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Low Levels of Cyclin D and Nonfunctional Rb Protein Affect cdk6 Association with Cyclin-Dependent Kinase Inhibitor p27^{Kip1}

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p27^{Kip1} associates with cyclin/cdk complexes and inhibiting cdk activity, and overexpression of p27^{Kip1} induces G1 arrest. We found that p27^{Kip1} overexpression inhibits cdk2 kinase activity, but not cdk6 kinase activity in HeLa cells. The amount of p27^{Kip1} associated with cdk2 was significantly higher than that associated with cdk6. cdk6 complexes contained detectable amounts of p27^{Kip1} in all human cell lines examined, except in HeLa cells where p27^{Kip1} preferentially associated with cdk2. It appears that in HeLa cells overexpressed p27^{Kip1} fails to inhibit cdk6 kinase activity because of low binding affinity of cdk6 to p27^{Kip1}. The low binding affinity is due to a low level of the cdk6/cyclin D complexes. Functional inactivation of pRb has an effect on p27^{Kip1} association with cdk6/cyclin D complexes.

The progression of eukaryotic cells through the cell cycle is regulated by the activation and inactivation of cyclin/cdk (cyclin-dependent kinase) complexes (1–3). The catalytic activity of the cyclin/cdk complexes is rigidly controlled by several complicated mechanisms one of which involves a family of low molecular weight inhibitors (4). The inhibitor p27^{Kip1} is induced by a process linked to cell contact inhibition and upon treatment with TGFβ, staurosporine, or rapamycin. This inhibitor is known to bind tightly to cyclin E, A/cdk2 and cyclin D/cdk4, cdk6 complexes where it blocks the activation of the cyclin-dependent kinase (5–9). The kinase activity of fully functional cyclin/cdk complexes is thus directly inhibited by p27^{Kip1} (9). We have shown previously that the cdk2 binding domain of p27^{Kip1} is involved in the inhibition of the kinase activity of cdk2/cyclin complexes (10).

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Several recent reports have demonstrated that ectopic expression of p27^{Kip1} in mammalian cells leads to G1 phase arrest and that antisense oligonucleotides of p27^{Kip1} can prevent mitogen-starved fibroblast from becoming quiescent (6, 7, 11). Furthermore, the phenotype of p27^{Kip1} deficient mice is increased in size and increased cellular proliferation (12). Little is known, however, about the details of p27^{Kip1} mediated kinase inhibition and the effect of overexpression of p27^{Kip1}. In this study, we show that p27^{Kip1} overexpressed in HeLa cells failed to inhibit cdk6 kinase activity due to a low binding affinity of cdk6 for p27^{Kip1}.

MATERIALS AND METHODS

Cell culture and materials. The murine BW5147 thymoma cell line, MDA361, HeLa, U937, THP-1, HepG2, K562 cells were obtained from the ATCC (Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle medium containing 10% FCS, 20 mM Hepes, 100 μg/ml gentamicin (complete medium). Anti-cdk2, anti-cdk6 and anti-p27^{Kip1} antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-cyclin D was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal anti-Rb was purchased from Pharmingen (San Diego, CA). Lysine-rich histone H1 was purchased from United States Biochemical Corporation (Cleveland, OH) and protein G-agarose from Calbiochem (La Jolla, CA).

Immunoprecipitation, Western blotting and binding assay. Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM Mops, 2 μg/ml proteinase inhibitor E64, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. For immunoprecipitation, 150 μg of cellular lysate was reacted with 2 μg of antibody at 4°C for 1 h. The immune complexes were recovered by the addition of protein G-agarose beads. For the binding assay, 5 μg of GST-p27^{Kip1} fusion proteins was combined with 30 μl of glutathione conjugated Sepharose beads and incubated for 30 min at 4°C. The beads were mixed with 150 μg of HeLa lysate or MDA361 lysate in 200 μl of binding buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) and incubated for 1 h at 4°C. The samples were then washed and resuspended in Laemmli buffer, boiled for 5 min, and analyzed on 10% SDS polyacrylamide gel. The

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proteins were electrotransferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA). Detection of specific proteins was carried out with an ECL kit according to manufacturer's instructions. The preparation of the GST-p27^{Kip1} fusion protein has been described previously (10).

Kinase assay. Immunoprecipitates prepared from 100 μg of cell lysate, as described above, were assayed for histone H1 and Rb-p56 kinase activity (13). Briefly, immune complexes were incubated with HB buffer (60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 15 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 80 mM NaCl, 2 μg/ml proteinase inhibitor E64, 5 mM EGTA, 25 mM Mops pH 7.2) containing 50 μM ATP, 5 μCi [γ-^{32}P] ATP (10 Ci/mM: NEN Dupont, Wilmington, DE), and 1 μg of the Rb-p56 protein at 30°C for 30 min. The reactions were terminated by addition of 15 μl of SDS–PAGE sample buffer. The cdk7 kinase assay was conducted by the same ways as described above expect that GST-cdk2 fusion protein was used as substrate. Protein phosphorylations were analyzed by 10% SDS–PAGE and detected by autoradiography after exposure at −70°C.

Reverse transcription-polymerase chain reaction (RT-PCR) and DNA sequence analysis. Total RNA was extracted from 10 million HeLa cells and MDA361 cells using the Ambion Total RNA kit according to the manufacturer's specifications. Two micrograms of total RNA was reverse transcribed (RT superscript, GIBCO) using random hexamers. The cDNA in 2 μl of each reaction was then amplified using 1 unit *Taq* polymerase and the following cdk6 oligonucleotide primers: 5′-TAGTTGAGCACCCCCCGGGCGC-3′(upstream primer), 5′-TAAAGTTAGTTTGAATTCTCTGTCTG-3′ (downstream primer). The upstream oligonucleotide primer contains a *Sma*I site and the downstream primer an *Eco*RI site. PCR proceeded for 30 cycles under the following conditions: annealing at 57°C (45 s), extension at 72°C (1 min), and denaturation at 94°C (45 s) using a Perkin–Elmer Thermocycler. One-tenth of each reaction mixture was electrophoresed on 1% agarose. Sequencing was performed by the dideoxynucleotide chain termination procedure on double stranded plasmid DNA with Sequenase kit version 2.0 (Gibco BRL, Gaithersburg, MD).

RESULTS

p27^{Kip1} Overexpression Inhibits cdk2 Kinase Activity, but Not cdk6 Kinase in HeLa Cells

As shown previously (14), ectopic expression of p27^{Kip1} induces a G1 arrest that is associated with the inhibition of cdk2 kinase activity. Overexpression of cyclin E rescue p27^{Kip1} mediated cdk2 kinase activity and G1 growth arrest. In our effort to understand the physiological role of p27^{Kip1}, we transfected the mouse p27^{Kip1} gene into the human HeLa cells. To establish that p27^{Kip1} overexpression had no adverse effect on the cellular levels of cdk2 and cdk6, lysates from HeLa cells transient transfected with vector alone or vector containing p27^{Kip1} cDNA were allowed to react with specific anti-p27^{Kip1}, anti-cdk2, and anti-cdk6 antibodies and the immune complexes analyzed by Western blot (Fig. 1). The results showed that p27^{Kip1} overexpression did not effect cdk2 and cdk6 expression levels.

To determine whether there was an effect of p27^{Kip1} overexpression on cdk activity, we assayed the lysate derived from the transfected cells for cdk2 and cdk6 kinase activity. The catalytic activity of cdk2 and cdk6 immunoprecipitated from 70 μg of the transfected cell

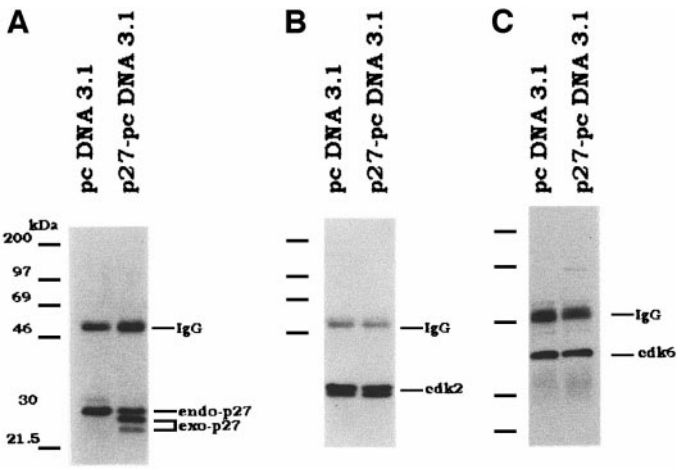

FIG. 1. Expression levels of p27^{Kip1}, cdk2, and cdk6 in cells transfected with p27^{Kip1} cDNA. Lysates (100 μg) of HeLa cells transiently transfected with vector alone or vector containing p27^{Kip1} cDNA were immunoprecipitated with specific anti-p27^{Kip1} (A), anti-cdk2 (B), and anti-cdk6 (C) and subjected to Western blot analysis. The data represent a typical experiment conducted two times with comparable results.

lysate was determined using Rb-p56 as substrate. Kinase activity of cdk2 was also determined using histone H1 as substrate (Fig. 2). Cells transfected with p27^{Kip1} exhibited threefold lower cdk2 kinase activity on Rb-p56 and histone H1 in comparison to just empty vector transfected cells. Interestingly, cdk6 kinase activity in the p27^{Kip1} transfected cells was not inhibited.

p27^{Kip1} Preferentially Associates with cdk2 in HeLa Cells

The results shown in Fig. 2 suggested that p27^{Kip1} had either no inhibitory activity that could effect cdk6 or a very low binding affinity for cdk6 in HeLa cells. In an effort to understand why the overexpressed p27^{Kip1} in HeLa cells failed to inhibit cdk6 kinase activity, we first tested the effect of various amounts of GST-p27^{Kip1} fusion protein added directly to immunoprecipitated cdk6 holoenzyme. As shown in Fig. 3, kinase activity was reduced significantly in presence of 5 μg of GST-p27^{Kip1} fusion protein. We then compared the levels of p27^{Kip1} associated with cdk2 and cdk6 in various cell lines. We employed one mouse cell line and 5 human cell lines. Equal amounts of lysates (150 μg) from murine BW5147, HeLa, MDA361, K562, U937, and HepG2 cells were immunoprecipitated with anti-cdk2 and anti-cdk6. The amounts of p27^{Kip1} associated with cdk2 and cdk6 were then analyzed by Western blot probed with anti-p27^{Kip1} (Fig. 4A). The protein amounts of cdk2, cdk6, and p27^{Kip1} were also confirmed by Western blot (Fig. 4B). As shown in Fig. 4B, there was no difference in the level of cdk6 expression between the cell lines. However, in all cell lines, except HeLa, the

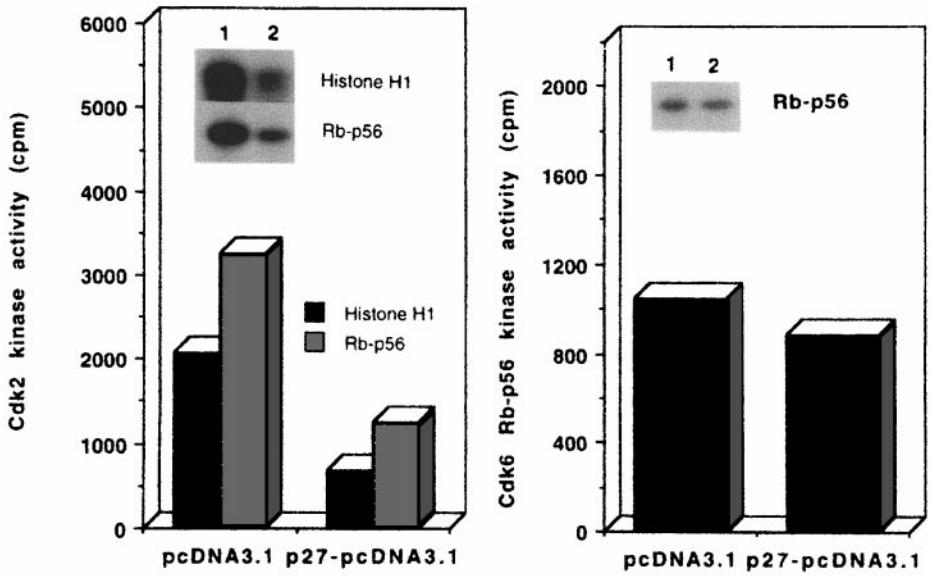

FIG. 2. $p27^{Kip1}$ overexpression inhibits cdk2 kinase activity, but not cdk6 kinase activity in HeLa cells. HeLa cells were grown to about 50% confluence and then transfected with vector alone (lane 1) or vector containing $p27^{Kip1}$ cDNA (lane 2). After 48 h, the cells were harvested and whole cell extracts were prepared. Equal amounts of whole cell lysate (70 μg) from transfected cells were treated with anti-cdk2 and anti-cdk6 and immunoprecipitates assayed for kinase activity using Rb-p56 and histone H1 as substrate. The reaction mixtures were analyzed by SDS–PAGE, and cdk2 and cdk6 kinase activities were determined by autoradiography (in box). The gel was then scanned for β emissions by phosphoimaging. The cdk2 and cdk6 kinase activities reported the mean of at least three experiments.

cdk6 complexes were associated with detectable levels of $p27^{Kip1}$. These results indicate that, in HeLa cells, cdk6 have a low binding affinity for $p27^{Kip1}$.

The association of $p27^{Kip1}$ with cdks is enhanced in the presence of a cyclin partner (15). To determine whether cyclin D effected $p27^{Kip1}$ association with cdk6 in HeLa cells, we looked at the levels of $p27^{Kip1}$ associated with cyclin D, cdk2, and cdk6 in HeLa cells and MDA361 cells. Equal amounts of lysate (150 μg) of HeLa cells and MDA361 cells were used in immunoprecipitation with anti-$p27^{Kip1}$ antibody. The immunoprecipitates then underwent Western blot analysis with anti-cdk2, anti-cdk6, anti-cyclin D, and anti-$p27^{Kip1}$ antibodies. The amount of cdk6 and cyclin D associated with $p27^{Kip1}$ in MDA361 immune complexes was significantly higher than that in the HeLa complexes (Fig. 5). The level of $p27^{Kip1}$ was confirmed by reprobing the erased membrane with anti-$p27^{Kip1}$ antibody. The data suggested that the monomeric cdks had less affinity for $p27^{Kip1}$ than the cyclin/cdk complexes. To further analyze why $p27^{Kip1}$ preferentially associated with cdk2 in HeLa cells, we assayed GST-$p27^{Kip1}$ binding to cdk2 and cdk6 in HeLa cell lysates and compared the results to MDA361 cell lysates as the control. GST-$p27^{Kip1}$ fusion protein (5 μg) was immobilized on glutathione-Sepharose beads and incubated with 150 μg of either HeLa or MDA361 cell lysate. After the beads had been washed, the bound proteins were analyzed by immunoblot using a cdk6 specific antibody (Fig. 6A). To identify the GST-$p27^{Kip1}$ associated cdk2, the same membrane was then stripped and reprobed with anti-cdk2 antibody (Fig. 6B). The GST-$p27^{Kip1}$ fusion protein exhibited decreased binding to HeLa cell cdk6 compared to MDA361 cdk6. However,

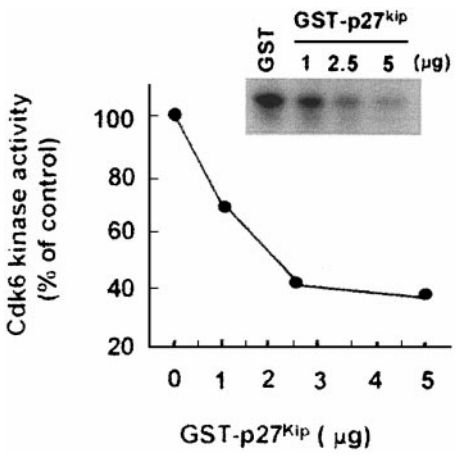

FIG. 3. $p27^{Kip1}$ inhibits cdk6 kinase activity. MDA361 whole cell lysate (70 μg) was allowed to react with anti-cdk6 and then added to an Rb-p56 kinase reaction mixture containing 5 μg GST alone or the indicated amounts (1, 2.5, and 5 μg) of GST-$p27^{Kip1}$. The reaction mixtures were analyzed by SDS–PAGE, and cdk6 kinase activity was determined by autoradiography. The bottom panel shows cdk6 Rb-p56 kinase activity. The gel was then scanned for β emissions by phosphoimaging, and the results are shown in the bottom panel. The cdk6 activities reported the mean of at least three experiments.

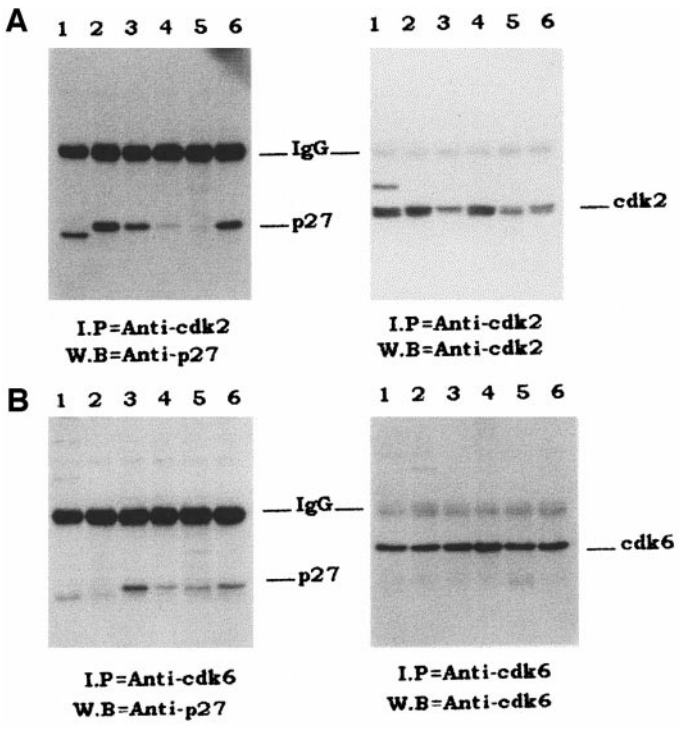

FIG. 4. Comparison of $p27^{Kip1}$ association with cdk2 and cdk6 in mouse and human cell lines. Equal amounts of lysate (150 μg) from murine BW5147 (lane 1), HeLa (lane 2), MDA361 (lane 3), K562 (lane 4), U937 (lane 5), and HepG2 (lane 6) were immunoprecipitated with anti-cdk2 (A) and anti-cdk6 (B). The amounts of $p27^{Kip1}$ associated with cdk2 and cdk6 were determined by Western blot analysis with anti-$p27^{Kip1}$ antibody. The cdk2 and cdk6 expression levels in each cell line were confirmed by reprobing the erased membrane with anti-cdk2 (A) and ant-cdk6 (B). The data represent a typical experiment conducted two times with comparable results.

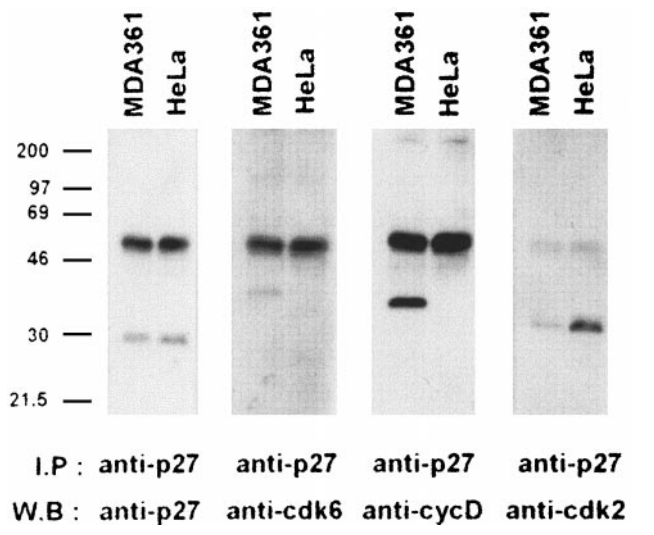

FIG. 5. Comparison of cdk2, cdk6, and cyclin D1 binding levels in $p27^{Kip1}$ immunoprecipitated complexes of HeLa and MDA361 cells. Equal amounts of lysate (150 μg) from HeLa cells and MDA361 cells underwent immunoprecipitation with anti-$p27^{Kip1}$. The levels of cdk6, cdk2, and cyclin D1 associated with $p27^{Kip1}$ were analyzed by Western blot probed with anti-cdk2, anti-cdk6, anti-cyclin D1, and anti-$p27^{Kip1}$. The data represent a typical experiment conducted two times with comparable results.

cdk2 binding to GST-$p27^{Kip1}$ showed completely opposite results, indicating that $p27^{Kip1}$ preferentially associated with cdk2 in HeLa cells. Therefore, the results suggest that the overexpressed $p27^{Kip1}$ in HeLa cells may fail to inhibit cdk6 kinase activity due to a lower cdk6 binding affinity for $p27^{Kip1}$.

The $p27^{Kip1}$ Binding Region of cdk6 Is Not Mutated in HeLa Cells

Recently, Wolfel *et al.* reported that, in human melanoma cells, a cdk4 mutant containing an arginine to cysteine substitution at the amino acid 24 position was unable to associate with p16 (16). A mutant from of cdk6 (R31C) with a mutation in the KARD domain failed to associate with p18, another cdk4 and cdk6 inhibitor (17). To further understand the nature of the decreased association of cdk6 with $p27^{Kip1}$ in HeLa cells, we wanted to determine if the cdk6 gene in HeLa cells was mutated in the $p27^{Kip1}$ binding domain. To address this question, we sequenced RT-PCR amplified DNA from HeLa cells and MDA361 cells which served as the positive control. The amplified RT-PCR products were then cloned into the pBS(+) vector and sequenced. We could not find a mutation in the region of the cDNA encoding amino acids 1–135 of cdk6 (negative data not shown). However, we can not rule out the possibility that cdk6 in HeLa cells might be mutated somewhere in the region down wards from amino acid 136 to the C terminus.

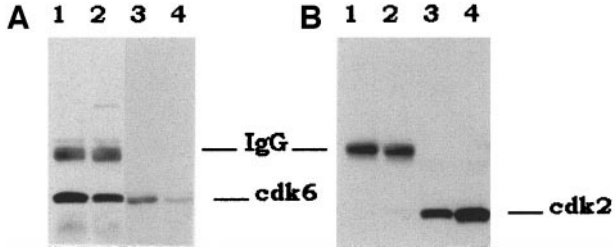

FIG. 6. $p27^{Kip1}$ preferentially associates with cdk2 in HeLa cells. (A) Equal amounts of lysate (150 μg) derived from HeLa cells (lane 1) and MDA361 cells (lane 2) were allowed to react with anti-cdk6. Equal amounts of lysate (150 μg) derived from HeLa cells (lane 3) and MDA361 cells (lane 4) were reacted with glutathione Sepharose beads conjugated with GST-$p27^{Kip1}$. The cdk6 protein bound to the glutathione Sepharose beads was determined after SDS–PAGE by immunoblot probed with anti-cdk6. (B) The amount of cdk2 protein bound to GST-$p27^{Kip1}$ was determined by stripping the membrane of the probe used in (A) by probing it with anti-cdk2. The data represent a typical experiment conducted two times with comparable results.

$p27^{Kip1}$ Association with cdk6 in Rb-Nonfunctional Cell Lines

As shown in Fig. 5, HeLa cells contain less or no cdk6/cyclin D complexes. Bates *et al.* reported that they observed cdk6/cyclin D complexes in many of the cells, but no complexes in cells lacking functional Rb protein (18). To test whether functional Rb protein had any effect on $p27^{Kip1}$ binding to cdk6, we employed one cell line (MDA361) with functional Rb as the positive control and 4 human cell lines (BT547, Du4475, HBL100, MDA468) lacking functional Rb. Equal amounts of lysate (150 μg) from human cells without functional Rb and from MDA361 cells were subjected to immunoprecipitation with anti-$p27^{Kip1}$ antibody. The immunoprecipitates were then analyzed by Western blot probed with anti-cdk6 and anti-$p27^{Kip1}$ antibody. Among the four human cell lines without functional Rb, we detected a cdk6/$p27^{Kip1}$ complex only in Du4475 (Fig. 7). To confirm the Rb status of the cell lines, Rb protein expression was determined by Western blot analysis with specific antibody to Rb protein.

DISCUSSION

Like most cyclin dependent kinase inhibitors, $p27^{Kip1}$ binds to cyclin/cdk complexes where it directly inhibits kinase activity (5–7). The inhibitory mechanism depends on the binding of $p27^{Kip1}$ to cdks. Other physiological functions of $p27^{Kip1}$ in the cell cycle remain to be identified. For example, recent reports suggest that $p27^{Kip1}$ acts as a kinase inhibitor at high concentrations, but functions as a facilitator of protein-protein interactions at low concentrations (19). This physiological function at low concentrations needs to be further studied. However, it is clear that for many cell types the concentration of $p27^{Kip1}$ is critical for cell cycle regulation. The results of the study presented here suggest that over-expressed $p27^{Kip1}$ in HeLa cells fails to inhibit cdk6 kinase activity due to the low binding affinity of cdk6 for $p27^{Kip1}$. Since the concentration of $p27^{Kip1}$ in the transfected HeLa cells was high, our data suggested that another factor may be involved in the inability of $p27^{Kip1}$ to inhibit cdk6 kinase activity in HeLa cells. We assume two possibilities. First, the $p27^{Kip1}$ binding site on cdk6 could be mutant, since several recent reports have demonstrated that mutations of cdk inhibitors and cdks can contribute to deregulated growth of certain cell lines and tumors (20–22). We therefore verified the DNA sequence of cdk6. Our results showed that cdk6 in HeLa cells is not mutated at least not in the sequence coding for amino acids 1 to 135. Furthermore, the cdk6 KARD domain, which is a critical binding site for p16 and p18 (17), does not appear to have an effect on $p27^{Kip1}$/cdk6 interaction in HeLa cells. A second possibility involves other

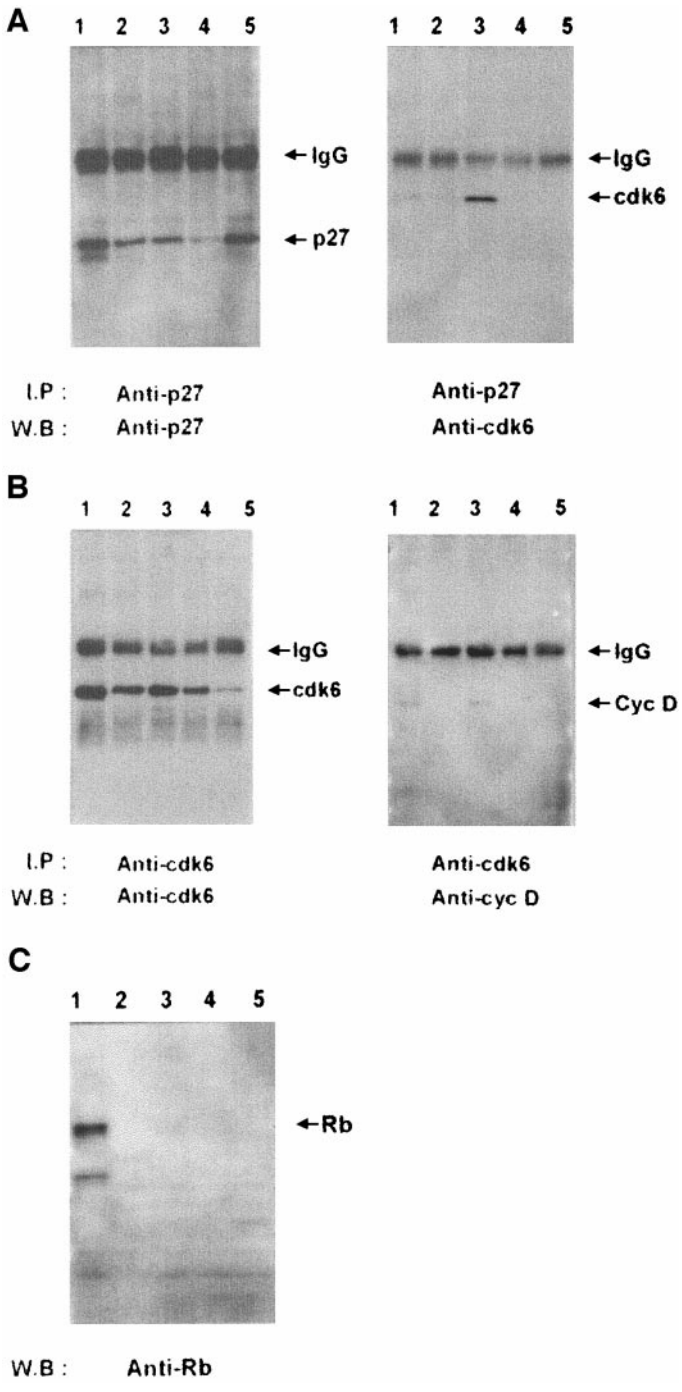

FIG. 7. $p27^{Kip1}$ association with cdk6 in Rb-nonfunctional cell lines. (A) Equal amounts of lysate (150 μg) from MDA361 (lane 1), BT549 (lane 2), and DU4475 (lane 3), HBL100 (lane 4), and MDA468 (lane 5) cells were immunoprecipitated with anti-$p27^{Kip1}$. The amount of cdk6 associated with $p27^{Kip1}$ was analyzed by immunoblot probed with anti-cdk6 antibody. (B) The cdk6 expression level of each cell lines was analyzed by immunoprecipitation and Western blot analysis with anti-cdk6 antibody. The level of cyclin D1 associated with cdk6 was determined by Western blot analysis with anti-cyclin D1. (C) Rb protein expression was determined by Western blot analysis with specific anti-Rb antibody. The data represent a typical experiment conducted two times with comparable results.

proteins that could be affecting the assembly of cdk6 and $p27^{Kip1}$ complexes. We and Haper *et al.* have reported that the association of $p27^{Kip1}$ with cdks is enhanced by the presence of a cyclin partner (13, 15). Cdk6 has been shown to be associated with cyclin D. The complex is thought to function in the G1 phase of the cell cycle. Tam *et al.* and Bartkov *et al.* have reported that low levels of cycle D1 and D3 correlate with functional inactivation of the retinoblastoma gene product (pRb) (23, 24). Cyclin D and cdk6 complexes are not detectable in cells harboring DNA tumor virus or in which the retinoblastoma gene has been deleted or mutated. Our HeLa cells and three other human cell lines (BT549, HBL100, MDA468) lacking functional Rb had no detectable $p27^{Kip1}$/cdk6 complexes. But Du4475 cells, although lacking Rb, did contain $p27^{Kip1}$/cdk6 complexes.

Taken together, the functional inactivation of pRb effects the $p27^{Kip1}$ association with cdk6/cyclin D complexes, because cell lines lacking functional pRb have low cyclin D expression levels or no cyclin D/cdk6 complexes. The correlation between lack of functional retinoblastoma protein and $p27^{Kip1}$ association with cdk6/cyclin D suggests the existence of a feedback loop between positive and negative acting components of the cell cycle regulation.

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