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Overexpression of Cyclin E and Cyclin-Dependent Kinase Inhibitor ($p27^{Kip1}$): Effect on Cell Cycle Regulation in HeLa Cells

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Transfection of the epithelioid cell line HeLa with $p27^{Kip1}$ resulted in an accumulation of cells in the G_1 phase of the cell cycle. Although the cellular level of cdk2 was not decreased as a result of this transfection, there was a significant decline in the cdk2 associated kinase activity. Restoration of the cdk2 kinase activity was proportional to the amount of cotransfected cyclin E plasmid DNA. Overexpression of cyclin E also reversed the $p27^{Kip1}$ mediated G_1 growth arrest. These findings suggest that the overexpression of cyclin E reverses the $p27^{Kip1}$ mediated G_1 growth arrest by binding the inhibitor restoring the cdk2 associated kinase activity necessary for the G_1/S transition.

Control of cell cycle progression in higher eukaryotes is regulated by a family of kinases that are associated with cyclin regulatory subunits (1-3). Progression through the mammalian cell cycle is facilitated by cyclin/cyclin dependent kinase (cdk) complexes each of which is activated at a specific point during the cell cycle. The activity of cdks is regulated by association with an activating cyclin, phosphorylation of cdk at a threonine residue (Thr 160/161) by cdk activating kinase (CAK), and the dephosphorylation of a tyrosine residue (Tyr 15) by the phosphatase cdc25 (4,5). Recently, low molecular weight protein inhibitors of cdk activities have been identified that are essential for the control of cdk activities in response to extra or intracellular signals (6,7). In mammalian cells, there are two classes of cdk inhibitor based on their structural and functional homologies. One class includes the INK-4 proteins: p15, p16, p18, and p19. These proteins prevent the formation of the cdk4 and cdk6/cyclin D complexes and contain ankyrin repeat sequences. The other class of cdk inhibitors include p21, p27 and p57 that preferentially associate with cyclin/cdk2 and cdk4 (6,7).

Cdk2 dominant negative mutant cells or cells microinjected with anti-cdk2 antibodies fail to progress to S phase, suggesting that cdk2 regulates cell proliferation by controlling cell cycle progression at a specific point in the G_1 phase (8-10). Cells arrested in G_1 as a result of contact-inhibition, serum deprivation, or exposure to rapamycin, staurosporine and transforming growth factor (TGF-β) expressed high level of $p27^{Kip1}$ and accumulate inactive cdk2/cyclin E complexes (11-14). The addition of excessive amounts of cyclin E to the lysates prepared from these G_1 arrested cells reestablished the kinase activity associated with cdk2 (15). In addition several studies have shown that the cyclins D and E are rate limiting in the progression of cells into S phase (16-19). Although overexpression of cyclin E is usually associated with a decrease in the length of G_1, it has recently been shown that overexpression of cyclin E induced high levels of $p27^{Kip1}$ and inhibition of cell growth in selected mammary epithelial cell lines (20,21). We show here that the HeLa epithelioid cell line transfected with $p27^{Kip1}$ arrest in G_1 and express reduced levels of cdk2 associated kinase activity both of which were reversible by cotransfection with cyclin E. During the course of this work, studies with murine fibroblasts arrested in G_1 by transfection with $p27^{Kip1}$ were rescued by transfection with cyclin E which correlated with restoration of the cdk2 associated kinase activity (22). Our studies reached similar conclusions regarding this aspect of those extensive investigations.

MATERIALS AND METHODS

Cell culture and materials. HeLa cells were obtained from ATCC (Rockville, MD). The culture medium used throughout these experi-

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Abbreviations: cdk, cyclin dependent kinase; TGFβ, transforming growth factor β; CAK, cdk activating kinase; PCR, polymerase chain reaction; Rb, retinoblastoma protein.

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ments was Dulbecco's modified Eagle's medium containing 10% FCS, 20 mM HEPES, 100 μg/ml gentamicin (complete medium). Anti-cdk2 and anti-p27^{Kip} antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Lysine-rich histone H1 was purchased from United States Biochemical Corporation (Cleveland, OH) and protein G-agarose from Calbiochem (La Jolla, CA).

Immunoprecipitaion, 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, proteinase inhibitor E64 2 μg/ml, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. For immunoprecipitation, 100 μg of cellular lysate were 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. Samples were then washed and suspended in Laemmi buffer, boiled for 5 min and analyzed on 10% SDS polyacrylamide gels. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Detection of specific proteins was carried out with the ECL Western blotting kit according to manufacturer's instructions.

Kinase assay. Immunoprecipitates prepared from 70 μg of cell lysate, as described above, were assayed for histone H1 and Rb-p56 kinase activity (23). Briefly, immune complexes were incubated with HB buffer (60 mM β-glycerophosphate, 15 mM p-nitrophenyl phosphate, 15 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 80 mM NaCl, proteinase inhibitor E64 2 μg/ml, 5 mM EGTA, 25 mM MOPS pH 7.2) containing 1 μg of the Rb-p56 protein (QED, San Diego, CA), 50 μM, 5 μCi [γ^{32}P] ATP at 30 °C for 30 min. The reactions were terminated by addition of 15 μl of SDS-PAGE sample buffer. Protein phosphorylations was analyzed by 10% SDS-PAGE and detected by autoradiography after exposure at −70°C.

DNA transfection. Mouse p27^{Kip1} transient expression vector (p27-pcDNA3.1) was constructed by subcloning a BamHI/EcoRI fragment containing the p27^{Kip1} open reading frame into the BamHI/EcoRI site of pcDNA3.1 (Invitrogen, San Diego, CA). HeLa cells were transfected with 4.0 μg of plasmid DNA using the Lipofectamine method (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's protocol. The cyclin E expression plasmid (cyl E-pcDNA3.1) was constructed by subcloning a PCR amplified BamHI/EcoRI fragment containing the cyclin E open reading frame into the BamHI/EcoRI site of pcDNA3.1. This cyclin E insert fragment was generated with the following oligonucleotide primer pair: 5′-TTTTGCAGGATCCAGATTGAAGAAATG-3′ (upstream primer), 5′-TTGGTGGAGAAGAATTCGGTGGT-3′ (downstream primer) Normalization of the transfection efficiency was determined by measurement of β-galactosidase activity, as described by the manufacturer (Promega, Madison, WI).

Flow cytometric analysis. Flow cytometric analysis was performed as described previously (23). Briefly, 2 days after transfection cells were treated with 3 mM EDTA, centrifuged and suspended in complete medium before staining with Hoechst 33342 (Molecular Probes, Eugene, OR).

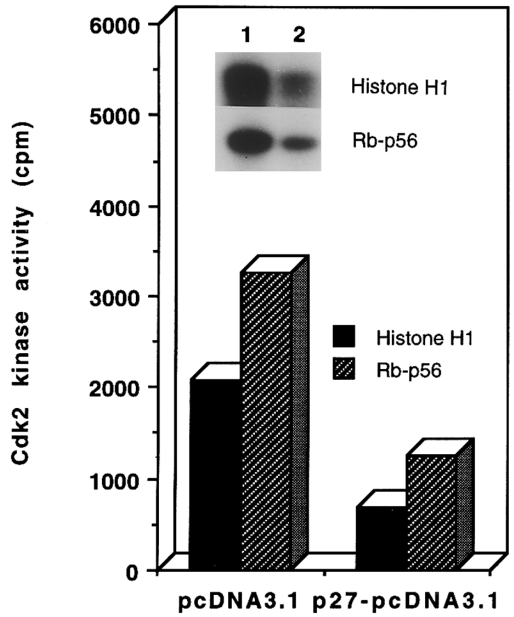

FIG. 2. Overexpression of p27^{Kip1} inhibits cdk2 kinase activity in HeLa cells. HeLa cells were grown to ~50% confluence, and then transfected with vector alone (lane 1) or vector containing p27^{Kip1} cDNA (lane 2). After 48 h, cells were harvested and whole cell extracts were prepared. An equal amount of whole cell lysates (70 μg) from transfected cells were immunoprecipitated with anti-cdk2 and assayed for kinase activity using Rb-p56 and histone H1 as substrate. The reaction mixtures were analyzed by SDS-PAGE and cdk2 kinase activity was determined by autoradiography (in box). The gel was then scanned for β emissions by phosphoimaging.

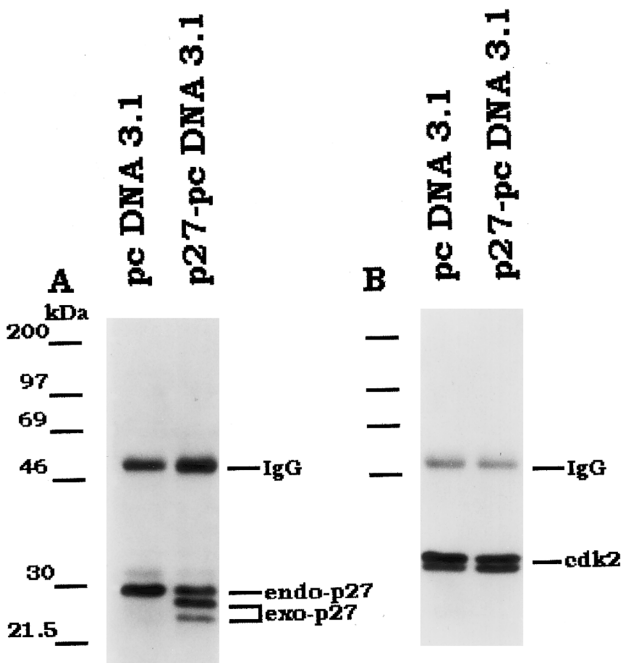

FIG. 1. Expression level of p27^{Kip1} and cdk2 in p27^{Kip1} cDNA-transfected cells. Lysates (100 μg) of HeLa cells transiently transfected with vector alone or vector containing p27^{Kip1} cDNA were immunoprecipitated with a specific anti-p27^{Kip1} (A) and anti-cdk2 (B) and analyzed by Western blot.

RESULTS AND DISCUSSION

p27^{Kip1} Overexpression Induces a G_1 Arrest That Is Associated with the Inhibition of cdk2 Kinase Activity

As shown previously, the intracellular level of cyclin-dependent kinase inhibitor p27^{Kip1} is elevated in resting

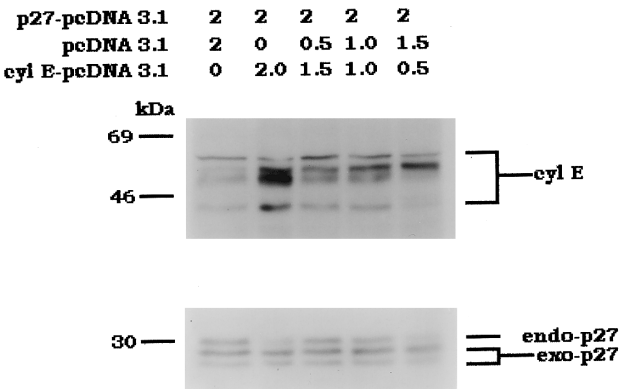

FIG. 3. Expression level of $p27^{Kip1}$ and cyclin E in $p27^{Kip1}$ and cyclin E cDNA cotransfected cells. HeLa cells were grown to ~50% confluence, and then cotransfected with a constant amount of $p27^{Kip1}$ cDNA (2 μg) and different amounts of cyclin E cDNA as indicated. After 48 hours, cells were harvested for the preparation of whole cell extracts. An equal amount of whole cell lysates (30 μg) from transfected cells were analyzed by Western blot with anti-cyclin E (upper panel) and anti-$p27^{Kip1}$ (bottom panel).

cells or nondividing cells and rapidly declines when cells are stimulated to enter the cell cycle (23). Ectopic expression of $p27^{Kip1}$ results in an arrest during G_1 (11,12) and antisense oligonucleotides of $p27^{Kip1}$ prevented mitogen-starved fibroblast from becoming quiescent (24). In order to determine the physiological activity of $p27^{Kip1}$, we transfected murine $p27^{Kip1}$ into the human HeLa cells. The expression level of $p27^{Kip1}$ was determined by Western blot analysis with a specific antibody to $p27^{Kip1}$ (Fig. 1A). The size difference between the human and murine forms was a convenient property for distinguishing the endogenous and exogenous $p27^{Kip1}$. To establish that the effect of $p27^{Kip1}$ overexpression had no adverse effect on the cellular levels of cdk2, lysates from HeLa cells transient transfected with vector alone or vector containing $p27^{Kip1}$ cDNA were immunoprecipitated with a specific anti-cdk2 and analyzed by Western blot (Fig. 1B). These results showed that $p27^{Kip1}$ overexpression did not effect cdk2 expression levels.

The $p27^{Kip1}$ associated G_1 arrest induced by a variety of treatments consistently correlates with the accumulation of inactive cdk2/cyclin E complexes (12-14, 25). To determine the effect of $p27^{Kip1}$ overexpression on cdk activity in HeLa cells, lysates derived from the transfected cell line were assayed for cdk2 kinase activity. The catalytic activity of immunoprecipitated cdk2 from the transfected cell lysates was determined using Rb-p56 and histone H1 as substrate (Fig. 2). Cells transfected with $p27^{Kip1}$ exhibited 3 fold lower cdk2 kinase activity for Rb-p56 and histone H1when compared with cells transfected by vector alone.

Cyclin E Rescue $p27^{Kip1}$-Mediated cdk2 Kinase Activity and G_1 Growth Arrest

The addition of recombinant cyclin E to lysates prepared from cells that have arrested in G_1 and which contain inactive cdk2/cyclin E complexes has been shown to restore the cdk2 kinase activity (15). To determine the effect of increased levels of cyclin E on cdk2 kinase activity in HeLa cells transfected with sufficient $p27^{Kip1}$ cDNA to retard G_1 progression, these cells were cotransfected with different amounts of cyclin E cDNA and the cdk2 kinase activity determined. The expression of cyclin E did not effect the expression level of $p27^{Kip1}$ in any of the cotransfections investigated (Fig. 3). The monoclonal anti-cyclin E used in these studies recognized at least four distinct forms of endogenous cyclin E in HeLa cells transfected with plasmid containing only the vector i.e. a high molecular weight band at 55 kDa and three bands at 50, 47, and 42 kDa. HeLa cells transfected with cyclin E cDNA resulted in the expression of the three lower molecular weight forms of cyclin E. The cyclin E expression level gradually increased in proportion to the cyclin E plasmid cDNA content transfected. Whole cell lysates prepared from these cell preparations were immunoprecipitated

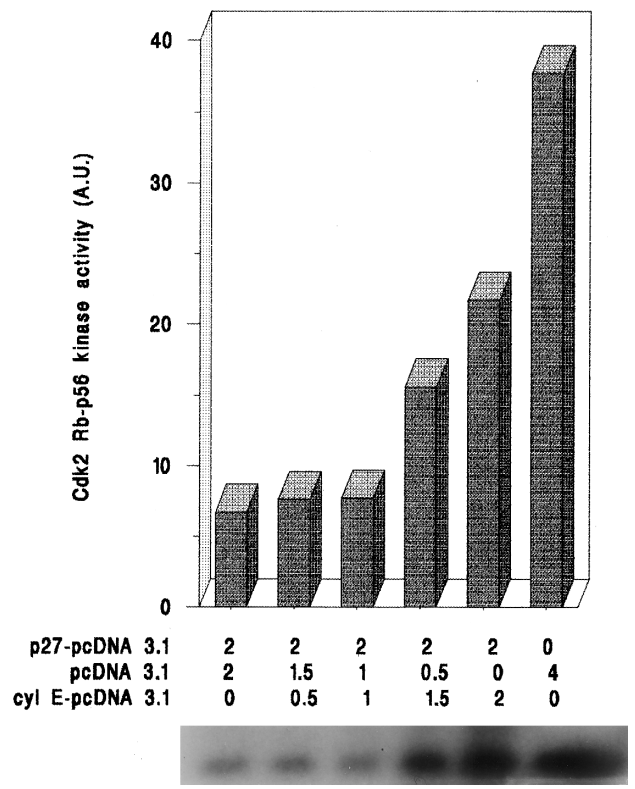

FIG. 4. Cdk2 Kinase Activation in HeLa Cells Cotransfected with Cyclin E and $p27^{Kip1}$. HeLa cells were grown to ~50% confluence, and then cotransfected with a constant amount of $p27^{Kip1}$ cDNA(2 μg) and different amounts of cyclin E cDNA as indicated. After 48 hours, cells were harvested whole cell extracts were prepared. An equal amount of whole cell lysates (70 μg) from transfected cells were immunoprecipitated with anti-cdk2 and assayed for kinase activity using Rb-p56 as substrate. The reaction mixtures were analyzed by SDS-PAGE and cdk2 kinase activity was determined by autoradiography. The gel was then scanned for β emissions by phosphoimaging.

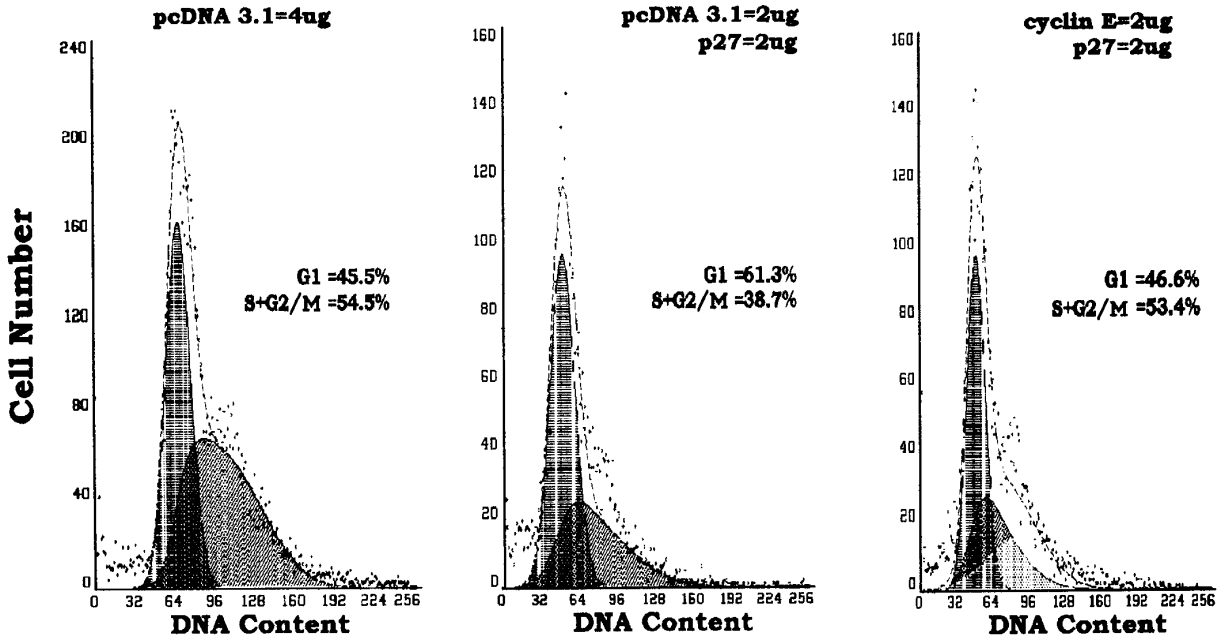

FIG. 5. Cell Cycle Analysis of HeLa Cultures After Transfection with Control. Vector, $p27^{Kip1}$, and Cotransfected with Cyclin E and $p27^{Kip1}$. HeLa cells were transfected with vector (pcDNA 3.1) containing no cDNA insert, vector containing $p27^{Kip1}$ cDNA or cotransfected with vectors containing $p27^{Kip1}$ cDNA and cyclin E cDNA. The transfected cells were cultured for 48 hours and then harvested for cell cycle analysis. The cells were stained with Hoechst 33342, and the DNA content was determined using flow cytometry as described in Material and Methods. The numbers indicate the percentage of cells in G_1 and $S+G_2$ phases of cell cycle.

with anti-cdk2 and assayed for kinase activity using recombinant truncated Rb as a substrate. HeLa cells transfected with $p27^{Kip1}$ cDNA expressed less than 20% of the kinase activity detected in cells transfected with the vector alone (Fig. 4). The level of cyclin E expressed in HeLa cells transfected with the lower amounts of cyclin E cDNA were unable to restore the cdk2 associated kinase activity. However lysates prepared from cells transfected with the higher levels of cyclin E cDNA expressed significantly higher levels of cdk2 kinase activity i.e. slightly greater than 50% of that observed in lysate from the control cell. These results indicated that increased levels of cyclin E are able to reverse the inhibitory effects of $p27^{Kip1}$ on cdk2 related kinase activity in vivo to a similar extent as that seen in vitro. The capability of cyclin E to reverse the inhibitory effects of $p27^{Kip1}$ is consistent with the binding properties $p27^{Kip1}$. The cdk2 binding domain of $p27^{Kip1}$ located within the amino acid sequence 53-85 (26) represents one of three domains that share homology with another member of the Kip/Cip family, $p21^{Cip1/Waf1}$. The crystal structure of the $p27^{Kip1}$ bound to the phosphorylated form of cdk2/cyclin A complex demonstrated independent binding sites for enzyme (residues 52-93) and cyclin (residues 25-49) (27). The cyclin binding domain of $p27^{Kip1}$ and $p21^{Cip1}$ is localized within this distal N-terminal region that contains the conserved LFG motif (residues 32-34 and 21-23 respectively) (27,28). Independent binding of $p27^{Kip1}$ by overexpressed cyclin E would effectively inactivate the inhibitory property of $p27^{Kip1}$ similar to that observed in vitro (15).

The constitutive (20,21) or conditional (19) overexpression of cyclin E in fibroblast has been shown to effect various cellular events associated with the G_1 phase of the cell cycle ie. decrease in cell size and length of G_1 and a reduced serum requirement for the G_1/S transition. Our studies suggest that the synthesis of cyclin E is involved in regulating signals that control cell proliferation and that cyclin E is rate-limiting with respect to the G_1/S transition. However the overexpression of cyclin E in a nontransformed mammary epithelial cell line resulted in an increase in cell size and lengthening of G_1 which correlated with an increase in the expression of $p27^{Kip1}$ (21). To correlate the results of cyclin E mediated recovery of cdk2 kinase activity with cell cycle regulation, HeLa cells were transfected with control vector and $p27^{Kip1}$ cDNA without or with cotransfection with cyclin E cDNA. After 48 h, the cells were harvested and analyzed for DNA content by flow cytometry (Fig. 5). In cells transfected with an equivalent amount of plasmid DNA without the $p27^{Kip1}$ cDNA insert, 45.5% of the cells were in the G_1 phase of the cell cycle. In those cells transfected with plasmid containing the $p27^{Kip1}$ cDNA, the number of cells in the G_1 phase of the cell cycle increased to 61.3%. Cotransfection of cyclin E cDNA into cells with $p27^{Kip1}$ cDNA reduced the number of cells in G_1 to approximately the same number as observed in those cells

transfected with only plasmid DNA. Taken together these results are consistent with the suggestion that overexpression of cyclin E reverses the $p27^{Kip1}$ mediated G_1 growth arrest by independently binding the inhibitor which restores the cdk2 associated kinase activity necessary for the G_1/S transition.

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