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Herbimycin A Induces G1 Arrest through Accumulation of $p27^{Kip1}$ in Cyclin D1-Overexpressing Fibroblasts

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The ansamycin antibiotic herbimycin A is a potent tyrosine kinase inhibitor and reduces the growth rate of various types of mammalian cells. When quiescent Rat6 fibroblast cells were treated with herbimycin A, serum-induced expression of cyclin D1 was inhibited, and this was associated with inhibition of G1 phase progression. However, herbimycin A also inhibited serum-induced G1 progression in derivatives of the Rat6 fibroblast cell line that stably overexpress a human cyclin D1 cDNA (R6ccnD1#4 cells), without affecting the expression levels of G1 cyclins. We found that herbimycin A prevented serum-induced downregulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, thereby leading to inactivation of the protein kinase activity of CDK2. These results suggest that herbimycin A inhibits a tyrosine kinase(s) that plays a role in degradation of the $p27^{Kop1}$ protein.

In eukaryotic cells, cell cycle progression is controlled by the sequential formation, activation, and subsequent inactivation of a series of specific cyclin-cyclin-dependent kinase (CDK) complexes. Cyclins D and E are expressed during the G1 phase in mammalian cells. Cyclin D associates with and activates CDK4 and CDK6 (1, 2). Expression of cyclin D depends on the presence of growth factors and may be a signal to cells that nutrient levels are sufficient for cell division (3). Cyclin E associates with CDK2 to form an active kinase (4, 5). The cyclin E concentration peaks at the G1 to S transition, suggesting a role of cyclin E in the initiation of DNA synthesis. The kinase activity of cyclin/CDK complexes is further regulated by two additional mechanisms (for review see 6–8). The first involves phosphorylation of CDKs (9). The complete activation of CDKs requires phosphorylation of a conserved threonine residue (residue 160 in CDK2) by the CDK-activating kinase (CAK). The second regulatory mechanism involves two classes of CDK inhibitors (CDKI) that bind to cyclin/CDK complexes and inhibit their catalytic activity. The INK4 class of CDKI proteins (p15, p16, p18, and p19) specifically bind to and inhibit the cyclin D-associated kinases CDK4 and CDK6 (10–12). Another class of CDKI, the Cip/Kip proteins ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$), are more promiscuous and inhibit the activity of several cyclin/CDK complexes, at least *in vitro* (13–21). The activated cyclinD1/CDK4 complex and the cyclin E/CDK2 phosphorylate the retinoblastoma protein (pRB), resulting in release of the transcription factor E2F, which then promotes the transcription of S phase-associated genes (22). However, the mechanisms that regulate the expression of CDKIs are not fully understood.

Herbimycin A was isolated as a substance which reversed the transformed morphology to normal when a temperature-sensitive RSV-transformed rat cell (ts/NRK) was treated with the drug at a permissive temperature (23). This was associated with a concomitant drastic reduction in intracellular $p60^{src}$ kinase activity due to inhibition of formation of the $p60^{src}$–heat shock protein 90 (HSP90) complex (24, 25). It was also reported that herbimycin A caused a pRb-dependent G1 block. Several mechanisms by which herbimycin A affects the pRb-dependent pathway have been proposed, including inhibition of cyclin D1 mRNA expression, inhibition of translation of cyclin D1 protein, and reduction of the stability of CDK6 (26–28). In the present study we have compared the effects of herbimycin A on cell cycle progression in Rat6 (R6) fibroblast cells

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(R6ccnD1#4) that stably overexpress cyclin D1 and in vector control cells (R6pl), using cell cultures synchronized by serum starvation and subsequent restimulation. We found that under these conditions herbimycin A inhibited both the increased expression of cyclin D1 and the degradation of $p27^{Kip1}$. Thus a herbimycin-sensitive tyrosine kinase(s) may be involved in both of these pathways.

MATERIALS AND METHODS

Materials. Herbimycin A was isolated from *Streptomyces* as described before (23). The polyclonal antibodies to cyclins D1, E, and CDK2 were obtained from UBI (Lake Placid, NY); $p27^{Kip1}$, from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); histone H1, from Boehringer-Mannheim; [^{3}H]thymidine and [^{35}S] protein labeling Mix, from Amersham Corp. (Arlington Heights, IL).

Cell synchronization. R6 cells were cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) and then for 48 h in medium containing 0.2% CS.

Western blotting. Cells were washed twice in ice-cold PBS and lysed in lysis buffer (50 mM Tris–HCl, pH 7.2, 125 mM NaCl, 0.5% NP-40, 0.1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice and centrifuged at 15,000 rpm for 15 min, to yield the soluble cell lysates. For immunoblotting, 50 μg proteins of cell lysates were subjected to 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred onto a PVDF membrane and then incubated with appropriate antibodies for 1 h. Enhanced chemiluminescence (Amersham) was used to visualize the immunoblot signals.

CDK assay. Cells were lysed in lysis buffer. After centrifugation, the clarified supernatant material was incubated with CDK2 antibody for 1 h at 4°C. Immune complexes were recovered with protein A–agarose and washed with lysis buffer. The immunoprecipitates were suspended in reaction buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM Na_3VO_4), and incubated with 1 μg of histone H1 and 5 μCi of [γ-^{32}P]ATP for 15 min at 30°C. The reaction product was analyzed by SDS–PAGE.

Detection of $p27^{Kip1}$ degradation. Cells were cultured for 24 h in DMEM supplemented with 10% calf serum (CS) and then for 24 h in medium without methionine and cysteine containing 0.2% CS and 100 μCi of ^{35}S-protein labeling mix (73% [^{35}S]methionine, and 22% [^{35}S]cysteine; NEN). Then the medium was changed to 10% CS DMEM with or without herbimycin A, and cells were further incubated for 16 h and lysed in lysis buffer. The cell lysate was immunoprecipitated with the $p27^{Kip1}$ antibody. The immunoprecipitates were analyzed by SDS–PAGE and fluorography.

RESULTS

Herbimycin A inhibits S-phase induction. When quiescent R6pl cells were stimulated with serum, they entered S phase at 16 h, as evaluated by [^{3}H]thymidine incorporation. As shown in Fig. 1A, herbimycin A inhibited serum-induced DNA synthesis at 0.1–1 μg/ml in a dose dependent manner, indicating that herbimycin A inhibited serum-induced G1/S progression. The accumulation of cells in G1 as a result of treatment with 1 μg/ml herbimycin A was confirmed by flow cytometric analysis (Fig. 1B). Quiescent Rat6 cells showed a sharp G0/G1 2C peak. After the addition of serum, by 20 h they displayed an S phase fraction and a G2/M 4C peak. The addition of herbimycin A completely inhibited the appearance of S- and G2/M-phase cells.

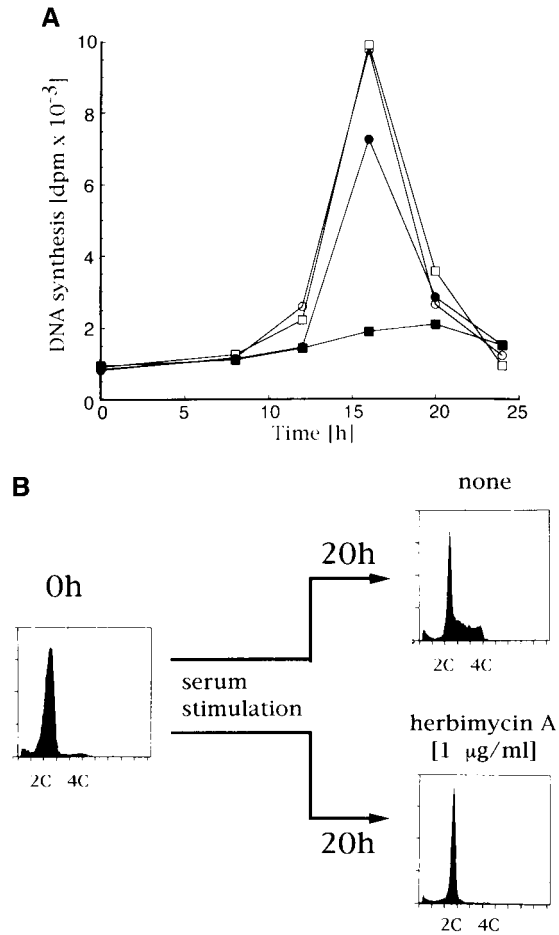

FIG. 1. Inhibition of S phase entry by herbimycin A in R6 pl cells. (A)Serum-starved cells were incubated with 5% serum and various concentrations of herbimycin A (○, 0 μg/ml; □, 0.1 μg/ml; ●, 0.3 μg/ml; and ■, 1.0 μg/ml). The cells were then labeled for 1 h with [^{3}H]thymidine at the indicated times after serum addition. Values are means ± SD of triplicate samples. (B) Serum-starved cells were stimulated with 5% serum in the presence or absence of 1 μg/ml of herbimycin A for 20 h. Then, the cells were stained with propidium iodide (PI) and examined for their DNA content by flow cytometer. 2C designates G0/G1 cells and 4C G2/M cells.

Herbimycin A inhibits expression of G1 cyclins and activation of CDK2 in R6pl cells. Because herbimycin A inhibited the G1/S progression of R6pl cells, we examined the protein levels of G1 cyclins and CDK2 in herbimycin A-treated R6pl cells (Fig. 2A). The expression of cyclin D1, which was induced by serum stimulation at about 10 h, corresponding to the early G1 phase, was strongly inhibited by the treatment with herbimycin A. Cyclin E was induced at about 15 h and herbimycin A also markedly inhibited its expression. CDK2 proteins were detected as a doublet, with the

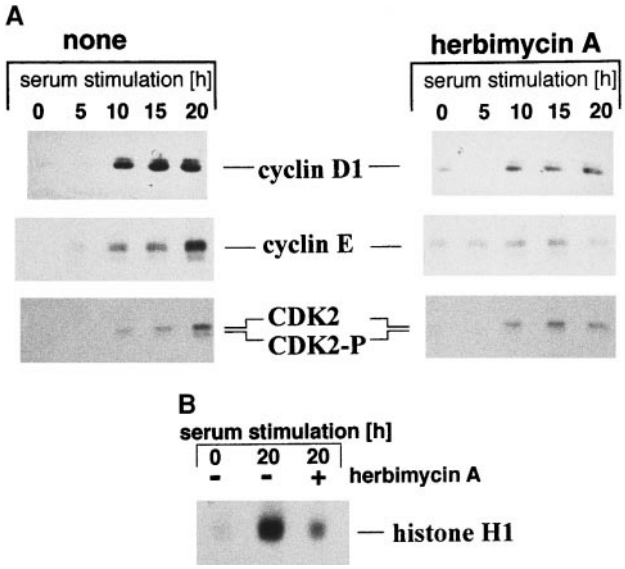

FIG. 2. Inhibition of serum-induced G1 cyclin expression and CDK2 phosphorylation by herbimycin A in R6pl cells. (A) Serum-starved cells were stimulated with 5% of serum in the presence or absence of 1 μg/ml of herbimycin A. Samples (50 μg proteins) were prepared at the indicated times (h) after serum addition and analyzed by Western blotting using appropriate antibodies. (B) Serum-starved cells (0 h) or cells 20 h after serum stimulation in the presence or absence of 1 μg/ml of herbimycin A (20 h) were lysed and CDK2 immunoprecipitates were assayed *in vitro* for histone H1 kinase activity.

lower band representing phosphorylation on the threonine 160 residue. Herbimycin A did not affect the protein level of CDK2, but it did reduce the level of phosphorylated CDK2 (Fig. 2A). Next, we examined the effect of herbimycin A on CDK2 activation. As shown in Fig. 2B, high histone H1 kinase activity in an immune complex prepared from R6pl cells with an anti-CDK2 antibody was detected at 20 h after serum addition. On the other hand, only very weak histone H1 kinase activity was detected with the CDK2 immunoprecipitates prepared from the cells treated with 1 μg/ml of herbimycin A during the stimulation with serum.

Effect of herbimycin A on G1 progression in cyclin D1 overexpressor cells. Because herbimycin A inhibited expression of G1 cyclins in R6pl cells (Fig. 2A), we examined the effect of herbimycin A on S phase entry in quiescent and serum stimulated R6ccnD1#4 cells that stably overexpress a human cyclin D1 cDNA. In these cells, not only cyclin D1 but also cyclin E and phosphorylated CDK2 were presented at relatively high levels at the 0-time point, and throughout the 15-h time course. Herbimycin A did not affect the expression levels of these proteins (Fig. 3A). However, serum-induced G1/S progression was completely inhibited by herbimycin A treatment of the cyclin D1-overexpressing cells (Fig. 3B), and the activation of CDK2 induced by serum stimulation was also inhibited by herbimycin A treatment in the overexpressor cells (Fig. 3C).

Herbimycin A inhibited serum-induced degradation of $p27^{Kip1}$. Because herbimycin A inhibited activation of CDK2 without causing a reduction in the levels of G1 cyclins in R6ccnD1#4 cells, we examined the level of expression of the CDK inhibitor $p27^{Kip1}$ protein following the restimulation of serum-starved cells. The $p27^{Kip1}$ protein was expressed at a relatively high level

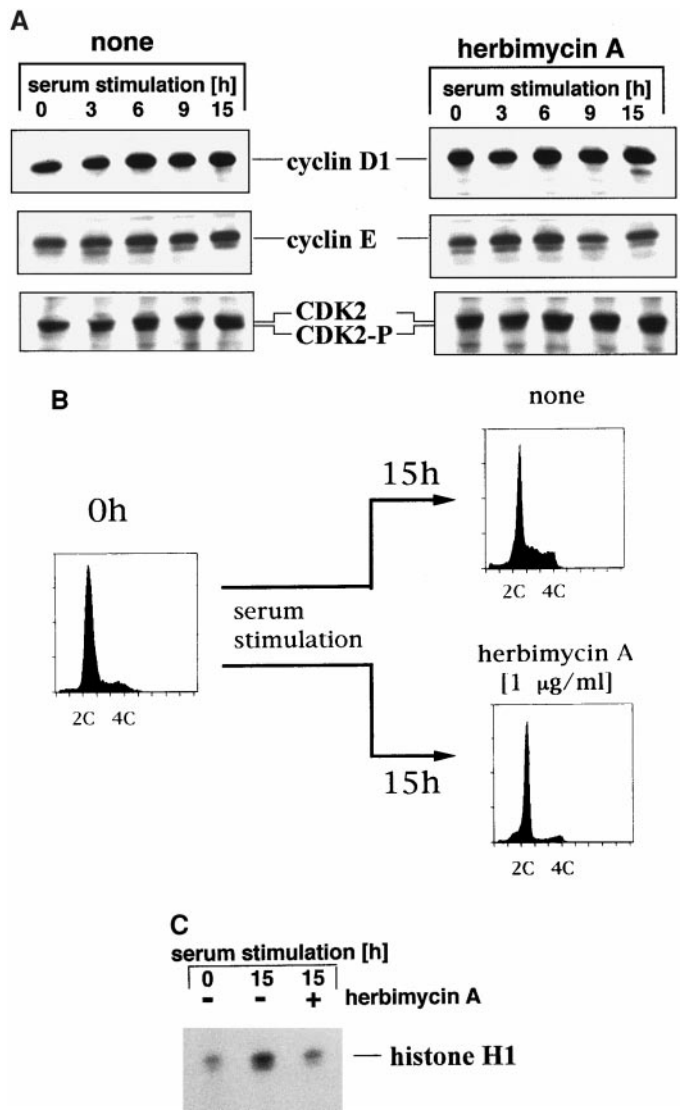

FIG. 3. Inhibition of S phase entry by herbimycin A in cyclin D1 overexpressing R6ccnD1#4 cells. (A) Serum-starved cells were stimulated with 5% serum in the presence or absence of 1 μg/ml of herbimycin A for 15 h. The cells were then stained with PI and examined for their DNA content by flow cytometer. (B) Serum-starved cells (0 h) or cells 15 h after serum stimulation in the presence or absence of 1 μg/ml of herbimycin A (15 h) were lysed and CDK2 immunoprecipitates were assayed *in vitro* for histone H1 kinase activity.

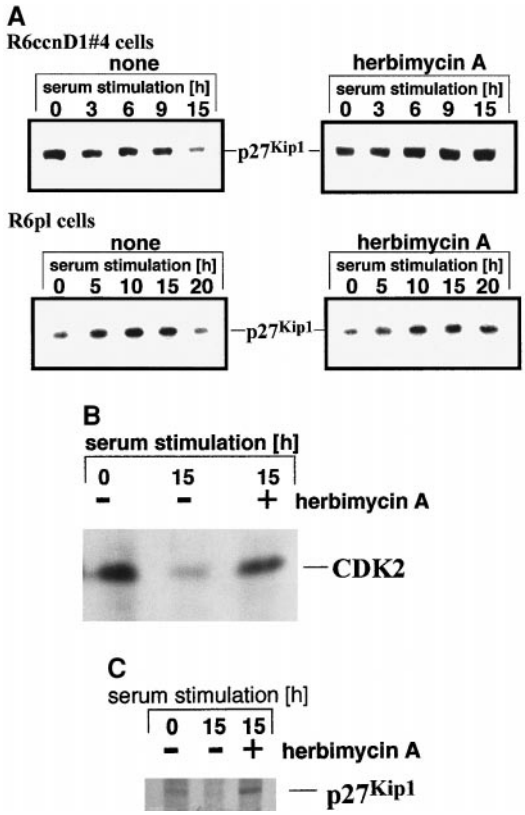

FIG. 4. Inhibition of serum-induced $p27^{Kip1}$ degradation by herbimycin A in R6ccnD1#4 cells. (A) Serum-starved cells were stimulated with 5% serum in the presence or absence of 1 μg/ml of herbimycin A. Samples (50 μg proteins) were prepared at the indicated times (h) after serum addition and analyzed by Western blotting using anti-$p27^{Kip1}$ antibody. (B) Serum-starved cells (0 h) or cells 15 h after serum stimulation in the presence or absence of 1 μg/ml of herbimycin A (15 h) were lysed and immunoprecipitates were prepared with anti-$p27^{Kip1}$ antibody and then subjected to SDS–PAGE and Western blot analysis with the CDK2 antibody. (C) Serum starved cells were labeled with [^{35}S]-labeled methionine and cysteine for 24 h. The cells were then stimulated with 5% serum in the presence or absence of 1 μg/ml of herbimycin A. At the indicated times [h], cells were lysed and immunoprecipitated with anti-$p27^{Kip1}$ antibody. Immunoprecipitates were and analyzed by SDS–PAGE and fluorography.

in the quiescent R6ccnD1#4 cells and began to decline at 15 h after serum stimulation, but in the herbimycin A-treated cells the level of this protein actually increased during this period (Fig. 4A). In R6pl cells, there was increased expression of $p27^{Kip1}$ at 5 h after release from serum starvation, and the level of $p27^{Kip1}$ declined at 20 h (Fig. 4A). The decline in this protein at 20 h in R6pl cells was also inhibited by herbimycin A. Furthermore, we found that there was increased binding of CDK2 to $p27^{Kip1}$ in the herbimycin A treated R6ccnD1#4 cells (Fig. 4B), resulting in inactivation of CDK2 activity. As shown in Fig. 4C, [^{35}S]-labeled $p27^{Kip1}$ seen in the quiescent R6ccnD1#4 cells largely disappeared at 15 h after serum stimulation, whereas in herbimycin A-treated cells, [^{35}S]-labeled $p27^{Kip1}$ was still detected at 15 h following serum stimulation.

DISCUSSION

Previous studies demonstrated that herbimycin A arrested several types of cells in the G1 phase of the cell cycle (26–28). In the present study we found that the serum-induced G1/S transition of quiescent R6 cells was also inhibited by herbimycin A (Figs. 1A and 1B). Herbimycin A inhibited the serum-induced expression of cyclins D1 and E, and the phosphorylation of CDK2 by CAK, thereby inhibiting CDK2 activation (Figs. 2A and 2B). We previously reported that in R6 cells stable overexpression of cyclinD1 leads to increased expression of cyclin E, and increased phosphorylation of CDK2, even under conditions of serum-starvation (29). Therefore, it appeared that the herbimycin A-inhibition of G1 progression might be due to inhibition of cyclin D1 expression. To confirm this, we examined the effect of herbimycin A on serum-induced G1 progression in cyclin D1-overexpressing R6 (R6ccnD1#) cells. Treatment of R6ccnD1#4 cells with herbimycin A did not affect the high level of expression of cyclin D1 in these cells (Fig. 3), indicating that the inhibition of cyclin D1 expression by herbimycin A seen in normal R6 cells was not due to translational inhibition. It is of interest that in the cyclin D1-overexpressing cells, although expression of G1 cyclins and phosphorylation of CDK2 were not affected by herbimycin A, CDK2 activation was still inhibited and there was inhibition of S phase entry (Fig. 3). These findings indicate that a mechanism other than inhibition of cyclin D1 expression is responsible for herbimycin A-induced G1 arrest. Quiescent cells are considered to require two mitogenic stimuli to reenter the cell cycle, induction of the synthesis of G1 cyclins and induction of $p27^{Kip1}$ degradation (29–31). We found that in the R6ccnD1#4 cells as well as in the R6pl cells, herbimycin A inhibited the usual decrease of $p27^{Kip1}$ seen after serum stimulation and instead $p27^{Kip1}$ accumulated (Fig. 4A). Accumulated $p27^{Kip1}$ in R6ccnD1#4 cells was bound to CDK2 (Fig. 4B). Metabolic labeling experiments showed that the accumulation of $p27^{Kip1}$ was due to inhibition of serum-induced degradation of $p27^{Kip1}$ (Fig. 4C). Previous studies indicated that the $p27^{Kip1}$ protein is degraded by the ubiquitin-proteasome pathway (28). Therefore, it is likely that herbimycin A inhibits $p27^{Kip1}$ degradation through inhibition of the ubiquitin-proteosome system. It has been reported that the expression of cyclins and CDK inhibitors is controlled by ERK2, p38, JNK (33, 34) and $p70^{S6}$ kinase (35). However, herbimycin A does not affect the activation of these kinases (data not shown). Herbimycin A is known to inhibit formation of the HSPs/src family tyrosine kinase complex, thus resulting in inactivation of tyrosine kinase activity (25). Therefore, it appears that a herbimycin A-sensitive tyrosine kinase(s) is involved in pathways of both serum-induced cyclin D1 expression and of $p27^{Kip1}$ degradation.

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