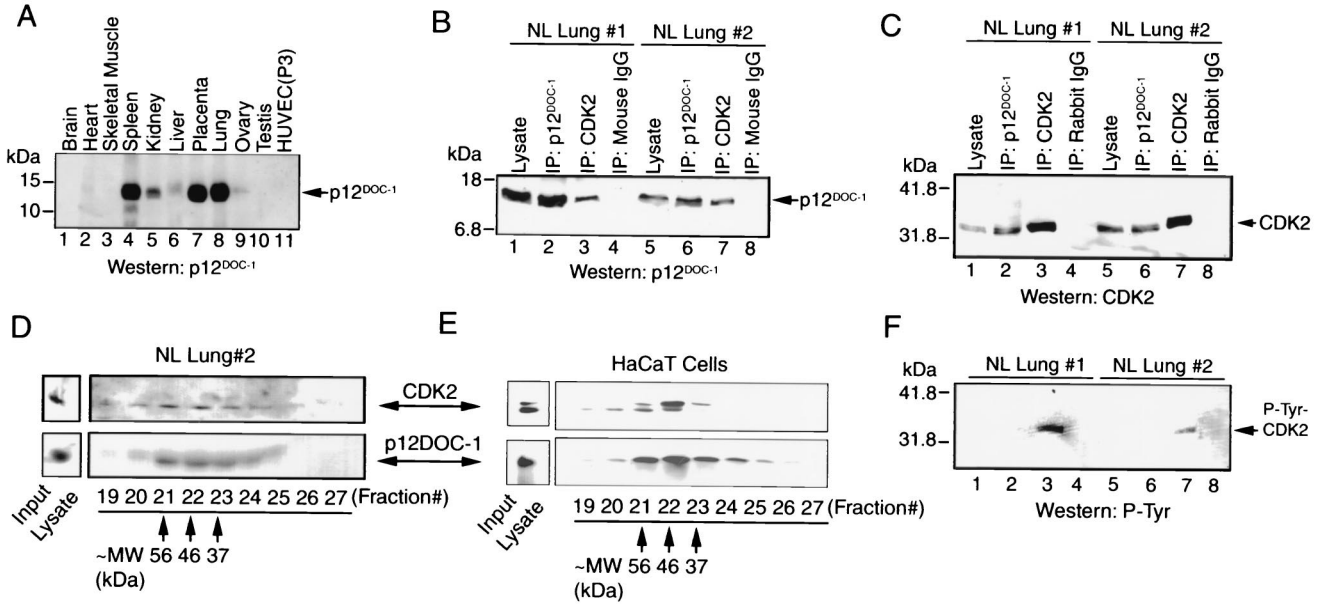

FIG. 3. Interaction of $p12^{DOC-1}$ and CDK2 in vivo. (A) Immunoblot to detect $p12^{DOC-1}$ in normal human tissue lysates. Thirty micrograms of tissue lysates was loaded onto each lane. HUVEC (P3) cells are third passage normal human umbilical endothelial cells. (B and C) Coprecipitation of $p12^{DOC-1}$ with CDK2 in human lung lysates from two donors. Panel B shows a $p12^{DOC-1}$ immunoblot using $p12^{DOC-1}$ Ab3; panel C shows a CDK2 immunoblot using anti-CDK2 antibody (C18520 Clone 55; Transduction Laboratories). Lanes 1 and 5, input lysate (25 μg); lanes 2 and 6, $p12^{DOC-1}$ immunoprecipitation; lanes 3 and 7, CDK2 immunoprecipitation; lanes 4 and 8, negative control using nonimmune mouse and rabbit immunoglobulin G for panels B and C, respectively. (D and E) Gel filtration chromatograph elution profiles of normal human lung lysate (#2) and HaCaT cells. Top panels show immunoblots for CDK2; bottom panels show immunoblots for $p12^{DOC-1}$. Thirty micrograms of total proteins was used for the respective input lysate lanes. Approximate molecular sizes (MW) of fractions 21, 22, and 23 are calibrated against known molecular size standards. (F) Phosphotyrosine immunoblot of the same membrane used for panel C to show that the CDK2 coprecipitated with the endogenous $p12^{DOC-1}$ detected in normal lung lysates was not tyrosine phosphorylated, suggesting that it is the monomeric nonphosphorylated p33CDK2.

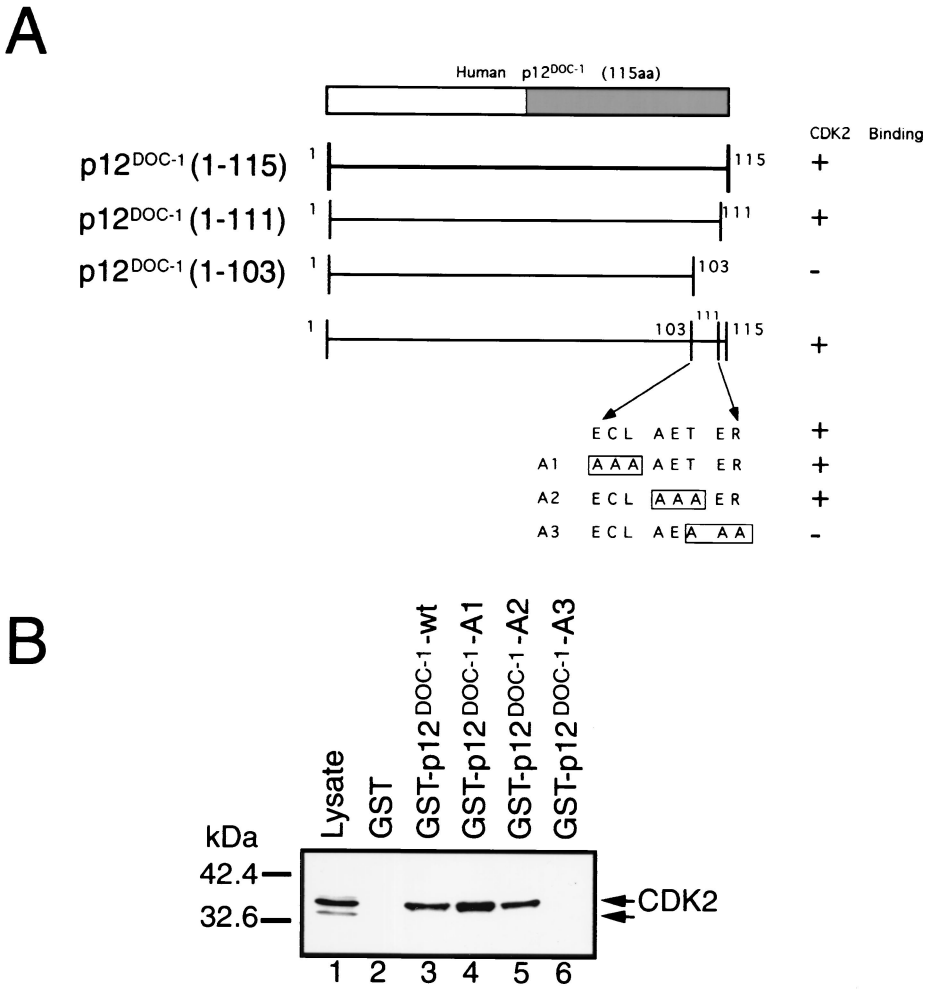

FIG. 4. Amino acids 109 to 111 are necessary for p12^{DOC-1}'s association with CDK2. (A) Schematic of the mutagenesis strategy. Mutants were created by the Stratagene QuickChange site-directed mutagenesis system. The gray box in the C terminus (amino acids 62 to 115) is a domain that is homologous to a domain in a *C. elegans* protein, Y43F4B.7, and a related protein, DOC-1R (15). (B) In vitro association of the A1, A2, and A3 mutants with monomeric CDK2 in 293 cell lysate. One microgram of GST or GST-p12^{DOC-1} wild-type or mutant protein was mixed with 200 μg of 293 cell lysate. The CDK2 immunoblot was done using anti-CDK2 antibody (C18520 Clone 55; Transduction Laboratories).

coelution of p12^{DOC-1} and CDK2 in fractions 21, 22, and 23. Note that p34CDK2 is the predominant form of CDK2 comigrating with p12^{DOC-1} in fractions 21 to 23. Some p33CDK2 apparently comigrates with p12^{DOC-1} as well, as a minor component. Thus, for both normal lung cells and the HaCaT cells, p12^{DOC-1} and CDK2 coelute in gel filtration. The elution profiles are very similar. The elution profile from the HaCaT cells further supports the comigration of p12^{DOC-1} with p34CDK2. In addition, in both lung cells and HaCaT cells, CDK2 was also detected in fractions 12 to 14 (data not shown), which may represent a high-molecular-mass (~200-kDa) CDK2 complex with other CDK2 binding proteins. Cyclins A and E coelute with CDK2 in fractions 12 to 14.

Amino acids 109 to 111 of p12^{DOC-1} are critical for CDK2 association. To verify the specificity of p12^{DOC-1}'s association with monomeric CDK2, we identified a domain in p12^{DOC-1} that is critical for its association with CDK2. Deleting the 12 amino acids [p12^{DOC-1}(1–103)] from the C-terminal region negates CDK2 association, and deleting the last 4 amino acid residues [p12^{DOC-1}(1–111)] retains CDK2 association (Fig. 4A). This thus identified the region from amino acid 103 to 111 as containing amino acids that are critical for association with CDK2. The eight amino acids in this region were mutagenized in sets of three (A1, A2, and A3). These mutants were expressed as GST fusion proteins and were used to determine their ability to associate with CDK2 in 293 cell lysate. Both A1 and A2, but not A3, mutants retained CDK2 association (Fig. 4B). The inability of the A3 mutant to interact with CDK2 was verified in cells by cotransfecting 293 cells with pCDK2 and either the FLAG-tagged-DOC-1 wild type (pFLAG-DOC-1-wt) or the A3 mutant (pFLAG-DOC-1-A3). Figure 2A and B show that the p12^{DOC-1}-A3 mutant is expressed but did not interact with CDK2 (lanes 5 to 8).

Ectopic expression of p12^{DOC-1} suppressed CDK2-associated kinase activities. Cellular CDK2 kinase activity at any moment is largely a reflection of intracellular levels of cyclin E-associated CDK2 (G_1/S) and cyclin A-associated CDK2 (S phase) (6–9). We evaluated the effect of ectopic p12^{DOC-1} expression on cellular CDK2 levels and CDK2-associated kinase activities in 293 cells. The p12^{DOC-1}-A3 mutant was similarly ectopically expressed in parallel experiments to determine if any observed alteration in CDK2-mediated biochemical activities requires association with p12^{DOC-1}. Figure 5A shows cellular levels of CDK2, FLAG-p12^{DOC-1}-wt,

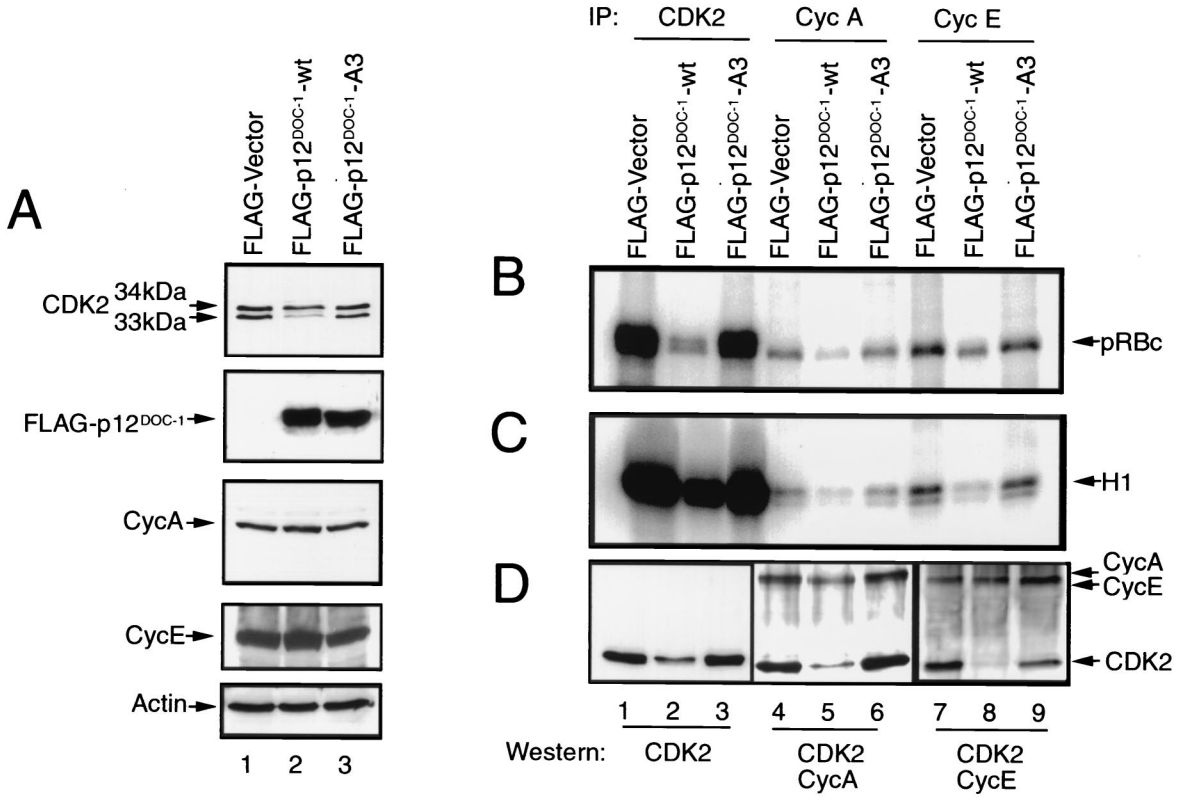

FIG. 5. Ectopic expression of p12^{DOC-1} and CDK2 kinase activity in 293 cells. (A) Cellular levels of CDK2, FLAG-p12^{DOC-1}-wt, FLAG-p12^{DOC-1}-A3, cyclin A, cyclin E, and actin in control vector and p12^{DOC-1} transfectants. The samples were run on long SDS-PAGE gels to resolve the 33- and 34-kDa CDK2 bands. (B and C) In vitro phosphorylation using GST-pRBc and histone H1, respectively, as substrates. (D) CDK2, cyclin A, and cyclin E immunoblots to show intracellular levels of these proteins in p12^{DOC-1}-wt (lanes 2, 5, and 8), p12^{DOC-1}-A3 (lanes 3, 6, and 9), and control transfectants (lanes 1, 4, and 7). Lanes 1, 2, and 3, immunoblot for CDK2; lanes 4, 5, and 6, immunoblot for CDK2 and cyclin A; lanes 7, 8, and 9, immunoblot for CDK2 and cyclin E. Signals were quantified by exposing the probed membranes to a quantitative imaging system (Fluor-S MAX MultiImager; Bio-Rad). IP, immunoprecipitation.

FLAG-p12^{DOC-1}-A3, cyclin A, cyclin E, and actin in control vector, p12^{DOC-1}-wt, and p12^{DOC-1}-A3 transfectants. Consistent with the in vitro biochemical data that p12^{DOC-1} associates predominantly with p34CDK2, the ratio of the p34CDK2 to the p33CDK2 forms of CDK2 is altered in the p12^{DOC-1} transfectants. In control vector transfectants, the ratio of p34CDK2 to p33CDK2 is ~1:1, while in the p12^{DOC-1} transfectants, the ratio is ~2:1. The ratio is restored to 1:1 in cells ectopically expressing the p12^{DOC-1}-A3 mutant, indicating that the ability of p12^{DOC-1} to associate with CDK2 is necessary for the observed CDK2 alteration. The steady-state cellular level of CDK2 is reduced to about half in the 293 cells overexpressing p12^{DOC-1}. The cellular levels of cyclins A and E are, however, similar in the control and p12^{DOC-1} (wild-type and A3) transfectants (Fig. 5A).

The cellular biochemical effect of ectopic p12^{DOC-1} expression on CDK2-associated kinase activities was examined by immunoprecipitating total CDK2, cyclin A-associated kinases (CDK2 and CDC2), and cyclin E-associated CDK2 from vector control, p12^{DOC-1}-wt, and p12^{DOC-1}-A3 transfectants. The immunoprecipitated complexes were used to phosphorylate known CDK2 substrates, pRBc (a carboxyl-terminal fragment of pRB) and histone H1, in separate reactions. Figure 5B shows that ectopic expression of p12^{DOC-1}-wt reduces the CDK2-mediated phosphorylation of pRBc by approximately fivefold (lane 2). Similar reductions were observed for cyclin A-associated kinases (approximately threefold; lane 5) and cyclin E-associated CDK2 (approximately threefold; lane 8). Figure 5C shows parallel phosphorylation reactions with histone H1 as the substrate, demonstrating that the ectopic expression of p12^{DOC-1} also suppressed CDK2-associated histone H1 kinase activity (approximately threefold; lane 2). Similar suppressions were seen in cyclin A-associated histone H1 kinase (approximately twofold; lane 5) and cyclin E-associated histone H1 kinase activities (approximately threefold; lane 8). Ectopic expression of the p12^{DOC-1}-A3 mutant showed kinase activity profiles similar to that of the control vector, indicating that association with p12^{DOC-1} is necessary for the observed alterations in CDK2-associated kinase activities (Fig. 5B and C, lanes 3, 6, and 9). These experiments have been independently repeated three times. Consistently we observed the relatively low levels of cyclin A- and cyclin E-associated CDK2 kinase activities in the 293 cells. Fig. 5D shows immunoblots to demonstrate the cellular levels of CDK2 alone and CDK2 associated with cyclins A and E in the transfectants. Cyclin A- and cyclin E-associated CDK2, however, are reduced by four- and fivefold, respectively (Fig. 5D, lanes 5 and 8). Steady-state cellular CDK2 levels are reduced by approximately twofold in p12^{DOC-1}-wt transfectants (Fig. 5D, lane 2). These results suggest that ectopic expression of p12^{DOC-1}-wt reduced CDK2-associated kinase activities, perhaps in part due to a decrease in CDK2 as a consequence of ectopic p12^{DOC-1}-wt expression. This may suggest a role of p12^{DOC-1} in targeting CDK2 for proteolysis. The reduction of CDK2 coprecipitating with cyclins A and E further suggests that the association of p12^{DOC-1} with monomeric nonphosphorylated

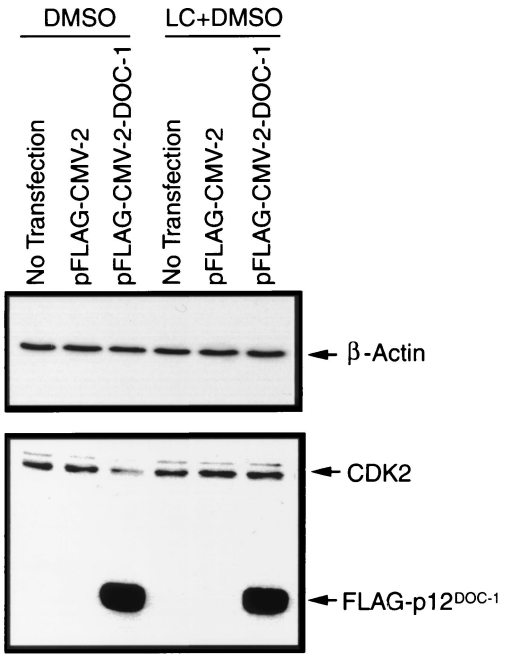

FIG. 6. $p12^{DOC-1}$ targets CDK2 for proteolysis. 293 cells were transfected with the pFLAG vector or pFLAG-DOC-1 in the presence or absence of the proteosome inhibitor *clasto*-lactacystin β-lactone solubilized in dimethyl sulfoxide (5 μM) for 24 h. Top panel, immunoblot for β-actin to quantify proteins loaded. Bottom panel, immunoblot for CDK2 and FLAG-$p12^{DOC-1}$.

CDK2 may interfere or compete with binding of cyclins A and E to CDK2. It should be noted that the $p12^{DOC-1}$-mediated suppression of CDK2-associated kinase activities is specific. In similar experiments involving immunoprecipitation of CDK4 and CDK6, ectopic expression of $p12^{DOC-1}$ did not alter the phosphorylation pattern of pRBc or that of histone H1 (data not shown). To test the possibility that the $p12^{DOC-1}$-mediated decrease in CDK2 levels took place through proteosome-dependent proteolysis, 293 cells were transfected in the presence or absence of the proteosome inhibitor *clasto*-lactacystin β-lactone. Figure 6 shows that the $p12^{DOC-1}$-mediated proteolysis of CDK2 was averted when the transfectants were grown in the presence of the proteosome inhibitor (5 μM; 24 h).

Ectopic expression of $p12^{DOC-1}$ suppresses CDK2-mediated cell cycle phenotypes. To gain insight into the potential role of $p12^{DOC-1}$ with CDK2-mediated cell cycle phenotypes, we examined the effect of ectopic expression of $p12^{DOC-1}$ in 293 cells. Our data predict that the effect of $p12^{DOC-1}$'s interaction with CDK2 will likely lead to the reduction of the intracellular pool of active CDK2, resulting in the negative regulation of CDK2-mediated cell cycle phenotypes (G_1/S transition, S phase progression, and DNA replication). We indeed observed suppression of CDK2-mediated cell cycle phenotypes in cells ectopically expressing $p12^{DOC-1}$ (Fig. 7). We consistently observed that ectopic $p12^{DOC-1}$ expression in cells (Fig. 7A) was associated with growth suppression (Fig. 7B), changes in the cell cycle profile ($\uparrow$ G_1 and $\downarrow$ S) ($P < 0.05$) (Fig. 7C), and reduction of tritiated thymidine incorporation ($P < 0.05$) (Fig. 7D).

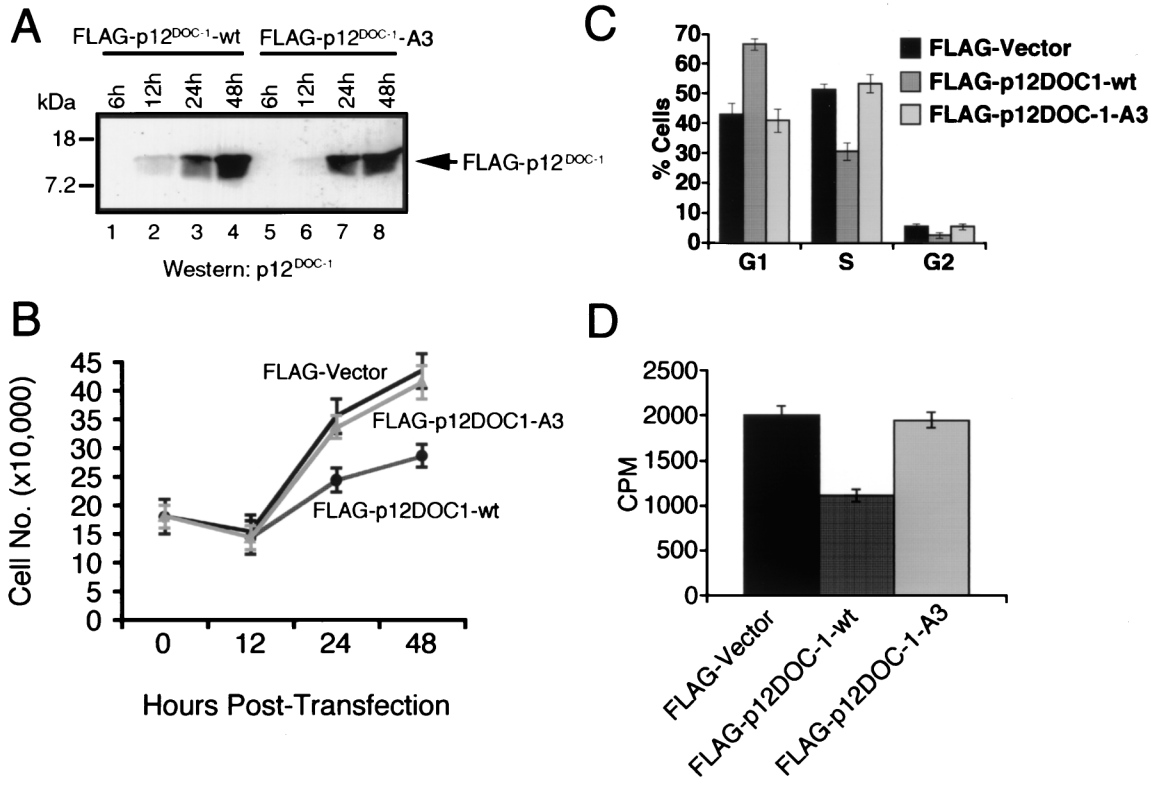

FIG. 7. Effect of ectopic expression of $p12^{DOC-1}$ on CDK2-mediated phenotypes in 293 cells. (A) Immunodetection of FLAG-$p12^{DOC-1}$ expression in 293 cells. (B) Effect of $p12^{DOC-1}$ ectopic expression on cell growth at 12, 24, and 48 h posttransfection. (C) Cell cycle positions at 48 h posttransfection. (D) Tritiated thymidine incorporation at 48 h posttransfection. For panels A and B, 293 cells were transfected with pFLAG vector or pFLAG-DOC-1 for the indicated time points and analyzed. For panels C and D, 293 cells were cotransfected with a neomycin expression vector (pcDNA3) and selected for 2 weeks in the presence of G418 at 400 μg/ml. Data are from three independent experiments. Transfection efficiency of 293 cells with Lipofectamine Plus (Life Technologies/Gibco-BRL, Grand Island, N.Y.) is ~70%.

DISCUSSION

We have identified a novel biological partner of CDK2, $p12^{DOC-1}$. The discovery was aided in part by the observation that overexpression of $p12^{DOC-1}$ is associated with suppression of growth in mammalian cells. Using a GST-$p12^{DOC-1}$ fusion protein, we have found it to interact with CDK2 in 293, HeLa, and A431 cells. The CDK2 that interacts with $p12^{DOC-1}$ in 293 cells was found to be the monomeric nonphosphorylated form. Mutation analysis revealed that amino acids 109 to 111 of $p12^{DOC-1}$ are critical for CDK2 association.

While the initial finding that $p12^{DOC-1}$ interacts with CDK2 was made with the 293, HeLa, and A431 cells, none of these cells expressed detectable endogenous $p12^{DOC-1}$. As indicated in Fig. 3A, the expression of $p12^{DOC-1}$ is restricted to normal tissues. No tumor tissues or transformed cell lines have been found to express $p12^{DOC-1}$ (35 were examined). Only recently have we found that the human epithelial cells HaCaT express detectable $p12^{DOC-1}$. The lack of detectable $p12^{DOC-1}$ in tumor cell lines and tissues is perhaps a reason why this low-molecular-weight CDK2-associated protein has not yet been found to interact with CDK2 by standard approaches, such as yeast hybrid screens or coprecipitation. In addition, it may be possible that the expression of $p12^{DOC-1}$ is associated with growth inhibition, such as quiescence.

Overexpression of $p12^{DOC-1}$ altered the cellular equilibrium of the inactive (p34) and active (p33) forms of CDK2, apparently by sequestering the inactive form and/or target CDK2 for proteolysis. This effect was nullified when the $p12^{DOC-1}$-A3 mutant was transfected into the 293 cells. The amino acids 109 to 111 were mutated in this $p12^{DOC-1}$-A3 mutant, which also ablated its binding to CDK2, suggesting that the association of $p12^{DOC-1}$ with CDK2 increases the inactive form of CDK2. Our data support $p12^{DOC-1}$'s specific association with the monomeric nonphosphorylated form of CDK2. $p12^{DOC-1}$ may act on the biochemical pathway of CDK2 activation at or before association with cyclins E and A. This idea is based on our data that neither cyclin E nor cyclin A was detected in the GST-$p12^{DOC-1}$ cellular lysate or the immunoprecipitated FLAG-$p12^{DOC-1}$–CDK2 complexes. In addition, $p12^{DOC-1}$ has no effect on CDK2-associated kinase activities following association with cyclin E or A in vitro (data not shown). Finally, $p12^{DOC-1}$ does not affect CDK2's downstream phosphorylation by WEE1 and CAK. WEE1 and CAK kinase activities are not affected in the presence of $p12^{DOC-1}$ (data not shown). The net effect of $p12^{DOC-1}$'s interaction with CDK2 is therefore likely to be the reduction of the intracellular pool of active CDK2, leading to the suppression of CDK2-mediated cell cycle phenotypes (G_1/S transition, S phase progression, and DNA replication).

Our data in Fig. 5 and 6 also support a role for $p12^{DOC-1}$ in targeting p33CDK2 for proteolysis. Ectopic expression of $p12^{DOC-1}$ in 293 cells causes a decrease of CDK2 through proteosome-dependent proteolysis (Fig. 6). These two mechanisms (sequestering p34CDK2 and targeting p33CDK2 for proteolysis), independently or in combination, can reduce cellular levels of p33CDK2 and the associated CDK2-mediated biological activities.

The $p12^{DOC-1}$-A3 mutant was identified by its inability to retain CDK2 association in vitro. While amino acids 109 to 111 (TER) are of importance in mediating CDK2 association, there are likely to be additional sites in $p12^{DOC-1}$ that are involved in CDK2 interaction. Chen et al. have recently aligned a number of cyclin-CDK-binding proteins ($p45^{SKP2}$, E2F-1, E2F-2, E2F-3, p107, p130, p21, p27, and p57) and identified an "RxL" motif in the C terminus that is of importance in cyclin-CDK binding (1, 2). Conversion of the "RxL" motif of p27 to "AxA" abolishes its interaction with cyclin-CDK. It is interesting to note that in both $p12^{DOC-1}$ and the related protein DOC-1R (15), there is an "RxL" motif seven amino acids N terminal to the identified "TER" CDK2 interaction site. The amino acid sequence in this region in $p12^{DOC-1}$ is RGLVRECLAETERNAR, and that for DOC-1R is RALVRECLAETERNAR. This region resides in the C-terminal domains of both proteins, which share significant homology with a domain in a *Caenorhabditis elegans* protein, Y43F4B.7. We have mutated the RAL region in $p12^{DOC-1}$ to AAA and found that the resultant $p12^{DOC-1}$(R112A/L114A) mutant binds to CDK2 no differently than wild-type $p12^{DOC-1}$ (data not shown), suggesting that this motif is likely not to be involved in cyclin-CDK2 association.

It will be of importance to know the region of CDK2 that $p12^{DOC-1}$ binds to. While we have not yet mapped the $p12^{DOC-1}$ interactive domains in CDK2, a set of nine CDK2 point mutants (for CDK2-30, point mutants V30A, A31F, and L32A; for CDK2-33, K33A; for CDK2-38, D38A and E40A; for CDK2-145, D145N; for CDK2-150, R150A, A151F, and F152A; for CDK2-159, Y159A and T160D; for CDK2-204, P204A, D206A, D208A, and D210A; for CDK2-217, R217A; and for CDK2-250, P250L) was obtained to determine whether any of these mutations will affect $p12^{DOC-1}$ binding (3). Expressing $p12^{DOC-1}$ and CDK2, wild type and mutants, by in vitro transcription and translations revealed that all nine CDK2 mutants and the wild type coprecipitated with $p12^{DOC-1}$ (data not shown). Thus, these nine mutations do not affect $p12^{DOC-1}$ binding. Efforts are in progress to use a conventional strategy similar to the one employed in this study to map the region in $p12^{DOC-1}$ to which CDK2 binds.

While we have demonstrated interaction of endogenous $p12^{DOC-1}$ and CDK2 in the normal lung lysates, the actual physiological relevance and contribution of $p12^{DOC-1}$ to CDK2 biology is currently being studied. We have recently identified cellular models (such as the HaCaT cells) that will permit the biochemical and functional interactions of endogenous $p12^{DOC-1}$ and CDK2 to be examined.

Our data support that $p12^{DOC-1}$ is a specific CDK2-associated protein capable of negative regulation of CDK2-associated activities. Our data suggest that $p12^{DOC-1}$ negatively regulates CDK2 through a mechanism that is different from that of the $p16^{INK4a}$ and $p21^{WAF1/CIP1/CAP20}$ families of CDK inhibitors (11). It is likely that $p12^{DOC-1}$ sequesters the monomeric form of CDK2 prior to its association with cyclins E and A and/or targets CDK2 for proteolysis. Studies are in progress to detail these mechanisms as well as the physiological relevance of the interactions between $p12^{DOC-1}$ and CDK2 in cells. It is unclear what upstream signals regulate $p12^{DOC-1}$. Perhaps regulatory signals in G_1/S transition, S phase progression, and DNA replication may feed into this regulation pathway. It is intriguing to note that while the $p21^{WAF1/CIP1/CAP20}$ family of CDK inhibitors is universal for CDKs and the $p16^{INK4a}$ family is specific for CDK4 and CDK6, $p12^{DOC-1}$ may be a specific CDK2-associated protein that suppresses CDK2 activities.

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