JOURNAL OF VIROLOGY, Jan. 2003, p. 1–9 Vol. 77, No. 1
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.1.1–9.2003
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Ubiquitin-Dependent Proteolysis of Cyclin D1 Is Associated with Coxsackievirus-Induced Cell Growth Arrest

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Received 25 July 2002/Accepted 19 September 2002

Coxsackievirus group B3 (CVB3) replication is influenced by host cell cycle status. However, the effect of CVB3 infection on cell cycle regulation and the mechanisms involved are not precisely defined. In this study, we examined cell cycle progression and regulation when the infection was initiated in late G_1 phase of the cell cycle. Analysis of cellular DNA synthesis in infected cells by thymidine incorporation assays showed a significant reduction in [^{3}H]thymidine uptake compared to that of sham-infected cells. To further clarify the effects of CVB3 on the host cell cycle, we examined the cell cycle regulatory proteins involved in G_1 progression and G_1/S transition. Infection resulted in dephosphorylation of retinoblastoma protein and reduced G_1 cyclin-dependent kinase activities, accompanied by decreased levels of G_1 cyclin protein expression (cyclin D1 and cyclin E). We further investigated the mechanisms by which CVB3 infection down-regulates cyclin D1 expression. Northern blotting showed that cyclin D1 mRNA levels were modestly increased following CVB3 infection, suggesting that cyclin D1 regulation occurs by a posttranscriptional mechanism. Viral infection resulted in only a 20 to 30% inhibition of cyclin D1 protein synthesis 3 h postinfection. However, the proteasome inhibitors MG132 and lactacystin prevent CVB3-induced cyclin D1 reduction, indicating that CVB3-induced down-regulation of cyclin D1 is facilitated by ubiquitin-proteasome proteolysis. Finally, using GSK3β pathway inhibitors, we showed that the reduction of cyclin D1 is GSK3β independent. Taken together, our results demonstrate that CVB3 infection disrupts host cell homeostasis by blocking the cell cycle at the G_1/S boundary and induces cell cycle arrest in part through an increase in ubiquitin-dependent proteolysis of cyclin D1.

Coxsackievirus group B3 (CVB3), an enterovirus of the family *Picornaviridae*, is a common human pathogen associated with various diseases, such as myocarditis, meningitis, and pancreatitis (7, 30, 48). It has been suggested that early virus and host cell interactions can determine the degree of viral replication and the progression of target organ injury. Like most viruses, CVB3 has evolved a variety of mechanisms to optimize cellular conditions to benefit its own replication. We have previously shown that the extracellular signal-regulated kinase (ERK) is activated during CVB3 infection and that inhibition of such activity blocks CVB3 replication. It has also been reported that CVB3 modifies host gene expression to optimize its replication (34, 44, 50). As part of such manipulation of host cells, CVB3 may modify the host cell cycle regulatory machinery to facilitate its replication. Indeed, during the preparation of this study, Feuer et al. (14) showed that coxsackievirus replication and persistence were affected by cell cycle status. Although it was proposed that virus-induced shutdown of host protein synthesis led to cell growth arrest (14), the effect of CVB3 infection on cell cycle control and the precise molecular mechanisms involved are not fully characterized.

The cell cycle is controlled at various biological checkpoints by cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (31, 39, 40). Cell cycle progression is triggered by the activation of a series of CDKs; such activation is determined by their associations with various inhibitors and cyclins. G_1 cyclins, including cyclin D and cyclin E, in association with CDK4 and -6 and CDK2, respectively, play important roles in cell cycle control at the G_1/S boundary. Previous studies have suggested that cyclin D1 is regulated at both the transcriptional and the posttranscriptional levels (1, 8, 17, 22, 45). Activation of the ERK pathway increases cyclin D1 mRNA levels (4). Mitogens may increase the rate of cyclin D1 translation by activation of the translation initiation factor (45). Further, cyclin D1 protein turnover can be regulated by degradation via the ubiquitin-proteasome pathway. It was reported that phosphorylation of cyclin D1 by glycogen synthase kinase 3β (GSK3β) on threonine 286 is required for its ubiquitination and subsequent degradation by the 26S proteasome (8, 9).

In addition to the G_1 cyclins, CDK inhibitors play an important role in the regulation of the activities of CDKs during cell cycle progression (35, 39, 40). CDK inhibitors (including p27 and p21) inhibit the kinase activity of cyclin D/CDK4 and -6 and cyclin E/CDK2 by binding directly to cyclin/CDK complexes.

As a major target of cyclin D/CDK4 and -6 and cyclin E/CDK2 complexes, retinoblastoma protein (Rb) plays a role that is critical for cells to progress from G_1 to S phase. Rb exists in its unphosphorylated form, which can bind and inhibit the E2F transcription factor during G_0 and early G_1 phases. It then becomes phosphorylated by cyclin D/CDK4 and -6 and cyclin E/CDK2 during mid- to late G_1 phase. Once phosphorylated, Rb releases E2F, which is involved in initiating the

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transcription of genes whose products are necessary for the initiation of DNA replication during S phase.

In this study, we attempted to explore the role of CVB3 in cell cycle regulation and the mechanisms responsible for CVB3-induced cell growth arrest. We demonstrated that CVB3 infection significantly reduces host cell DNA synthesis, which was accompanied by decreased levels of cyclin D1, cyclin E, CDK2, and CDK4 activities and reduced phosphorylation of Rb, indicating that CVB3 infection results in G_1 phase cell cycle arrest. We further identify ubiquitin-proteasome as the major pathway responsible for CVB3-induced cyclin D1 reduction.

MATERIALS AND METHODS

Cells, virus, and materials. HeLa CG cells were obtained from Charles Gauntt (University of Nebraska Medical Center, Omaha). They were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). CVB3 (Nancy strain) was propagated in HeLa cells and stored at −80°C. Prior to infection, the virus titer was routinely determined by plaque assay on HeLa cell monolayers as previously described (29). UV-irradiated virus was prepared as described previously (2).

Most supplies were purchased from Sigma Chemical Co. Polyclonal phospho-Rb antibody was purchased from New England Biolabs. Polyclonal cyclin D1, CDK2 (M2), CDK4 (H-22), p27 antibodies, and monoclonal cyclin E, p21, and p53 antibodies were obtained from Santa Cruz Biotechnology, and monoclonal Rb antibody was obtained from PharMingen. Antibody against β-catenin was obtained from BD Transduction Laboratories. Rb-C fusion protein was purchased from Cell Signaling Biotechnology. Protein A-agarose was from Roche Molecular Biochemicals. MG132, lactacystin, and polyclonal anti-ubiquitin antibody were purchased from Calbiochem.

Cell synchronization and virus infection. Subconfluent cultures of HeLa cells were synchronized in G_0 phase by serum starvation. The cell monolayers were washed once with phosphate-buffered saline (PBS) and then incubated with serum-free medium at 37°C for 24 h. The starved cells were restimulated with medium containing 10% FCS for 16 h and then infected with CVB3. After 16 h of serum stimulation, the cells were in late G_1 phase, which was verified by flow cytometry analysis (data not shown). HeLa cells were infected at a multiplicity of infection of 10 with CVB3 or sham treated with PBS for 1 h. The cells were washed with PBS and cultured in Dulbecco's modified Eagle's medium containing 10% FCS.

[^{3}H]thymidine incorporation. At different time points postinfection (p.i.), 0.5 μCi of [^{3}H]thymidine/ml was added to the cells and incubated for 2 h. The cells were washed twice with cold 10% trichloroacetic acid and dissolved in 0.5 N NaOH for 10 min, and then [^{3}H]thymidine incorporation was determined by scintillation counting.

Western blot analysis. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. The membrane was blocked for 1 to 2 h in a nonfat dry milk solution (5% in Tris-buffered saline) containing 0.1% Tween 20. The blot was then incubated for 1 h at room temperature with primary antibody, followed by incubation with secondary horseradish peroxidase-conjugated antibody for 1 h. Immunoreactive bands were visualized through enhanced chemiluminescence (Amersham).

CDK assay. CDK2 or CDK4 was immunoprecipitated from cell lysates with specific anti-CDK2 or anti-CDK4 antibody in the presence of protein A-agarose overnight at 4°C. The immunocomplexes were washed three times with lysis buffer and twice with Rb kinase buffer (120 mM HEPES–NaOH [pH 7.5], 120 mM $MgCl_2$) and then incubated in kinase buffer containing 1 μg of Rb-C fusion protein, 10 μM ATP, and 10 μCi of [γ-^{32}P]ATP (250 μCi/ml; Amersham) at 30°C for 30 min. The reaction was stopped by the addition of SDS sample buffer. The samples were boiled for 5 min and then separated on an SDS–10% PAGE gel. The gels were dried, and the phosphorylated substrates were visualized by autoradiography.

26S proteasome activity. Fresh cytoplasmic extracts were used to measure 26S proteasome activity as described previously (19). Two hundred micrograms of cytoplasmic protein was added to an assay buffer (20 mM Tris-HCl [pH 8.0], 1 mM ATP, and 2 mM $MgCl_2$) in the presence of the synthetic fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC (SLLVY-AMC; Calbiochem) in a final volume of 1 ml. The tubes were incubated at 30°C for 30 min. The fluorescence product AMC in the supernatant was measured at a 460-nm emission wavelength, using a fluorometer.

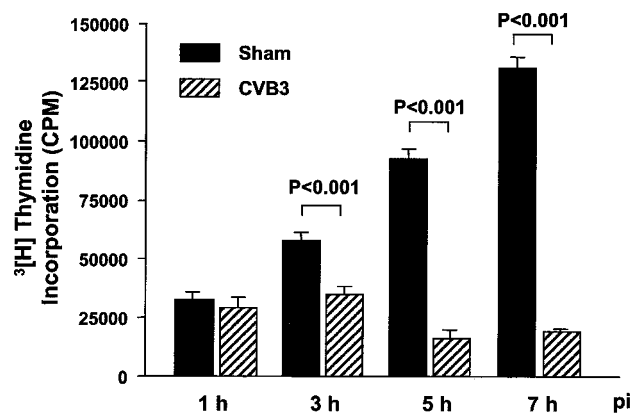

FIG. 1. CVB3 infection inhibits cellular DNA synthesis. HeLa cells were growth arrested in serum-free medium for 1 day, restimulated by the addition of 10% serum for 16 h, and subsequently infected with CVB3 or sham infected. At different times after infection, samples were collected and DNA synthesis was analyzed by [^{3}H]thymidine incorporation and expressed as counts per minute. The data shown are means ± SE (n = 4). Significance was determined by Student's t test. Similar results were obtained in two independent experiments.

RNA isolation and Northern blot analysis. After viral infection, total RNA was extracted from CVB3-infected or sham-infected HeLa cells at selected times using TRIzol reagent (Life Technologies). Five micrograms of each RNA sample was separated on a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane (Zeta-Probe; Bio-Rad). The membranes were hybridized overnight with ^{32}P-labeled human cyclin D1 cDNA probe (a 925-bp fragment; a generous gift from James Roberts, Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, Wash.) prepared by random primer labeling (Amersham). After washes, the membranes were subjected to autoradiography. The cyclin D1 mRNA levels were quantified and normalized against the levels of 28S rRNA.

Cyclin D1 metabolic labeling. Cyclin D1 biosynthesis was determined by metabolic labeling. At different times post-CVB3 infection, the culture medium was replaced with methionine-free medium containing 300 μCi of [^{35}S]methionine/ml. The cells were labeled for 30 min and then collected. Five hundred micrograms of protein from each sample was immunoprecipitated with anti-cyclin D1 antibody, followed by SDS-PAGE separation and visualization by autoradiography.

Statistical analysis. Statistical analysis was performed using the paired Student's t test. Data were reported as the mean ± standard error (SE). A P value of <0.05 was considered significant.

RESULTS

CVB3 infection blocks host cell DNA synthesis. To examine the effects of CVB3 on cell proliferation, we synchronized HeLa cells in G_0 by serum starvation for 1 day and then induced cell cycle progression by the readdition of serum. The cells were either sham or CVB3 infected at 16 h post-restimulation with serum. Samples were collected at various times after infection for determination of DNA synthesis by [^{3}H]thymidine incorporation (Fig. 1). In sham-infected cells, thymidine uptake continually increased from 1 through to 7 h p.i. (17 to 23 h after the readdition of serum). In contrast, in virus-infected cells, serum-stimulated DNA synthesis was significantly reduced compared to that in sham-infected cells. Cell numbers did not significantly change in the infected cells throughout the time course of the experiment (data not shown). This finding indicates that CVB3 infection almost

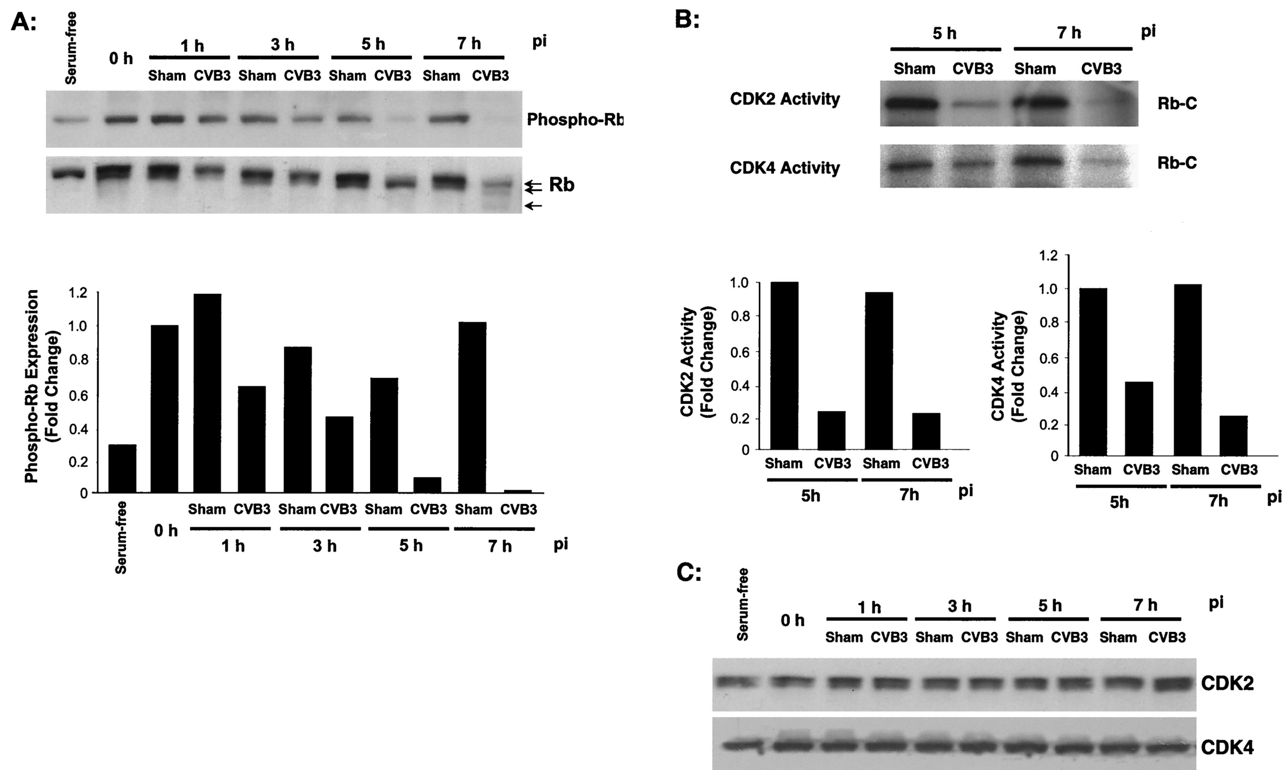

FIG. 2. CVB3 infection prevents Rb hyperphosphorylation and activation of G_1 cyclin kinases. HeLa cells were synchronized by serum starvation, restimulated with serum, and then infected with CVB3 as described in the legend to Fig. 1 or sham infected. (A) Cell lysates were collected and examined by Western blot analysis for hyperphosphorylated Rb (top) and for the levels and relative mobilities of Rb (bottom). Phospho-Rb expression was quantitated by densitometric analysis using NIH ImageJ version 1.27z and normalized to the activity at 0 h p.i., which was arbitrarily set to a value of 1.0. The data represent one of three independent experiments. (B) CDK2 and CDK4 were immunoprecipitated from cell lysates, and kinase activities were determined by an immune complex kinase assay using Rb-C as a substrate. CDK2 and CDK4 activities were quantitated by densitometric analysis and normalized to the sham infection at 1 h p.i. as described above. The data represent one of two independent experiments. (C) Cell lysates were collected, and the expression of CDK2 and CDK4 was examined by Western blotting. The data represent one of three independent experiments.

completely blocks new DNA synthesis by 3 h p.i., prior to CVB3-induced apoptosis, which occurs around 9 h p.i.

CVB3 infection prevents Rb phosphorylation and activation of G_1 cyclin kinases. The results of the DNA synthesis analysis indicate that CVB3-infected cells did not progress beyond the G_1/S boundary. To further explore how CVB3 affects host cell DNA synthesis, we examined the expression of several key cell cycle regulators. Western blotting was performed for hyperphosphorylated Rb using a phosphospecific Rb antibody (Fig. 2A, top) and for levels and mobilities of Rb protein using an Rb antibody (Fig. 2A, bottom). As shown in Fig. 2A, top panel, Rb phosphorylation was markedly increased in serum-stimulated cells compared with serum-starved cells. There was a significant decrease in the Rb phosphorylation of serum-stimulated cells following CVB3 infection at 3 h p.i. At 5 and 7 h p.i., there was a complete ablation of Rb hyperphosphorylation. The phosphorylated forms of Rb have slower mobility in SDS-PAGE gels. Sham-infected cells in the presence of serum showed both hyperphosphorylated and unphosphorylated forms of Rb (Fig. 2A, bottom). Serum-starved cells or CVB3-infected cells in the presence of serum failed to induce hyperphosphorylated Rb, which is consistent with the results using the phosphorylated Rb antibody. These results further indicate that CVB3 infection results in a loss of Rb phosphorylation. Interestingly, we observed a cleavage of Rb during the late phase of viral infection, i.e., 7 h p.i. Thus, at 7 h p.i., the decrease in Rb phosphorylation may also be due to decreased Rb protein levels.

Rb phosphorylation is dependent on the cyclin D/CDK4 and -6 and cyclin E/CDK2 activities (31). We next examined the effects of CVB3 infection on G_1-phase CDK activity. HeLa cells were synchronized by serum starvation and then released into G_1 phase by the readdition of serum. CDK2 and CDK4 activities were analyzed by an in vitro kinase assay using the Rb-C terminal fusion protein as a substrate. As shown in Fig. 2B, we observed a significant reduction in CDK2 and CDK4 activities in CVB3-infected cells compared to those in sham-infected groups at 5 and 7 h p.i. To determine whether the inhibition of CDK2 and CDK4 activities following CVB3 infection was due to loss of CDK2 and CDK4 protein expression, respectively, we examined the levels of CDK2 and CDK4 by Western blotting (Fig. 2C). We did not observe altered protein expression in CVB3- and sham-infected HeLa cells, which suggests that CVB3 targets cyclin-CDK complex formation as opposed to CDK proteins themselves.

CVB3 infection leads to the loss of G_1-phase cyclin proteins.

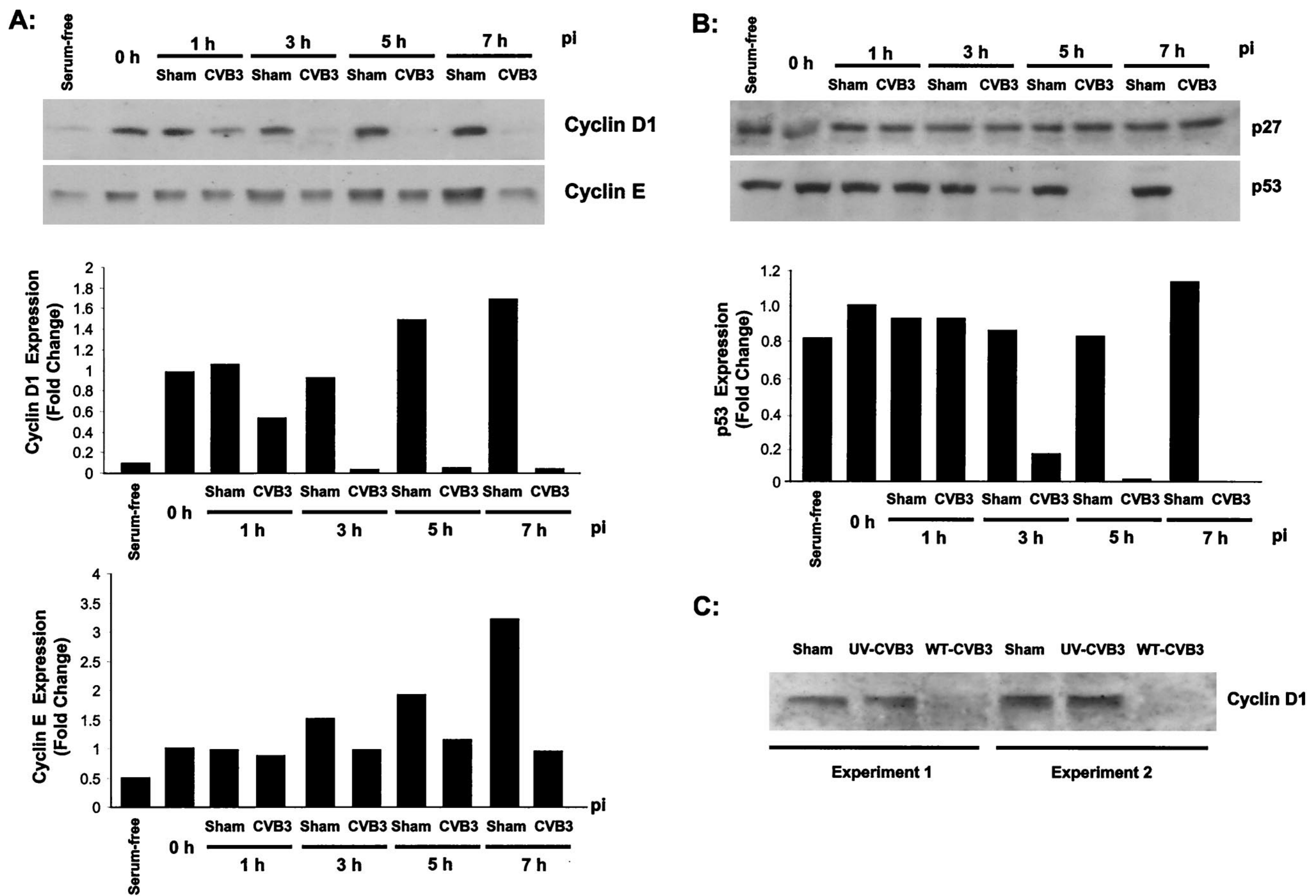

FIG. 3. CVB3 infection leads to loss of G_1 cyclin proteins. HeLa cells were treated as described in the legend to Fig. 1. (A) Expression of cyclin D1 and cyclin E was detected by Western blotting and quantitated by densitometric analysis as described in the legend to Fig. 2A. The data represent one of three independent experiments. (B) Expression of p27 and p53 was examined by Western blotting, and p53 protein expression was quantitated as described in the legend to Fig. 2A. The data represent one of three independent experiments. (C) Synchronized HeLa cells were restimulated with serum for 16 h and then infected with either wild-type virus (WT-CVB3) or UV-irradiated virus (UV-CVB3). Cell lysates were collected 5 h p.i., and cyclin D1 expression was determined by Western blotting.

To understand the mechanism of CDK inhibition following CVB3 infection, we next examined the levels of cyclin D1 and cyclin E. As Fig. 3A shows, cyclin D1 and cyclin E protein expression was markedly increased in response to serum stimulation. Levels of cyclin D1 were significantly decreased in CVB3-infected cells compared with those in sham-infected cells as early as 1 h p.i. By 3 h post-CVB3 infection, cyclin D1 protein expression was undetectable. In sham-infected groups, cyclin E expression began low at 0 h (16 h post-serum stimulation) and dramatically increased at 7 h p.i. (23 h post-serum stimulation). In contrast, cyclin E expression was decreased after CVB3 infection in the presence of mitogenic stimulation. These results reveal that kinase activity inhibition is due at least in part to a loss of cyclin D1 and cyclin E proteins during CVB3 infection.

Since kinase activities of CDK can be negatively regulated by CDK inhibitors (40), CVB3 might decrease CDK activities through these inhibitors. We next examined the levels of CDK inhibitors, p21 and p27, following CVB3 infection. p21 levels were consistently undetectable (data not shown), whereas p27 levels remained unchanged in both sham- and CVB3-infected cells, as shown in Fig. 3B. Thus, it is unlikely that the CDK inhibitors p21 and p27 are involved in the observed inhibition of CDK2 and CDK4 activities, although we cannot rule out the possibility, for example, that p27 may switch from binding cyclin D1/CDK4 to binding cyclin E/CDK2 following loss of cyclin D1.

p53 is induced by a variety of cellular stresses, such as DNA damage and viral infection. The accumulation of p53 prevents G_1/S transition through initiation of p21 expression (24, 27). Since p21 was undetectable in both sham- and CVB3-infected cells, to further determine the effects of CDK inhibitors on CDK activities, we examined the expression of p53, an upstream regulator of p21 (16, 27). Levels of p53 were decreased at 3 h post-viral infection and were undetectable by 5 h p.i. (Fig. 3B), which is consistent with a recent report that p53 was degraded following poliovirus infection of HeLa cells (46).

To determine whether CVB3-induced reduction of cyclin D1 is dependent on viral protein products, we used UV-irradiated virus. Such inactivated virus fails to express viral proteins but is capable of binding to the cell receptor and entering the cell (2). As shown in Fig. 3C, 5 h of infection with UV-irradiated virus failed to reduce cyclin D1 protein expression compared to that following wild-type CVB3 infection, which

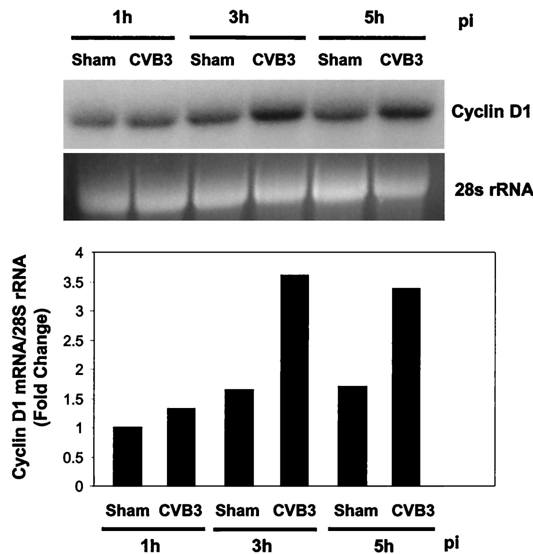

FIG. 4. CVB3 infection leads to a modest increase in cyclin D1 mRNA. Synchronized HeLa cells were restimulated with serum for 16 h and then infected with CVB3 or sham infected. RNA was extracted at the indicated times following viral infection, and cyclin D1 mRNA levels were determined by Northern blotting and normalized with the 28S rRNA. The results were quantitated by densitometric analysis and normalized to sham infection at 1 h p.i. as described in the legend to Fig. 2. The data represent one of two independent experiments.

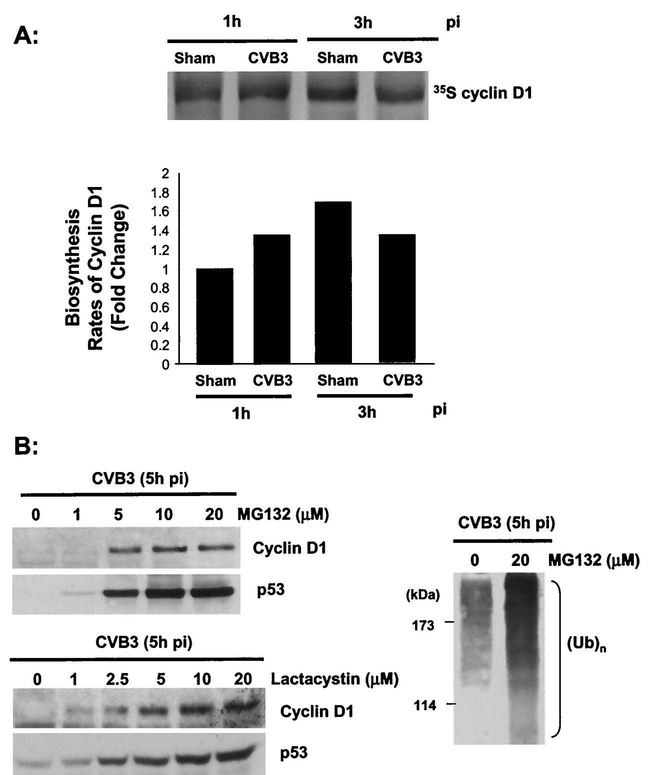

FIG. 5. CVB3 accelerates proteolytic degradation of cyclin D1. HeLa cells were synchronized and restimulated as described in the legend to Fig. 1. (A) At 1 and 3 h post-CVB3 or sham infection, the cells were metabolically labeled with [^{35}S]methionine for 30 min, and immunoprecipitated cyclin D1 was resolved by SDS-PAGE and visualized by autoradiography. Cyclin D1 biosynthesis was quantitated by densitometric analysis and normalized to the sham infection at 1 h p.i. as described in the legend to Fig. 2. The data are representative of three independent experiments. (B) Cells were preincubated with increasing concentrations of proteasome inhibitors, MG132 and lactacystin, for 30 min and then infected with CVB3. Five hours after infection, the cell lysates were analyzed for cyclin D1, p53, and ubiquitin expression by Western blotting. The masses of protein markers are indicated. The data are representative of three independent experiments. $(Ub)_n$, polyubiquitin.

suggests that viral replication and viral protein products are required for CVB3-induced inhibition of cyclin D1 expression.

Cyclin D1 is degraded via the ubiquitin-proteasome pathway. We next investigated the mechanisms of cyclin D1 downregulation following CVB3 infection. Cyclin D1 expression is essential for cell cycle progression from G_1 to S phase and can be regulated at three levels: transcription (1, 17), translation (45), and proteolysis (8, 22). We first determined the cyclin D1 mRNA level during CVB3 infection by Northern blotting. Figure 4 shows that CVB3 infection led to a modest induction of cyclin D1 mRNA as early as 3 h p.i., and the level remained elevated at 5 h p.i. Such an observation suggests that posttranscriptional regulation of cyclin D1 may contribute to the decreased level of cyclin D1 following CVB3 infection.

We then examined the rates of biosynthesis and degradation of cyclin D1. Following immunoprecipitation of [^{35}S]methionine-labeled infected HeLa cells, the levels of translation of cyclin D1 were determined by autoradiography. As shown in Fig. 5A, the rates of biosynthesis of cyclin D1 remained constant at 1 h p.i. and were reduced by 20 to 30% at 3 h p.i. Compared to decreased expression of cyclin D1 by 1 h p.i. and nonexistent cyclin D1 protein expression by 3 h p.i. (Fig. 3A), the extent of cyclin D1 biosynthesis reduction is much more conservative, suggesting that decreased cyclin D1 translation levels are likely not the major cause of cyclin D1 down-regulation during CVB3 infection. Previous studies have found that cyclin D1 is largely regulated by proteolysis via the ubiquitin-proteasome pathway (8, 9). To investigate whether CVB3 affects cyclin D1 protein stability, we examined the effects of proteasome inhibitors, MG132 and lactacystin, on cyclin D1 expression. MG132 is a nonspecific, potent, and reversible proteolysis inhibitor. Lactacystin is highly specific and inhibits the proteasome irreversibly by covalently modifying the proteasome β-subunit (25). Cyclin D1 protein expression in CVB3-infected cells was restored after the addition of either MG132 or lactacystin in a dose-dependent manner (Fig. 5B, left), which suggests that CVB3 infection facilitates the ubiquitin-proteasome processes of cyclin D1. The effectiveness of the proteasome inhibitor was proved by the accumulation of multiubiquitinated proteins (Fig. 5B, right).

Cyclin D1 proteolysis is mediated by two steps: phosphorylated cyclin D1 is first targeted for polyubiquitination and then is degraded by the 26S proteasome. To further elucidate which steps CVB3 infection particularly targets, we first examined the effect of viral infection on the ubiquitination of cyclin D1. [^{35}S]methionine-labeled cell extracts were immunoprecipitated with an anti-cyclin D1 antibody followed by protein separation, transfer, and autoradiography. As shown in Fig. 6A, left, we observed multiple bands ranging from 68 to 80 kDa in CVB3-infected cells. To determine whether these bands actually rep-

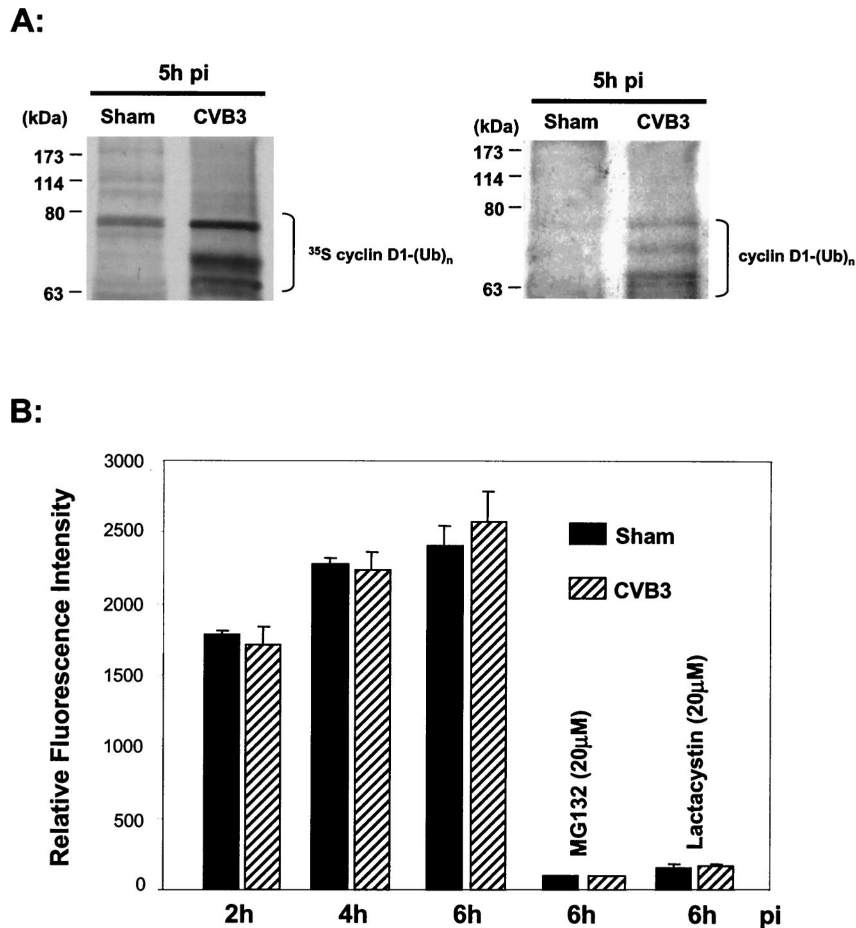

FIG. 6. CVB3 facilitates ubiquitination of cyclin D1. (A) [^{35}S]methionine-labeled cyclin D1 was analyzed as described in the legend to Fig. 5A. Following separation by SDS-PAGE, the gels were transferred and visualized by autoradiography. On the left is shown the upper portion of the autoradiogram. The same membrane was then examined for ubiquitin expression by Western blotting (right). The masses of protein markers are indicated. (B) 26S proteasome activity following CVB3 infection. At different times after CVB3 or sham infection in the presence or absence of proteasome inhibitors, cell lysates were collected and proteasome activity was measured as described in Materials and Methods using the fluorogenic substrate SLLVY-AMC. The results are means ± SE of three independent experiments.

resent multiubiquitinated cyclin D1, Western blotting was performed on the same membrane using an anti-ubiquitin antibody. This showed a similar pattern of immunoreactive bands (Fig. 6A, right), suggesting that CVB3 infection targets the first step of cyclin D1 proteolysis, i.e., the ubiquitination process.

It has been reported that proteasome activities were increased in response to cytokine stimulation and virus infection (18, 19). To determine whether CVB3 could also regulate proteasome activity, we measured proteasome cleavage of a fluorogenic substrate. Pretreatment with the proteasome inhibitor MG132 or lactacystin almost completely inhibited proteasome activity in both sham- and CVB3-infected cells (Fig. 6B). However, we did not observe significant changes in 26S proteasome cleavage activity throughout the course of viral infection in the two groups. This result indicates that CVB3-induced degradation of cyclin D1 does not appear to be related to alteration in proteasome activities.

CVB3-induced cyclin D1 degradation is independent of GSK3β activities. Previous evidence supports a role for GSK3β in the regulation of cyclin D1 proteolysis. GSK3β phosphorylates cyclin D1 on Thr-286 and triggers proteasomal degradation of cyclin D1 (8, 9). CVB3 infection activates multiple intracellular signaling pathways, including GSK3β (unpublished data). To determine whether GSK3β is involved in cyclin D1 degradation during CVB3 infection, we examined the effects of the GSK3β inhibitor lithium chloride on cyclin D1 expression. It is well established that the degradation of β-catenin requires GSK3β activity (43). As expected, exposure to CVB3 for 5 h caused a significant decrease in β-catenin protein expression, and the addition of 30 mM LiCl induced an accumulation of β-catenin (Fig. 7). However, treatment with LiCl was not able to prevent CVB3-induced down-regulation of cyclin D1 and p53 (Fig. 7), indicating that proteolysis of cyclin D1 by CVB3 infection is GSK3β independent.

DISCUSSION

In this study, we investigated the effects of CVB3 on DNA synthesis and the mechanism by which CVB3 dysregulates the cell cycle. CVB3 infection of cells in late G_1 phase resulted in a dramatic reduction in DNA synthesis and a significant inhi-

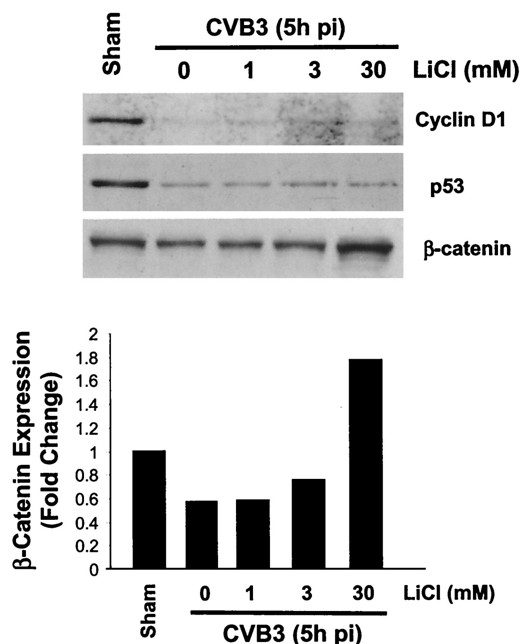

FIG. 7. CVB3-mediated cyclin D1 proteolysis is independent of GSK3β activity. Serum-restimulated HeLa cells were preincubated with different concentrations of the GSK3β inhibitor LiCl for 30 min and then infected with CVB3 or sham infected. Five hours after CVB3 infection, the cell lysates were analyzed for cyclin D1, p53, and β-catenin expression by Western blotting. The Western blotting results for β-catenin were quantitated by densitometric analysis and normalized to the sham-infected cells as described above. The results were similar in two independent experiments.

bition of cyclin E/CDK2 and cyclin D/CDK4 activities, which are necessary for Rb phosphorylation. We further found that inhibition of CDK activity is dependent on the loss of G_1-phase cyclin proteins and independent of the CDK inhibitor status of the cell. Finally, we demonstrated that increased ubiquitin-dependent cyclin D1 degradation is linked to CVB3-mediated cell growth arrest.

CDK2 and CDK4 protein levels remain constant while their activities are reduced following virus infection. Thus, CVB3 appears to be targeting the formation and maintenance of the cyclin-CDK complexes, which are determined by G_1-phase cyclin expression and the activities of CDK inhibitors. Although recent studies have suggested that p21 and p27 may be involved in the up-regulation of cyclin D/CDK4 in mitogen-stimulated murine fibroblasts (6), it is generally believed that p21 and p27 are negative regulators of CDK activities. In this study, we observed that levels of p21 and p27 were either undetectable or unchanged, suggesting that CVB3-mediated inhibition of CDKs is p21 and p27 independent.

Analysis of G_1-phase protein expression indicated that inhibition of CDK activity was due to a loss of G_1-phase cyclin expression. We therefore focused our study on the mechanisms of virus-mediated reduction of cyclin D1. Cyclin D1 is a labile protein and forms a holoenzyme with its catalytic partner, CDK4. Cyclin D1 expression could potentially be regulated at the levels of both biosynthesis (transcription and translation) and protein stability. In response to mitogen stimulation, the cyclin D1 gene is transcriptionally induced by c-Myc, AP-1, and NF-κB (12). The ERK signaling pathway has also been shown to transcriptionally regulate cyclin D1 expression (4). We have previously found that ERK was activated during the course of CVB3 infection of HeLa cells (29). In this study, we showed increased levels of cyclin D1 mRNA by 3 and 5 h p.i., suggesting that induction of cyclin D1 transcript may be a consequence of virus-mediated ERK activation. Alternatively, cyclin D1 mRNA up-regulation may represent a compensatory response to decreased cyclin D1 protein levels.

Infection with poliovirus results in a shutdown of cap-dependent protein synthesis while allowing cap-independent translation of viral mRNA, which is mainly associated with viral protease 2A-mediated eukaryotic translation initiation factor eIF4G cleavage (13, 23). eIF4G is a central protein involved in the initiation of cap-dependent translation, since it binds to the 5′ end of capped mRNA and serves as a molecular bridge that enables mRNA to bind to 40S ribosomal subunits. Cleavage destroys its ability to function in cap-dependent translation initiation. Translation initiation factor has been implicated in the regulation of cell cycle and cyclin D1 expression (38, 41, 45). To determine whether CVB3 infection down-regulates cyclin D1 expression by a decrease in protein synthesis, we investigated the rates of biosynthesis of cyclin D1. However, in this report we showed that the translation of cyclin D1 was reduced by only 20 to 30% at 3 h p.i., suggesting that reduction of cyclin D1 biosynthesis is not the major cause, at least during the early stage of viral infection, of CVB3-induced cyclin D1 down-regulation.

Cyclin D1 is an unstable protein that is degraded by ubiquitin-dependent proteolysis. In the process of degradation by the ubiquitin-proteasome pathway, the protein substrate is first conjugated to multiple molecules of ubiquitin in a reaction involving ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3) (25). The polyubiquitinated substrate is then rapidly degraded by the 26S proteasome. In addition to altering protein synthesis, CVB3 might down-regulate cyclin D1 by stimulating ubiquitin-proteasome-mediated degradation. This was confirmed by our data showing that proteasome inhibitors, MG132 and lactacystin, blocked CVB3-mediated down-regulation of cyclin D1. Consistent with these data, multiple ubiquitin-cyclin D1 conjugates were observed in CVB3-infected cells but not in sham-infected cells. Further, our studies using oligonucleotide microarray technology (Affymetrix, Santa Clara, Calif.) have determined that ubiquitin-like protein is up-regulated in CVB3-infected HeLa cells (unpublished data). It has been suggested that phosphorylation of cyclin D1 by GSK3β on a single threonine residue positively regulates the proteasomal degradation of cyclin D1 (8, 9). The GSK3β inhibitor LiCl, which prevented CVB3-induced degradation of β-catenin, did not block cyclin D1 reduction. Such findings suggest that GSK3β does not appear to be involved in the regulation of cyclin D1 during CVB3 infection. Future studies to identify the protein kinase(s) that regulates this process will elucidate the precise mechanism by which CVB3 degrades cyclin D1.

It has been shown that CVB3 infection in vitro triggers apoptosis and cell death (5). The detailed mechanisms by which CVB3 induces cell death are still unclear, although the mitochondial pathway has been implicated in early cell death (submitted for publication). The tumor suppressor protein p53

has been shown to play a critical role in cell cycle arrest and apoptosis by activating several target genes, including those for Bax, p21, and gadd45 (24, 27). DNA tumor viruses have evolved mechanisms to both trigger and inhibit apoptosis which involve binding and inactivation of the tumor suppressor protein p53 (47, 49). In this study, we showed that expression of p53 was markedly reduced following CVB3 infection, suggesting that the CVB3-induced cell death pathway is unrelated to the p53 pathway. Furthermore, CVB3 may have developed certain mechanisms to inhibit apoptosis by inactivating the p53 pathway. It was reported recently that p53 is degraded by poliovirus protease 3C and that this degradation does not appear to involve the ubiquitin-proteasome pathway (46). However, in this study we clearly showed that the inhibition of ubiquitin-proteasome attenuated p53 degradation by CVB3, a virus closely related to poliovirus.

It is not clear why CVB3 prevents host cells from proliferating. Many viruses have been shown to either promote or prevent cell cycle progression to maximize their own replication. For example, tumor viruses that replicate in the nuclei of host cells have evolved strategies to provide an environment that is more favorable for their replication. Such viruses include simian virus 40 (26), adenovirus (3, 32), and human T-cell leukemia virus (33), which have been reported to stimulate host cell entry into S phase to facilitate replication of the viral genome. In contrast, many other viruses, such as human immunodeficiency virus type 1 (15, 36), herpes simplex virus (11, 42), and human cytomegalovirus (10, 28), maximize virus production by preventing cell proliferation and progression of the cell cycle. CVB3 is an RNA virus and encodes several proteins essential for viral RNA synthesis, including an RNA-dependent RNA polymerase, suggesting that cellular S-phase factors are not required for efficient infection. Indeed, we have shown that CVB3 infection of cells in G_1 phase prevents those cells from entering S phase. It is conceivable, therefore, that a CVB3-induced cell cycle block may create an environment favorable for viral replication, one that requires the takeover of the host replicative apparatus and utilization of host biological materials. This hypothesis has most recently been confirmed while this paper was in preparation. Feuer et al. (14) demonstrated that cells arrested at G_1 or G_1/S phase produced high levels of infectious CVB3. Early virus-mediated manipulation of cell cycle progression prior to the induction of apoptosis may be part of a resourceful viral strategy to conserve cellular energy and materials for maximum replication. Such disruption may also initiate apoptotic signals, causing further injury and eventually facilitating efficient progeny release. The success of CVB3 replication in terminally differentiated cardiomyocytes suggests that a lack of cell proliferation may benefit virus growth. Cell cycle disruptions in this setting may contribute to the pathogenesis of chronic infection in the late stages of the infectious process (20, 21, 37). Increased understanding of the interactions between CVB3 infection and host cell cycle regulation may provide new insights into viral replication and viral pathogenicity and may lead to new avenues for therapeutic intervention in CVB3-induced diseases.

ACKNOWLEDGMENTS

This work was funded by grants from the Heart and Stroke Foundation of British Columbia and Yukon (B.M.M.) and the Canadian Institutes of Health Research (B.M.M.) and doctoral traineeships from the Canadian Institutes of Health Research and Heart and Stroke Foundation of Canada (B.Y.) and the Michael Smith Foundation for Health Research (B.Y.).

We thank Reinhard Kandolf (University of Tübingen, Tübingen, Germany) for providing the original stock of CVB3.

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