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GL331 Induces Down-Regulation of Cyclin D1 Expression via Enhanced Proteolysis and Repressed Transcription

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ABSTRACT

GL331 is a novel podophyllotoxin-derived compound. In this study, GL331 induced human lung adenocarcinoma cell line CL1-5 growth arrest before death during the initial 24-h incubation period. We found that GL331 had no inhibitory effect on the expression of cyclins E, A, B1, CDK 4, and CDK 2; instead, its cell growth-inhibitory effect was partly attributable to an early down-regulation of cyclin D1 expression and in turn the reduction of retinoblastoma protein phosphorylation. GL331 enhanced the proteolysis of cyclin D1, and a proteasome inhibitor was able to block GL331-caused cyclin D1 reduction, suggesting that GL331-stimulated cyclin D1 degradation was through proteasomal processes. Additionally, GL331 reduced cellular cyclin D1 mRNA level down to 45% of control in 4 h and further to around 20% in 12 h. However, GL331 did not accelerate the disappearance of cyclin D1 mRNA under the condition of transcription blockage induced by actinomycin D. It was reported that a certain region in the 3′-untranslated region (UTR) of cyclin D1 mRNA mediated the mRNA degradation upon extracellular stresses. Herein, transient transfection studies demonstrated that the 3′-UTR insertion did not confer the susceptibility of luciferase reporter gene to the GL331 treatment. Together, these data suggested that GL331 did not decrease the stability of cyclin D1 mRNA. On the other hand, we found that GL331 specifically inhibited the cyclin D1 promoter-driven luciferase reporter activity. Western blot analyses showed that GL331 decreased the level of phosphorylated extracellular signal-regulated kinase (Erk), with no effect on p38 or c-Jun NH_2-terminal kinase. Furthermore, GL331's inhibition of cyclin D1 promoter was attenuated by ectopic Erk-2 overexpression. These data suggested that GL331 inhibited cyclin D1 gene transcription via the Erk signaling pathway. In summary, we report that GL331 induced an early decline of cyclin D1 expression by dual mechanisms: 1) enhancement of protein turnover and 2) repression of Erk-mediated gene transcription.

Eukaryotic cell cycle progression is a tightly monitored process involving the sequential activation of cyclin-dependent kinases (CDKs; Morgan, 1992; Elledge, 1996). The activity of CDKs is regulated through their interaction with specific cyclins and CDK inhibitors. Among the D-type cyclins, cyclin D1 plays an important role in regulating G_1 progression (Ohtsubo and Roberts, 1993; Sherr, 1993). Cyclin D1 binds and activates CDKs 4 and 6, which can phosphorylate Rb, a critical event required for G_1-S transition (Weinberg, 1995; Dyson, 1998). Overexpression of cyclin D1 shortens the G_1 phase and occurs in many types of human cancer, whereas inhibition of cyclin D1 expression blocks G_1-S transition (Quelle et al., 1993; Musgrove et al., 1994; Tam et al., 1994). A unique characteristic of cyclin D1 is that it is expressed in low abundance in quiescent cells, but quickly accumulates upon the stimulation with serum or mitogens and then remains relatively constant throughout the cell cycle. It was reported that mitogen-activated protein kinase (MAPK) cascades are involved in the modulation of cyclin D1 expression (Lavoie et al., 1996). The extracellular signal-regulated kinase (Erk) pathway up-regulates the expression of cyclin D1 by increasing its promoter activity. In contrast, the p38 signaling pathway inhibits cyclin D1 promoter activity (Lavoie et al., 1996). It has been found that some anticancer agents known to induce cell growth arrest result in reduced cyclin D1 expression through multiple mechanisms. Flavopiridol was reported to inhibit cyclin D1 gene transcription (Carlson et al., 1999), whereas retinoic acid and irradiation enhanced the proteolysis of cyclin D1 protein (Langenfeld et al., 1997; Agami and Bernards, 2000). Recently, prostaglandin A_2 (PGA_2) was found to down-regulate cyclin D1

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ABBREVIATIONS: CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; PGA_2, prostaglandin A_2; UTR, untranslated region; Topo II, topoisomerase II; CDC, cell division cycle protein; JNK, Jun NH_2-terminal kinase; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline-Tween 20; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *N*-CBZ-L-L-L-AL, *N*-carbobenzyloxy-leucine-leucine-leucine-aldehyde.

expression by enhancing the turnover of cyclin D1 mRNA (Lin et al., 2000). In that study, a segment (K12) in the 3′-untranslated region (UTR) of cyclin D1 mRNA was identified containing critical *cis*-acting element(s) responsible for PGA_2-triggered cyclin D1 mRNA degradation.

GL331 (Genelabs, Inc., Redwood City, CA) is a semisynthetic compound derived from a plant toxin podophyllotoxin (for review, see Whang-Peng and Huang, 1997). Podophyllotoxin derivatives, represented by etoposide (VP-16), have been used as chemotherapeutic agents to treat cancers such as lymphoma, leukemia, testicular carcinoma, small cell lung cancer, nonsmall cell lung cancer, breast, and other malignancies (Lock and Ross, 1987; Liu, 1989). GL331 shares many structural and biochemical properties with VP-16, and it is effective in killing cancer cells resistant to VP-16 treatment (Chang et al., 1991; Huang et al., 1999, 2000). Besides acting as a topoisomerase II (Topo II) poison to induce DNA damage, GL331 was found to trigger apoptosis through dysregulating the activity of cyclin B1/CDC 2 complex (Huang et al., 1996b, 1997). The phase I clinical trial of GL331 has defined the maximal tolerated dose as 300 mg/m^2; furthermore, a phase II clinical trial protocol has been proposed for lung cancer. We in advance studied the effects of GL331 on human lung cancer cells. In this report, we observed that GL331 induced human lung adenocarcinoma cell line CL1-5 growth arrest before death during the initial 24-h incubation period. Furthermore, we explored GL331's effects on the expression of cyclins D1, E, A, and B1. The data indicated that this potential anticancer agent caused an early and selective decline of cellular cyclin D1, which was accompanied by impaired Rb phosphorylation, a process linked to causation of growth arrest. Our series studies demonstrated that GL331-induced down-regulation of cyclin D1 expression was through both the enhancement of cyclin D1 proteolysis and the inhibition of cyclin D1 gene transcription. Furthermore, we found that GL331 inhibited the Erk activity but had little or no effect on JNK and p38. Ectopic overexpression of Erk-2 was able to attenuate GL331's inhibition of cyclin D1 promoter activity, suggesting that GL331 inhibited cyclin D1 gene transcription through the Erk signaling pathway.

Materials and Methods

Cell Culture. Human lung adenocarcinoma CL1-5 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, and were cultured at 37°C in a humidified atmosphere containing 5% CO_2 (Chu et al., 1997). The transfected CL1-5 cells stably expressing cyclin D1 promoter-driven luciferase were maintained in the above-described medium plus 100 μg/ml hygromycin B.

Antibodies. The antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (anti-cyclins A, B1, D1, E, CDKs 2 and 4, Rb, Erk, phospho-Erk, p38, phospho-p38, JNK, and phospho-JNK), Cell Signaling (Beverly, MA) (anti-phospho-Rb), and Sigma Chemical (St. Louis, MO) (anti-α-tubulin).

Flow Cytometric Analysis. Flow cytometry was performed as described previously (Huang et al., 2000). Untreated or GL331-treated CL1-5 cells were trypsinized and fixed with prechilled 80% (v/v) ethanol. After centrifugation, cell pellets were resuspended in 0.5% Triton X-100 for 5 min. The suspensions of permeabilized cells were further treated with 1 ml of 50 μg/ml propidium iodide plus 0.5% (w/v) of RNase A. Ten minutes later, the DNA content of cell samples was analyzed by the FACStar flow cytometer with an argon laser tuned to the 488-nm line for excitation (BD Biosciences, San Jose, CA).

Western Blot Analysis. Cells treated as indicated in the figure legends were washed twice with PBS and collected using scrapers followed by low-speed centrifugation. Total cell lysate was prepared by the method described previously (Huang et al., 1997). The protein concentrations of cell lysates were determined by the method of Bradford (1976). Aliquots (40 μg) of cell lysates were resolved by 12% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene membranes (Amersham Pharmacia Biotech, Piscataway, NJ). After blocking with PBST (PBS plus 0.1% Tween 20) plus 5% nonfat milk, the blots were incubated with indicated antibody (in PBST plus 5% milk) at 4°C for 12 h. The blots were then washed three times with PBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were again washed three times with PBST, and the protein band signals were obtained by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Cyclin E-Associated CDK 2 Kinase Assay. Cell lysates were prepared by using a mild lysis method (Huang et al., 1997). Equal amounts of cell lysates were incubated with anti-cyclin E antibody plus protein A-Sepharose at 4°C for 15 h with constant shaking. The immunoprecipitates were then washed four times with lysis buffer and twice with kinase buffer (20 mM Tris-Cl, pH 7.4, 7.5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 μg/ml bovine albumin serum). Finally, the immunoprecipitates were resuspended in kinase buffer plus 30 μM ATP, 50 μCi [γ-^{32}P]ATP (7000 Ci/mmol; Amersham Pharmacia Biotech) and 5 μg of histone H1 (Roche Molecular Biochemicals, Indianapolis, IN). The kinase reactions were performed at room temperature for 30 min. After being resolved by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene membranes, the radioactive histone H1 bands were detected and printed by PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Protein Stability Assay. Cells were incubated in methionine-free RPMI-1640 medium plus 10% dialyzed fetal bovine serum for 30 min and then labeled with 100 μCi/ml [^{35}S]methionine for 30 min. After labeling, cells were washed with PBS twice and then incubated with normal culture medium in the absence or presence of GL331 at 37°C for 15, 30, or 45 min. Cells were then washed with PBS and lysed with lysis buffer (Carlson et al., 1999). Each lysate (4×10^6 cpm) was preadsorbed with mouse preimmune serum plus protein A-Sepharose for 1 h at 4°C with gentle shaking (Huang et al., 1996a). The supernatants were collected and were immunoprecipitated with anti-cyclin D1 antibody plus protein A beads at 4°C for 16 h. The mixtures were then subjected to centrifugation and the supernatants were removed. The remaining protein A beads were washed five times with lysis buffer, mixed with loading buffer, and resolved in 12% SDS-polyacrylamide gels. After electrotransferred onto polyvinylidene membranes, the signals were detected by PhosphorImager. A parallel Western blot analysis was performed to confirm the cyclin D1 signals. The protein concentration was determined using the Bio-Rad (Richmond, CA) protein assay reagent.

Northern Blot Analysis. Total RNA was isolated from CL1-5 cells by using TRIzol according to the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA). Twenty micrograms of total RNA was resolved in 1% agarose gels containing 6.7% formaldehyde and then transferred onto nylon membranes. Cyclin D1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were labeled by Redi Prime II random priming system (Amersham Pharmacia Biotech). Hybridization was performed as described previously (Lin et al., 2000). The signals were detected and quantified by PhosphorImager.

Plasmid Construction. To determine the effects of specific regions derived from the 3′-UTR of cyclin D1 mRNA on the expression of chimeric luciferase reporter, the polymerase chain reaction-amplified DNA fragments comprising the cyclin D1 cDNA nucleotides 1022 through 2403 and 1712 through 2870 (designated as B2 and K4, respectively) were subcloned into the *Xba*I site of the vector pGL3-

promoter (Lin et al., 2000) to generate pGL3-B2 and pGL3-K4 plasmids.

Transfection. For the study of GL331's effect on cyclin D1 promoter activity, exponentially growing cells were transiently transfected with 1 μg of −1745CD1luc [a kind gift from Dr. R. G. Pestell (Northwestern University, Chicago, IL) Albanese et al., 1995] or pRC-luc (our unpublished construct). For assessment of the influence of specific regions, derived from the coding region and the 3′-UTR of cyclin D1 mRNA, on the expression of chimeric luciferase reporter, cells were transiently transfected with 1 μg of pGL3-promoter, pGL3-A1 (previously named pGL3-CR; Lin et al., 2000), pGL3-B2, pGL3-K4, and pGL3-K12 (Lin et al., 2000), respectively. Cotransfection with a β-galactosidase plasmid served as the control for normalization of transfection efficiency. To generate the cells stably expressing luciferase reporter driven by cyclin D1 promoter, CL1-5 cells were transfected with 10 μg of −1745CD1luc and 1 μg of pCEP4 (Invitrogen). Transfected cells were selected against the medium containing 150 μg/ml hygromycin B. The selected clones were pooled and maintained in the medium containing 100 μg/ml hygromycin B. For the study of the effect of Erk signaling on cyclin D1 promoter, cells stably expressing cyclin D1 promoter-driven luciferase were divided into two groups. One was transfected with pSRα-HA-Erk2 [a kind gift from Dr. M. Karin (University of California, San Diego, CA) Liu et al., 1996], and the other one with pSRα-HA-β-galactosidase. Cells were then either treated with GL331 or left untreated. All the transfections were performed using the Effectene reagent following the manufacturer's protocol (QIAGEN, Hilden, Germany).

Luciferase Reporter Assay. Cells were transfected with constructs as indicated in the figure legends and were either treated with GL331 or left untreated. Luciferase assays were performed using Luciferase assay kit (Promega, Madison, WI). Briefly, cells were washed with PBS after treatments and lysed using the provided lysis buffer. After centrifuged at 14,000 rpm for 5 min, the supernatants were collected. Twenty microliters of lysates was added to 100 μl of luciferase substrate. Arbitrary units of luminescence were detected with a Lumat LB 9507 luminometer (PerkinElmer Berthold, Boston, MA).

Results

GL331 Decreased Cellular Cyclin D1 Level and CDK 4 Activity. We studied the GL331's effect on the growth of CL1-5 cells. Cells were treated with GL331 and the growth curve was compared with that of untreated cells. As shown in Fig. 1A, 10 μM GL331 potently inhibited the growth of CL1-5 cells. Trypan blue exclusion assay revealed no significant change in the numbers of both alive and dead cells in the initial 24 h of treatment. Our data suggested that GL331 caused growth arrest in as early as 12 h. The parallel flow cytometric analysis demonstrated that GL331 perturbed CL1-5 cell cycle progression. Most of the cells were arrested in G_1 and S phases after 12 h of GL331 treatment (Fig. 1B). Longer treatment also generated similar cell distribution pattern (data not shown). To study whether GL331 treatment results in the reduction of cellular levels of cyclin D1 as well as other cell cycle regulators, cells were treated with GL331 and were subjected to Western blot analyses. The data showed that cellular cyclin D1 protein level was reduced dramatically (>50%) in 4 h of GL331 treatment (Fig. 2A). Meanwhile, we found little increase in the level of cyclin E protein and no change in the levels of cyclins A and B1 proteins (Fig. 2A). We further examined the effect of reduced cyclin D1 level on the phosphorylation of Rb. The Ser-780 of Rb protein is the phosphorylation site of cyclin D1-associated CDK 4 kinase. Our results showed that as the cyclin D1 level decreased with the treatment, the phosphorylation at the Ser-780 of Rb was inhibited, whereas the levels of CDK 4 and total Rb proteins remained constant (Fig. 2B). In addition, the in vitro histone H1 kinase assay suggested that the activity of cyclin E-associated CDK 2 kinase was not significantly changed by GL331 treatment (Fig. 2C). These data indicated that GL331 had a selective inhibitory effect to the expression of cyclin D1, which was reflected on the loss of CDK 4 kinase activity.

GL331 Enhanced the Degradation of Cyclin D1 Protein. To address the mechanism(s) underlying GL331-induced decrease of cyclin D1, we first determined whether GL331 enhanced the degradation of cyclin D1 protein. Therefore, a pulse-chase experiment was performed. CL1-5 cells were pulse-labeled with [^{35}S]methionine and then incubated with excess cold methionine with or without concomitant treatment of GL331 for 15, 30, or 45 min. Cyclin D1 proteins were immunoprecipitated, resolved by SDS-polyacrylamide gels, and the signals were detected by PhosphorImager. The data showed that GL331 significantly increased the turnover rate of cyclin D1 (Fig. 3A). The half-life of cyclin D1 (normally around 40 min) was reduced to less than 15 min after treat-

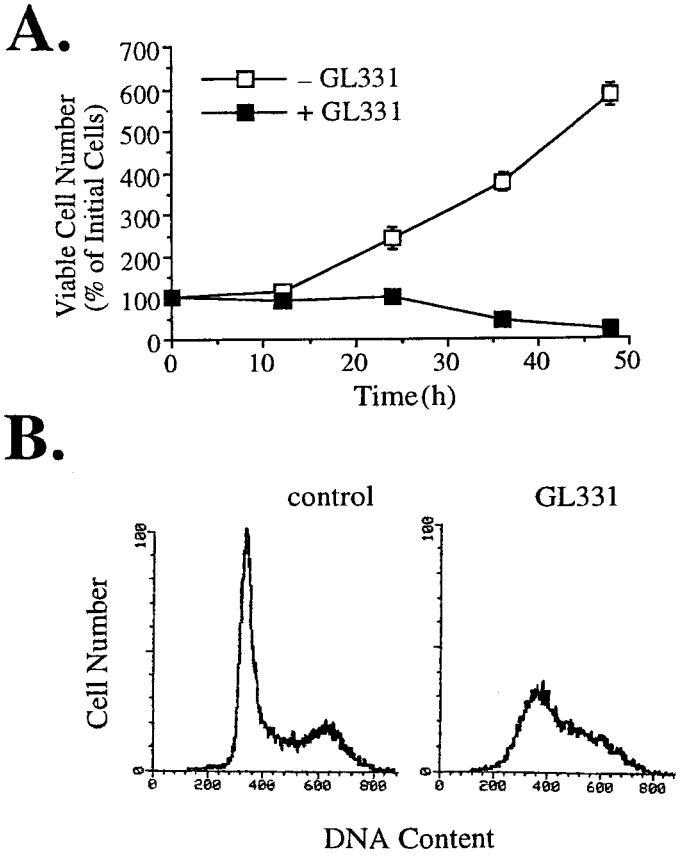

Fig. 1. GL331 induced CL1-5 cell cycle arrest. A, GL331 induced CL1-5 cell growth arrest before death. Cells were either treated with 10 μM GL331 or left untreated. At the times indicated, cells were trypsinized and harvested for counting the cell number. Living or dead cells were judged by trypan blue exclusion or not. It was noted that the numbers of alive and dead cells were not significantly altered by GL331 during the initial 24-h incubation period. Data represent the mean ± S.D. of three independent experiments. B, flow cytometric analysis of DNA content in the CL1-5 cells treated with or without 10 μM GL331 for 12 h. It was noted that most of GL331-treated cells were arrested in G_1 and S phases. The representative data from at least three independent experiments are shown.

ment. We also found that a proteasome inhibitor, *N*-carbobenzyloxy-leucine-leucine-leucine-aldehyde (*N*-CBZ-L-L-L-AL), was able to reverse the reduction of cyclin D1 protein caused by GL331 (Fig. 3B). These results demonstrated that GL331 enhanced the degradation of cyclin D1 protein, which might be through proteasomal processes.

GL331 Did Not Enhance the Degradation of Cyclin D1 mRNA. Besides the cyclin D1 protein level, we also analyzed the level of cyclin D1 mRNA in CL1-5 cells treated with GL331. We found that GL331 reduced the expression of cyclin D1 in a time course- and concentration-dependent manner (Fig. 4). Ten μM GL331 reduced the level of cyclin D1 message down to 45% of control in 4 h and further down to around 20% of control in 12 h. The steady-state level of mRNA is determined by the balance between its synthesis and degradation. To examine whether GL331-induced loss of cyclin D1 mRNA was attributed by the enhancement of mRNA degradation, we treated cells with a de novo RNA synthesis inhibitor, actinomycin D, and then examined the degradation of cyclin D1 mRNA (Fig. 5). The data showed that GL331 treatment did not further decrease the levels of actinomycin D-resistant cyclin D1 mRNA, suggesting that GL331 was unlikely to enhance the turnover of cyclin D1 mRNA. To investigate this possibility, we constructed a series of chimeric luciferase reporter constructs that contained the luciferase gene ligated with different DNA segments derived from the 3′-UTR of cyclin D1 mRNA (Fig. 6A). The 3′-UTR of cyclin D1 mRNA has been recently demonstrated to play an important role in PGA_2-induced cyclin D1 mRNA

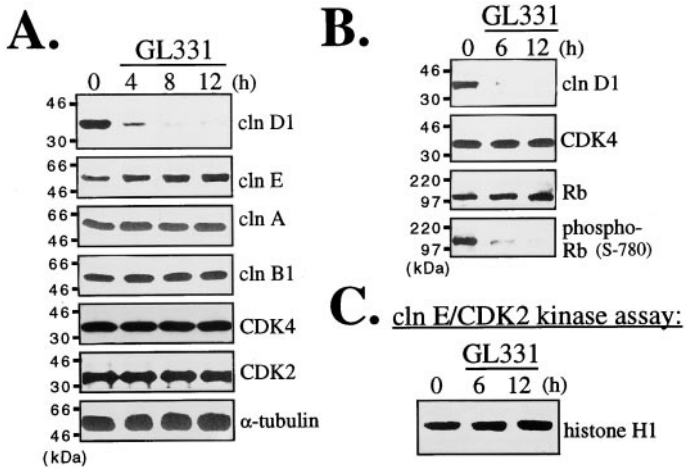

Fig. 2. GL331 selectively decreased cellular cyclin D1 protein level, which was accompanied by the reduction of Rb phosphorylation. A, CL1-5 cells were either treated with 10 μM GL331 for 4, 8, or 12 h or left untreated, and were subjected to Western blot analyses of the levels of cyclins D1, E, A, B1, CDK 4, CDK 2, and α-tubulin. B, cells were either treated with 10 μM GL331 for 6 h, 12 h, or left untreated. The levels of cyclin D1, CDK 4, total Rb, and the Ser-780-phosphorylated Rb were determined by Western blot analyses. C, cells were either treated with 10 μM GL331 for 6 h, 12 h, or left untreated. The cell lysates were subjected to determination of cyclin E-associated CDK 2 kinase activity by in vitro histone H1 kinase assay as described under *Materials and Methods*.

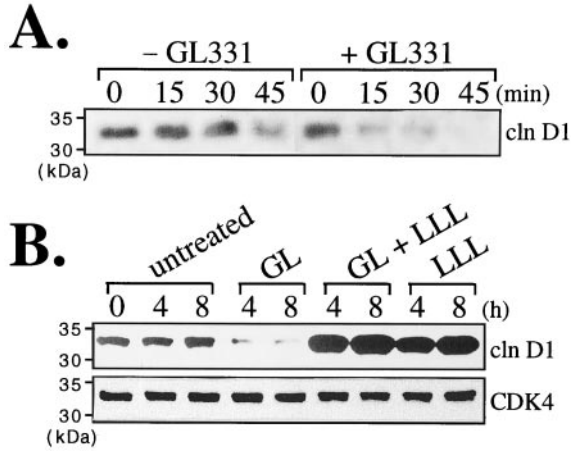

Fig. 3. GL331 enhanced the degradation of cyclin D1 protein. A, CL1-5 cells were pulse-labeled with [^{35}S]methionine as described under *Materials and Methods*. After labeling, cells were incubated with normal culture medium with or without 10 μM GL331 and harvested at the times indicated. Equal cpm of cell lysates were immunoprecipitated by anti-cyclin D1 antibody. The immunoprecipitates were further resolved by SDS-polyacrylamide gels, and the protein bands representing cyclin D1 were detected by PhosphorImager densitometer (Molecular Dynamics). B, cells were either treated with GL331 (GL), *N*-CBZ-L-L-L-AL (LLL), GL331 plus *N*-CBZ-L-L-L-AL (GL + LLL) for 4 or 8 h, or left untreated. Whole cell lysates were then prepared, and Western blot analyses were performed to detect the levels of cyclin D1 and CDK 4 proteins.

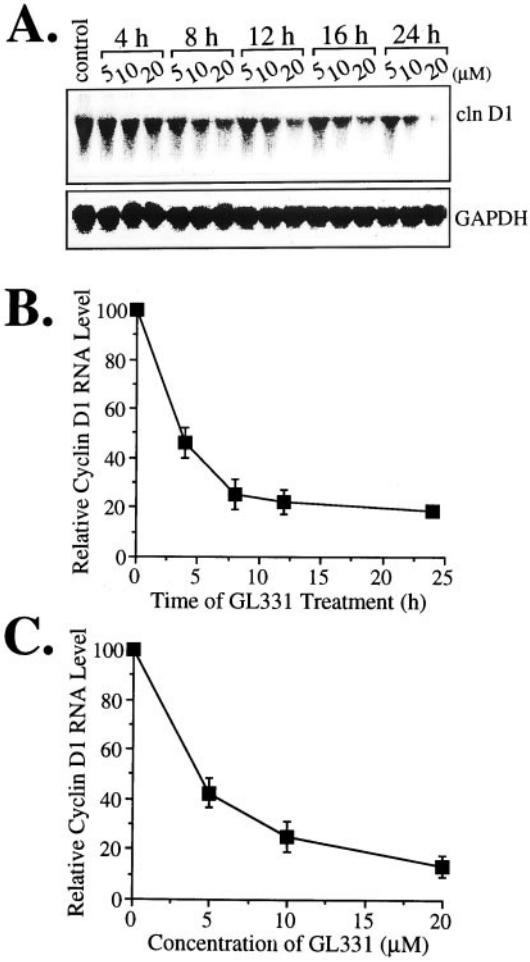

Fig. 4. GL331 decreased cyclin D1 mRNA level in CL1-5 cells. A, CL1-5 cells were either left untreated or were treated with 5, 10, or 20 μM GL331 for the time indicated. Total RNA was then isolated and the levels of cyclin D1 and GAPDH mRNA were determined by Northern blot analyses. B, time-dependent effect of 10 μM GL331 on the cyclin D1 mRNA expression was presented by normalizing the cyclin D1 mRNA levels to the GAPDH mRNA levels. C, after CL1-5 cells were treated for 8 h, the dose-dependent effect of GL331 on the expression of cyclin D1 mRNA was presented by normalizing the cyclin D1 mRNA levels to the GAPDH mRNA levels. All data represent the mean ± S.D. of three separate experiments.

degradation. After transiently transfecting cells with the chimeric reporter constructs, we found that none of the cyclin D1 mRNA segments could confer the susceptibility of luciferase reporter to GL331 treatment (Fig. 6B). Taken together, these data suggest that GL331 did not enhance the degradation of cyclin D1 mRNA.

GL331 Inhibited Cyclin D1 Promoter at Least through the Erk Signaling Pathway. To address whether GL331 inhibited the activity of cyclin D1 promoter, CL1-5 cells were transiently transfected with a reporter construct harboring luciferase gene driven by cyclin D1 promoter. An RC-RNase promoter-driven luciferase reporter construct was used for comparison. GL331 decreased cyclin D1 promoter activity to 60 and 42% of that of untreated in 4 and 8 h, respectively (Fig. 7). However, the RC-RNase promoter activity was just a little perturbed by GL331, indicating the selective inhibitory effect of GL331 on the activity of cyclin D1 promoter. MAPK pathways have been reported involved in regulating the activity of cyclin D1 promoter. To examine whether GL331 exploited the MAPK pathways to repress the cyclin D1 promoter, we performed Western blot analyses to detect the phosphorylation status of Erk, JNK, and p38 kinases. The results showed that GL331 did not affect the levels of total Erk and phosphorylated JNK and p38 kinases, but it efficiently decreased the phosphorylation level of Erk kinase (Fig. 8A), suggesting that GL331 inhibited the Erk activity with little or no effect on JNK and p38. To further address the involvement of Erk signaling cascade in GL331's inhibitory effect on cyclin D1 promoter, the CL1-5 cells stably expressing cyclin D1 promoter-linked luciferase reporter were further transiently transfected with a pSRα-HA-Erk2 plasmid or with a pSRα-HA-β-galactosidase control construct. The result showed that ectopic expression of Erk-2 kinase attenuated the inhibitory effect of GL331 on cyclin D1 promoter (Fig. 8B). These data suggested that GL331 inhibited the activity of cyclin D1 promoter at least through the Erk signaling pathway.

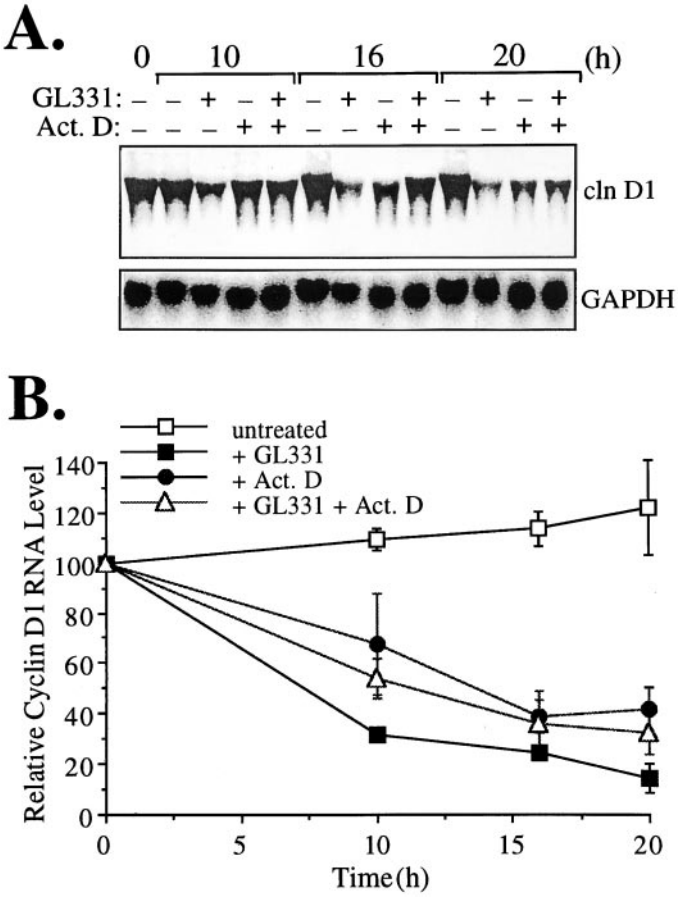

Fig. 5. GL331 did not enhance the degradation of actinomycin D-resistant cyclin D1 mRNA. A, cells were either left untreated or treated with GL331 (10 μM), actinomycin D (1 μg/ml), or combined GL331 with actinomycin D for the time periods indicated. Total RNA was then isolated and subjected to Northern blot analyses. B, cyclin D1 mRNA levels were quantitated and normalized to the GAPDH mRNA levels. Data represent the mean ± S.D. of three independent experiments.

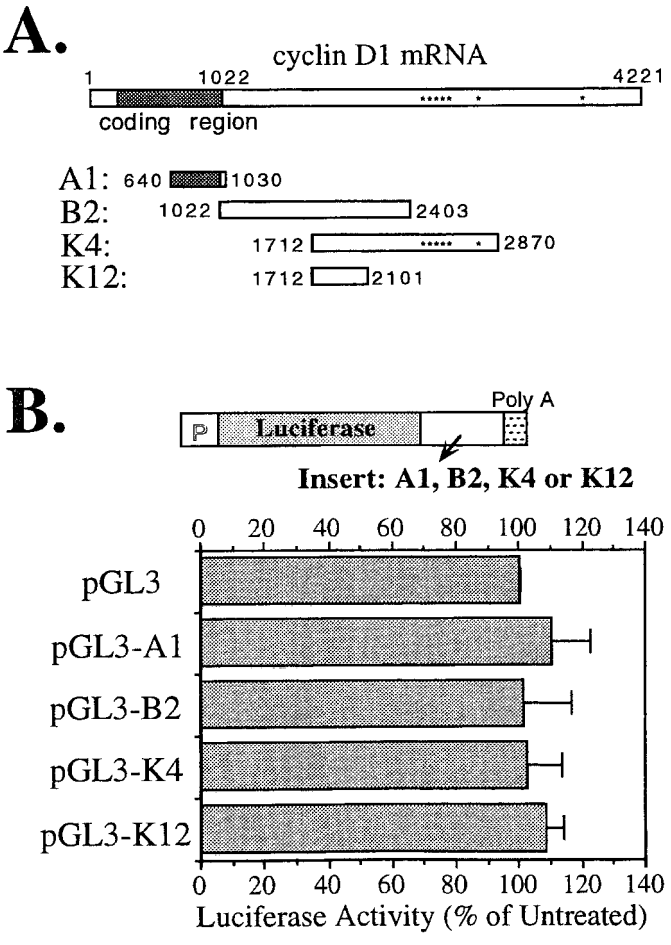

Fig. 6. Cyclin D1 mRNA segments did not render the chimeric luciferase reporter responsive to GL331. A, schematic representation of the full-length cyclin D1 cDNA and various segments derived from the coding region and 3′-UTR used in this study. The AUUUA motif is designated as *. B, impact of various regions of cyclin D1 mRNA on the expression of a chimeric luciferase reporter construct after GL331 treatment. One microgram of each pGL3-promoter, pGL3-A1, pGL3-B2, pGL3-K4, and pGL3-K12 were transiently transfected into CL1-5 cells. The β-galactosidase plasmid (0.2 μg) was cotransfected as a control. Cells were then treated with or without 10 μM GL331 for 6 h and collected for luciferase activity assay. The effect of GL331 on the expression of each vector was evaluated from the ratio of GL331 treated to untreated. The resulting (+GL331/−GL331) values of the chimeric construct groups were then calculated relative to that of the empty vector group (to which the value of 100% was assigned). Data represent the mean ± S.D. of six independent experiments.

Discussion

Cyclin D1 is a critical regulator for G_1 progression and G_1-S transition of the cell cycle. Ectopic expression of cyclin D1 shortens the G_1 phase, whereas inhibition of cyclin D1 expression blocks G_1-S transition (Quelle et al., 1993; Musgrove et al., 1994; Tam et al., 1994). Cyclin D1 may also contribute to tumorigenesis: it is overexpressed in many human tumor types, and when cotransfected with other oncogenes, is able to transform human fibroblast cells (Hinds et al., 1994; Lovec et al., 1994; Westwick et al., 1998). Therefore, cyclin D1 serves as a good target for cancer therapeutic approaches. In this report, we studied the growth-inhibitory effect of GL331 on CL1-5 cells, a highly invasive human lung adenocarcinoma cell line (Chu et al., 1997). We demonstrate that GL331 caused growth arrest of CL1-5 cells in G_1 and S phases of cell cycle (Fig. 1), and that GL331 was able to induce an early and selective decline of cyclin D1 expression (Fig. 2A), which was suggested as the "initiation" step to cause cell growth arrest (Agami and Bernards, 2000). Accompanying the loss of cyclin D1, the activity of CDK 4 was decreased as indicated by the reduction of CDK 4-directed Ser-780 phosphorylation of Rb (Fig. 2B). On the other hand, GL331 had no, if any, effect on the expression of cyclins E, A, B1, CDK 2, and CDK 4 as well as the cyclin E/CDK 2 kinase activity. The decline of cyclin D1 and the loss of phosphorylated Rb could therefore partly account for GL331-elicited cell cycle arrest (Fig. 1).

Our efforts to unveil the biological processes through which GL331 decreased cyclin D1 expression have revealed dual mechanisms. The data showed that GL331 not only enhanced the degradation of cyclin D1 protein (Fig. 3) but also reduced the levels of cyclin D1 mRNA (Fig. 4). Based on the finding that GL331-induced degradation of cyclin D1 was blocked by a proteasome inhibitor, regulation at the protein level seemed to involve a proteasomal proteolytic pathway. This phenomenon is similar to a recent finding that induced proteolysis is the main mechanism to cause early decrease of cyclin D1 level in irradiated cells (Agami and Bernards, 2000). However, GL331 also caused early loss of cyclin D1 mRNA (55% reduction in 4 h), suggesting that down-regulation of cyclin D1 mRNA also played a critical role, like accelerated proteolysis, to mediate the early decline of cyclin D1 in GL331-treated cells. The steady-state level of mRNA is determined by the dynamic equilibrium of its synthesis and degradation. Perturbation of either the synthesis or degradation of mRNA will affect the cellular mRNA level. Our data showed that GL331 was not likely to enhance the degrada-

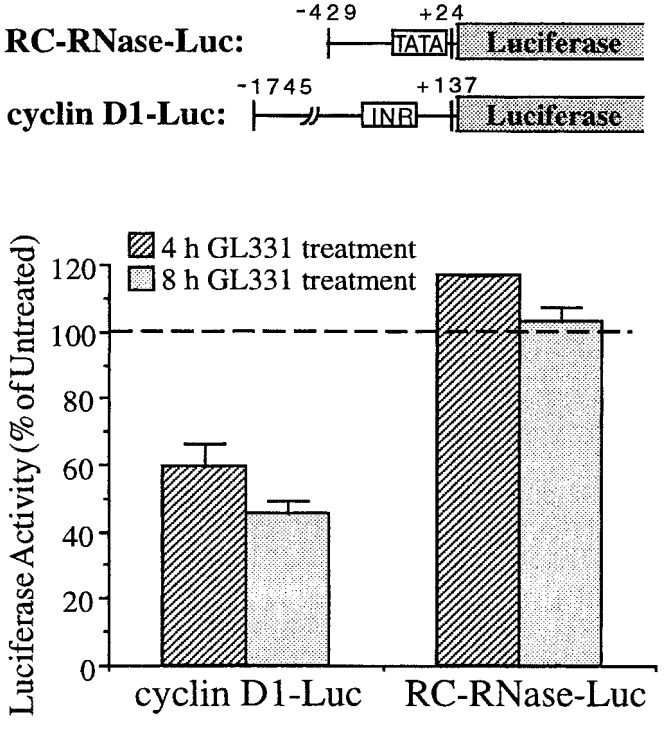

Fig. 7. GL331 inhibited the promoter activity of cyclin D1. Cells were transiently transfected with 1 μg of luciferase reporter gene driven either by the full-length cyclin D1 promoter (cyclin D1-Luc) or RC-RNase promoter (RC-RNase-Luc) along with 0.2 μg of β-galactosidase DNA as an internal control. Transfected cells were then either treated with 10 μM GL331 for 4 h, 8 h, or left untreated. Luciferase activities were measured and normalized to β-galactosidase activities. Data represent the mean ± S.D. of six separate experiments. TATA, TATAAA site; INR, initiator element.

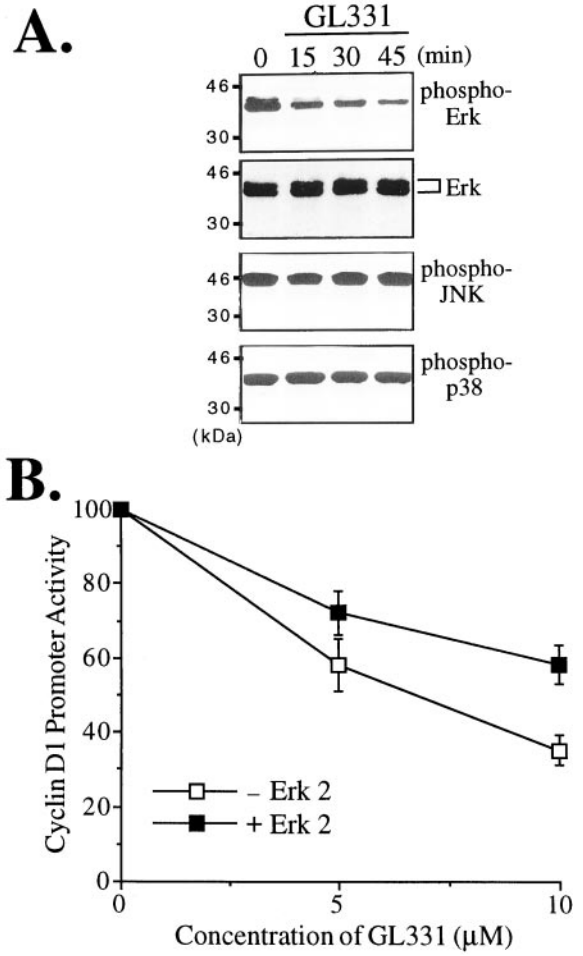

Fig. 8. Erk signaling pathway was involved in GL331's inhibitory effect on cyclin D1 promoter activity. A, CL1-5 cells were either left untreated or treated with 10 μM GL331 for 15, 30, or 45 min, and then subjected to Western blot analyses of the phosphorylation status of Erk, p38, and JNK. B, CL1-5 cells stably expressing −1745CD1luc reporter were divided into two groups. One was transiently transfected with 2 μg of pSRα-HA-Erk2 expression vector, and the other one with 2 μg of pSRα-HA-β-galactosidase construct. The cells of each group were then treated with 5 or 10 μM GL331 for 4 h and were subjected to luciferase activity assay. Data represent the mean ± S.D. of three independent experiments. Each experiment was performed in triplicate.

tion of cyclin D1 mRNA. The lack of the involvement of post-transcriptional event in the GL331-triggered decrease of cyclin D1 mRNA was supported by two findings. First, GL331 was not able to enhance the degradation of actinomycin D-resistant cyclin D1 mRNA (Fig. 4). The second finding is that different segments derived from the cyclin D1 mRNA did not render the ligated luciferase reporter susceptible to GL331's action. The special *cis*-acting elements located on the 3′-UTRs of mRNAs play a key role in modulating the stability of mRNAs encoding cell cycle regulators, cytokines, and transcription factors (Brewer, 1991; Gorospe et al., 1993; Buzby et al., 1996; Wang et al., 2000a,b). It is believed that these elements interact with RNA-binding proteins and confer the transcript to degradation. Emerging findings have suggested that this RNA-protein interaction also participates in modulating the half-life of cyclin D1 mRNA under stress. Recent study of the PGA_2-mediated degradation of cyclin D1 mRNA revealed that the K12 region of cyclin D1 mRNA served as a binding site for AUF1, and when cloned and ligated with the luciferase reporter gene, it could mediate the down-regulation of chimeric luciferase reporter upon PGA_2 treatment (Lin et al., 2000). In considering that the K12 segment of cyclin D1 mRNA may only mediate transcript degradation in response to PGA_2 treatment, we amplified several cDNAs to cover part of the coding region (A1) and most of the 3′-UTR of cyclin D1 mRNA (B2, K12, and K4) and constructed a series of chimeric luciferase reporters (pGL3-A1, pGL3-B2, pGL3-K12, and pGL3-K4). Cells were transiently transfected with each of these reporter plasmids and were treated with GL331. The results showed that both the 3′-coding region and 3′ UTR of cyclin D1 mRNA did not render the chimeric luciferase reporter susceptible to GL331 treatment (Fig. 6), suggesting that the RNA degradation function of the *cis*-acting element was not enhanced by GL331 treatment. We therefore concluded that GL331 was not likely to down-regulate the expression of cyclin D1 through enhanced mRNA turnover.

Next, we examined the GL331's effect on the transcription of cyclin D1, and the data clearly showed that GL331 repressed the activity of cyclin D1 promoter (Fig. 7). A parallel study with RC-RNase promoter-driven reporter indicated that GL331's inhibitory effect on cyclin D1 promoter was a selective event. Our studies support the notion that GL331 down-regulated the expression of cyclin D1 partly by inhibiting its transcription. This finding raises the question: Through what signaling pathway(s) does GL331 repress the transcription of cyclin D1? Several signaling cascades have been reported to participate in the regulation of the transcription and translation of cyclin D1. Serum stimulation of the phosphatidylinositol 3-kinase/Akt signaling pathway enhances the synthesis of cyclin D1 protein (Muise-Helmericks et al., 1998), whereas the MAPK and the signal transducer and activator of transcription-5 pathways have been found to be involved in the transcription of cyclin D1 (Lavoie et al., 1996; Matsumura et al., 1999). Mitogen stimulates the Erk signaling cascade to up-regulate the cyclin D1 promoter activity, whereas the p38 signaling cascade does the opposite (Lavoie et al., 1996). To address GL331's impact on the transcription of cyclin D1, we further examined whether the MAPK pathways were affected by GL331. By measuring the phosphorylation status of Erk, p38, and JNK kinases, we found that GL331 reduced the Erk activity but had little or no effect on the p38 and JNK activity, suggesting that GL331 could inhibit the transcription of cyclin D1 through repression of the Erk but not stimulation of the p38 activities. Finally, transient transfection of Erk-2 expression plasmid into the cells stably expressing the cyclin D1 promoter-driven luciferase showed that ectopic expression of Erk-2 attenuated GL331's inhibitory effect on the cyclin D1 promoter. These data indicated that GL331 induced the down-regulation of cyclin D1 mRNA expression at least through the Erk signaling pathway.

The results have helped us to expand the spectrum of GL331's action to include the expression of cyclin D1, which in turn serves as molecular pharmacological evidence to address GL331's anticancer potential. It is noteworthy that GL331 is a Topo II inhibitor, capable of inducing Topo II-mediated DNA breaks (Chang et al., 1991; Kuo et al., 1998) and is expected to have an impact on gene transcription (Lock and Ross, 1987; Liu, 1989). However, our results suggest that GL331's acting as a Topo II poison cannot fully account for its effect on cyclin D1 promoter because the RC-RNase promoter was not affected so much by GL331. The mechanism underscoring this action selectivity is currently unknown. Studies are being undertaken to locate the region of the cyclin D1 promoter responsible for the inhibition by GL331. Several transcription factors have been found participating in the transcription of cyclin D1 gene. β-Catenin stimulates the cyclin D1 promoter activity and may have a role in the development of colon cancer (Tetsu and McCormick, 1999). In chondrocytes, activating transcription factor-2 complexes with cAMP responsive element-binding protein to activate the transcription of cyclin D1 (Beier et al., 1999). Whether these transcription factors are involved in mediating GL331's effect on the transcription of cyclin D1 is a question to be answered.

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