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Involvement of Down-Regulation of Cdk2 Activity in Hepatocyte Growth Factor–Induced Cell Cycle Arrest at G1 in the Human Hepatocellular Carcinoma Cell Line HepG2

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Hepatocyte growth factor (HGF) induces growth stimulation of a variety of cell types, but it also induces growth inhibition of several types of tumor cell lines. We previously investigated the intracellular signaling pathway involved in the antiproliferative effect of HGF on the human hepatocellular carcinoma cell line HepG2. The results suggested that the HGF-induced proliferation inhibition is caused by cell cycle arrest, which results from the retinoblastoma tumor suppressor gene product pRb being maintained in its active hypophosphorylated form *via* a high-intensity ERK signal. In this study, we examined the molecular mechanism of the HGF-induced cell cycle arrest in HepG2 cells. Cyclin A/Cdk2 complexes phosphorylated serine residues on pRb crucial for the G1 to S phase transition in proliferating HepG2 cells, and HGF treatment inhibited the phosphorylation. The expression of cyclin A was decreased and the expression of a Cdk inhibitor $p21^{Cip1}$ was increased in HGF-treated HepG2 cells, and these changes were prevented by pretreatment with a low concentration of a MEK inhibitor. These results suggest that the decrease in cyclin A expression and increase in $p21^{Cip1}$ expression through a high-intensity ERK signal by HGF lead to suppression of the phosphorylation of pRb by Cdk2, which contributes to the cell cycle arrest at G1 in HepG2 cells by HGF. Furthermore, the expression of E2F-1, a member of the E2F transcription factor family, was decreased in HGF-treated HepG2 cells, suggesting that the decrease in E2F-1 expression may also contribute to the cell cycle arrest at G1.

Key words: cell cycle arrest, cyclin A/Cdk2, HepG2, HGF, $p21^{Cip1}$.

Abbreviations: Cdk, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal–regulated kinase; FBS, fetal bovine serum; HGF, hepatocyte growth factor; MEK, mitogen-activated protein kinase/ERK kinase; TGF-β1, transforming growth factor β1.

Hepatocyte growth factor (HGF) is a heparin-binding glycoprotein originally described as a potent mitogen for hepatocytes in primary culture (*1–4*). Subsequent studies have revealed that HGF stimulates the proliferation of other cell types (*5–7*). On the other hand, the proliferation of a number of tumor cell lines is inhibited by treatment with HGF (*8–11*), although HGF stimulates the proliferation of some tumor cell lines (*6*, *9*, *10*, *12*). The opposing effects of HGF on the proliferation of cells are transduced through the activation of the same high-affinity receptor, the c-*met* protooncogene product (the c-Met receptor) (*13–16*). Thus, the downstream signaling pathways of the c-Met receptor might be involved in the opposing effects of HGF. However, the molecular mechanism leading to the proliferation inhibition of tumor cell lines by HGF has yet to be studied extensively, although some mechanisms have been proposed (*17–19*).

We previously investigated the intracellular signaling pathway involved in the antiproliferative effect of HGF on the human hepatocellular carcinoma cell line HepG2. HGF induced strong activation of ERK in HepG2 cells. A reduction of this strong activation to a weak activation by a low concentration of MEK inhibitors restored the proliferation of HepG2 cells inhibited by HGF. In addition, expression of constitutively activated Ha-Ras, which induces a strong activation of ERK, led to the proliferation inhibition of HepG2 cells, and this inhibition was suppressed by the MEK inhibitor. Furthermore, HGF treatment and the expression of constitutively activated Ha-Ras changed the ratio of the hyperphosphorylated form of the retinoblastoma tumor suppressor gene product pRb to the hypophosphorylated form, and this change was inhibited by the same concentration of the MEK inhibitor needed to suppress the proliferation inhibition. Based on these results, we suggested that the level of ERK activity determines the opposing proliferation responses of HepG2 cells, and that the HGF-induced proliferation inhibition is caused by cell cycle arrest, which results from pRb being maintained in its active hypophosphorylated form *via* a high-intensity ERK signal in HepG2 cells (*20*). Other groups reported that the expres-

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sion of cyclin-dependent kinase (Cdk) inhibitors was induced in HepG2 cells treated with HGF, suggesting that the HGF-induced proliferation inhibition of HepG2 cells is caused by cell cycle arrest (*17*, *18*).

In this study, we examined the molecular mechanism by which HGF arrests the cell cycle *via* a high-intensity ERK signal in HepG2 cells. We found that cyclin A/Cdk2 complexes phosphorylated serine residues on pRb crucial for G1 to S phase transition in proliferating cells, and HGF treatment inhibited the phosphorylation. We also found that the expression of cyclin A was decreased and the expression of a Cdk inhibitor $p21^{Cip1}$ was increased in HepG2 cells treated with HGF, and that these changes were prevented by pretreatment with a low concentration of a MEK inhibitor. These findings suggest that the decrease in cyclin A expression and increase in $p21^{Cip1}$ expression through a high-intensity ERK signal by HGF lead to suppression of the phosphorylation of pRb by Cdk2, which contributes to the cell cycle arrest at G1 in HepG2 cells. Furthermore, we found that the expression of E2F-1, a member of the E2F transcription factor family, was decreased in HepG2 cells treated with HGF, suggesting that the decrease may also contribute to the cell cycle arrest at G1 in HepG2 cells by HGF.

EXPREMENTAL PROCEDURES

Reagents—Reagents were obtained as follows: anti-pRb (Clone G3-245) and anti–cyclin E (Clone HE67) antibodies from BD Pharmingen; an anti–cyclin A (Clone 25) antibody from BD Transduction Laboratories; anti-Cdk2, anti-Cdk4, anti-Cdk6, anti–cyclin A, anti–cyclin D2, anti-$p21^{Cip1}$, and anti–E2F-1 antibodies from Santa Cruz Biotechnology; anti–phosphorylated pRb at Ser780, 795, and 807/811 specific antibodies, an anti–pRb-COOH-terminal antibody and horseradish peroxidase–conjugated anti–rabbit immunoglobulin from Cell Signaling Technology; horseradish peroxidase–conjugated anti-mouse and anti-rabbit immunoglobulins from Amersham Pharmacia Biotech; recombinant human HGF from the Research Center of Mitsubishi Chemical Corp.; recombinant human TGF-β1 from R & D Systems; full-length retinoblastoma protein from QED Bioscience Inc.; recombinant fusion protein of pRb residues 701–928 and maltose binding protein from Cell Signaling Technology; PD98059 from Calbiochem.

Cell Culture and Cell Proliferation Assay—HepG2 cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Cells were seeded at a density of 5×10^4 cells/well in 12-well plates and cultured with DMEM containing 10% FBS for 1 day. They were subsequently treated with HGF (50 ng/ml) or TGF-β1 (2 ng/ml) and cultured for 4 days. The cells were harvested after trypsinization, and the number of cells was counted using a hemocytometer.

Cell-Cycle Analysis by Flow Cytometry—Cells were seeded at a density of 5×10^4 cells/well in 12-well plates and cultured with DMEM containing 10% FBS for 1 day. They were subsequently treated with reagents and cultured further. The cells were harvested after trypsinization and washed twice with phosphate-buffered saline. They were incubated with flow reagent (0.1% sodium citrate, 0.1% Triton X-100, 50 μg/ml propidium iodide, and 1 μg/ml RNase A) for an appropriate period at room temperature. Cell cycle distribution was analyzed using FACSCalibur equipped with CellQuest software (BD Biosciences).

Preparation of Cell Extracts and Immunoprecipitation—Cells were washed with cold phosphate-buffered saline containing 0.01% EDTA and 0.2 mM Na_3VO_4 before being lysed with cold lysis buffer (20 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 20 mM β-glycerophosphate, 1 mM EGTA, and 0.5% NP-40) containing 1 μg/ml leupeptin and 1 mM phenylmethyl sulfonate fluoride. The lysates were cleared by centrifugation, and the protein concentration in the lysates was measured using the BCA protein assay reagent (Pierce). Equal amounts of cell lysate were incubated with antibody and 20 μl of a 50% slurry of protein A-Sepharose (Amersham Pharmacia Biotech) for 3 h or overnight at 4°C. The immune complexes were precipitated and washed with lysis buffer.

Immunoblot Analysis—The cell lysates and precipitated immune complexes were boiled in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromphenol blue, and 5% 2-mercaptoethanol). The samples were then separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with the primary antibody for 1 h at room temperature or overnight at 4°C and then with horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were visualized with an enhanced chemiluminescence Western blotting detection system (ECL, Amersham Pharmacia Biotech) or Phototope-HRP Western Detection Kit (Cell Signaling Technology).

In Vitro Kinase Assay—Cells were washed with cold phosphate-buffered saline containing 0.01% EDTA and 0.2 mM Na_3VO_4 before being lysed with cold lysis buffer. The lysates were cleared by centrifugation, and the protein concentration in the lysates was determined using the BCA protein assay reagent (Pierce). The protein (1

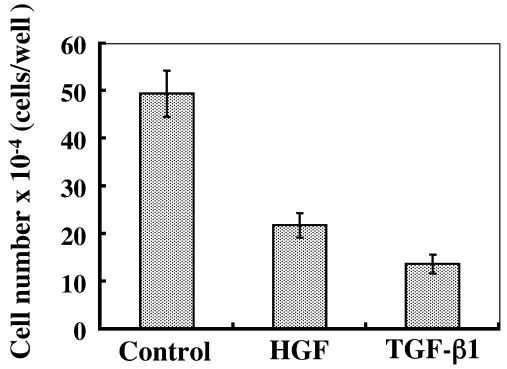

Fig. 1. **Effect of HGF or TGF-β1 on the proliferation of HepG2 cells.** Cells were seeded at a density of 5×10^4 cells/well (12-well plates) in DMEM with 10% FBS (DMEM/FBS). After 24 h, the medium was replaced with fresh medium, and cells were cultured in the absence (Control) or presence of HGF (50 ng/ml) or TGF-β1 (2 ng/ml). After 4 days of culture, cell numbers were counted in the presence of trypan blue. The viable cell number is indicated. Each value represents the mean ± SD of triplicate determinations from a representative experiment.

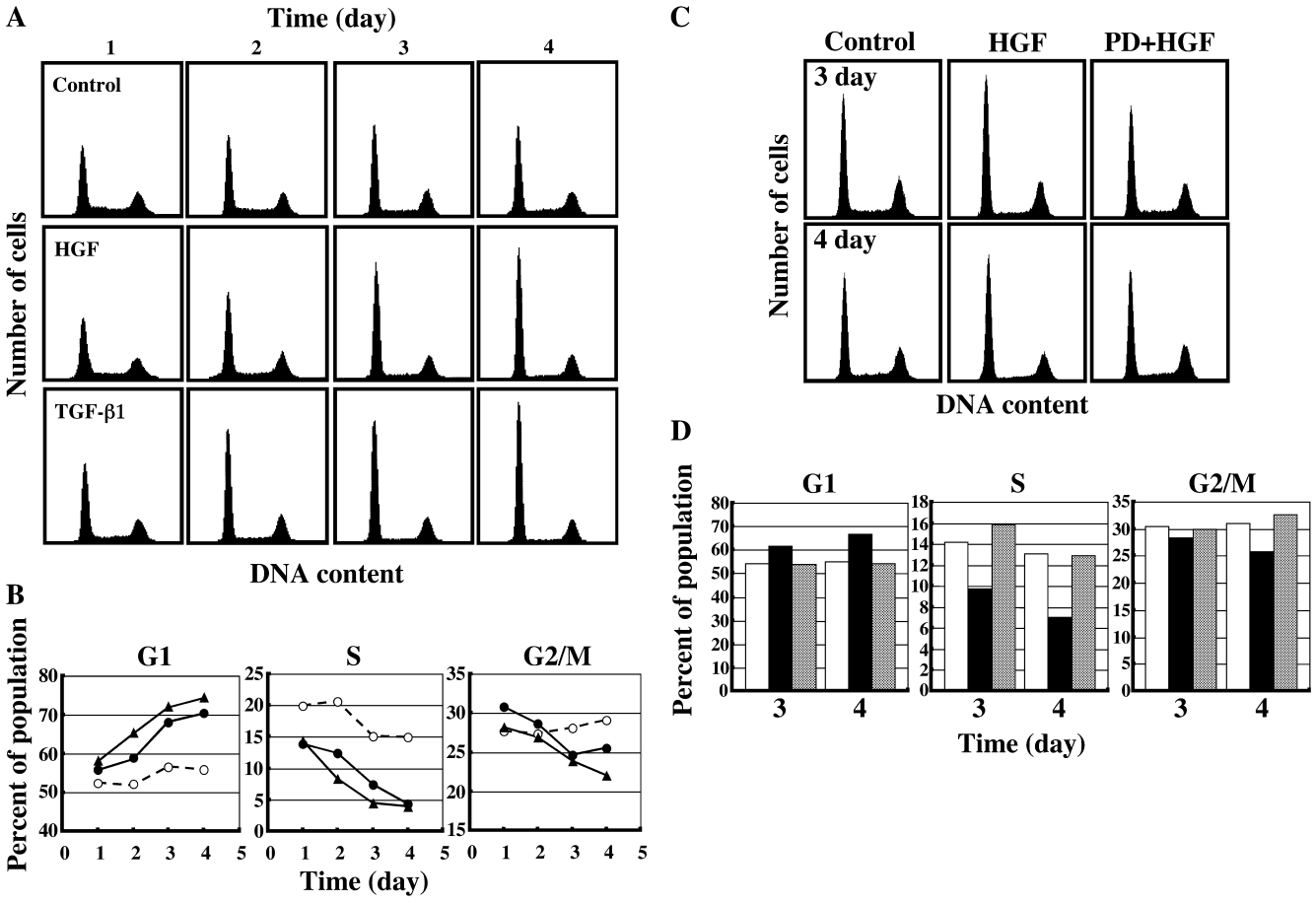

Fig. 2. **Cell-cycle distribution of HepG2 cells after treatment with HGF or TGF-β1.** A, cells were seeded at a density of 5×10^4 cells/well (12-well plates) in DMEM/FBS. After 24 h, the medium was replaced with fresh medium, and cells were cultured in the absence (Control) or presence of HGF (50 ng/ml) or TGF-β1 (2 ng/ml). At the indicated intervals, cells were collected, stained with propidium iodide and analyzed by flow cytometry. B, the percentage of cells in G1, S and G2/M phases is indicated. Cells were cultured in the absence (open circles) or presence of HGF (solid circles) or TGF-β1 (solid triangles). The percentages of cells in each phase of the cell cycle were calculated from a sample of 20,000 cells, using the program CellQuest. C, cells were seeded at a density of 5×10^4 cells/well (12-well plates) in DMEM/FBS. After 24 h, the medium was replaced with fresh medium, and cells were preincubated with or without PD98059 (10 μM) for 1 h, after which HGF (50 ng/ml) was added. Cells were cultured with or without HGF for 3 or 4 days, then collected, stained with propidium iodide and analyzed by flow cytometry. D, the percentage of cells in G1, S and G2/M phases is indicated. Cells were cultured in the absence (white bars) or presence of HGF (black bars) or PD98059 and HGF (gray bars). The percentages of cells in each phase of the cell cycle were calculated from a sample of 20,000 cells, using the program CellQuest.

mg) from the supernatant was incubated with antibodies and 20 μl of a 50% slurry of protein A–Sepharose for 3 h at 4°C. The immune complexes were precipitated and washed with lysis buffer and with reaction buffer (20 mM HEPES-NaOH, pH 7.5, 1 mM dithiothreitol, 10 μM Na_3VO_4, 2 mM β-glycerophosphate, and 100 μM EGTA) containing 1 μg/ml leupeptin and 1 mM phenylmethyl sulfonate fluoride. The precipitates were incubated in 30 μl of reaction buffer containing 1 μg of full-length retinoblastoma protein (pRb) or 1.5 μg of a recombinant protein of pRb residues 701–928 and maltose binding protein (Rb-C fusion protein), 24 μM ATP, and 12 mM $MgCl_2$ at 30°C for 30 min. The reaction was stopped by adding 30 μl of 2× Laemmli sample buffer. The mixture was heated at 99°C for 5 min. The proteins were separated by SDS-PAGE. The phosphorylation of full-length pRb or Rb-C fusion protein was analyzed by immunoblotting.

RESULTS

Effect of HGF or TGF-β1 on the Proliferation of HepG2 Cells—TGF-β1 is known to inhibit the proliferation of HepG2 cells by arresting the cell cycle at G1 (*21*). To explore the molecular mechanism of the HGF-induced cell cycle arrest in HepG2 cells, we compared the effect of HGF with that of TGF-β1. Our previous results showed that the antiproliferative effect of HGF on HepG2 cells first appeared at 3 days and became marked at 4 days (*20*). Fig. 1 shows the effect of HGF or TGF-β1 on the proliferation of HepG2 cells at 4 days after treatment. TGF-β1 inhibited the serum-induced proliferation of HepG2 cells more effectively than HGF. We previously showed that the proliferation inhibition of HepG2 cells by HGF is mediated through strong activation of ERK, and that a reduction of this strong activation to a weak activation by a low concentration (10 μM) of the MEK inhibitor PD98059 restored the proliferation of HepG2 cells inhib-

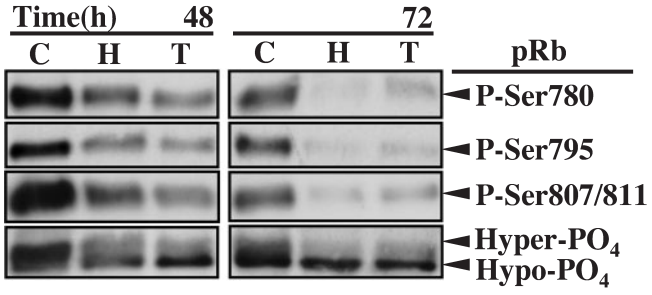

Fig. 3. **Immunoblot analysis of the phosphorylation of pRb at Ser780, Ser795, and Ser807/811 in HepG2 cells treated with HGF or TGF-β1.** Cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 2 days of culture, the medium was replaced with fresh medium and HGF (50 ng/ml) or TGF-β1 (2 ng/ml) was added. Cells were then cultured in the absence (C) or presence of HGF (H) or TGF-β1 (T), their lysates were prepared at the indicated times, and immunoprecipitation was performed using an anti-pRb antibody. The proteins were separated by SDS–6% PAGE and transferred to nitrocellulose membrane. The phosphorylation state of pRb was determined by immunoblot analysis using antibodies specific to pRb phosphorylated at Ser780, 795, and 807/811 (upper panels). Hyperphosphorylated pRb is indicated by a shift to a slower electrophoretic mobility on immunoblot analysis using an anti-pRb antibody (bottom panel). The bands detected with antibodies specific to pRb phosphorylated at Ser780, 795 and 807/811 correspond to the hyperphosphorylated pRb.

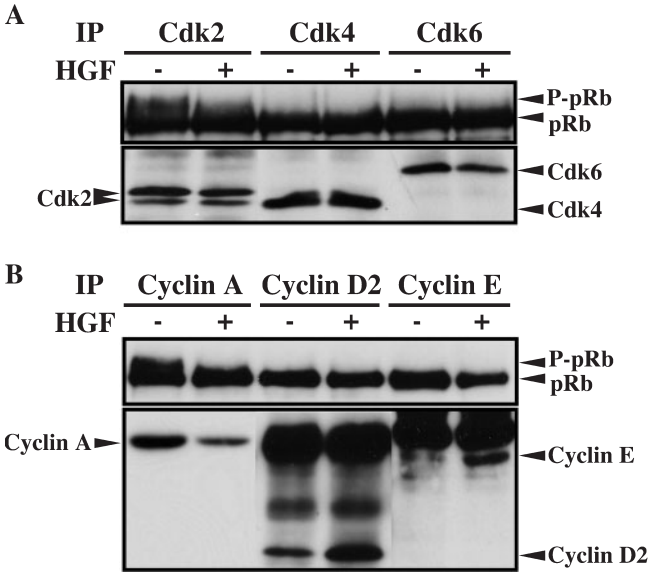

Fig. 4. **Immune complex kinase assay of Cdks and cyclins in HepG2 cells treated with HGF.** Cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 2 days of culture, the medium was replaced with fresh medium. Cells were then cultured in the presence or absence of HGF (50 ng/ml). After 2 days of culture, the cells were collected, and immune complex kinase assays were performed with anti-Cdk2, anti-Cdk4, and anti-Cdk6 antibodies (A), and anti–cyclin A, anti–cyclin D2, and anti–cyclin E antibodies (B) using full-length pRb as a substrate. The proteins were separated by SDS–8% PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with an anti-pRb antibody. The kinase activity of Cdk2, Cdk4, and Cdk6 (A) and cyclin A, cyclin D2, and cyclin E (B) was determined. Phosphorylated full-length pRb is indicated by a shift to a slower electrophoretic mobility (upper panels). Immunoprecipitated proteins were confirmed by immunoblot analysis using anti-Cdk2, anti-Cdk4, and anti-Cdk6 antibodies (A) and anti–cyclin A, anti–cyclin D2, and anti–cyclin E antibodies (B) (bottom panels).

ited by HGF (*20*). To examine whether the antiproliferative effect of TGF-β1 on HepG2 cells is also mediated through ERK signaling, we tested the effect of the MEK inhibitor PD98059. The inhibitor did not restore the proliferation inhibited by TGF-β1 (data not shown), suggesting that the proliferation inhibition of HepG2 cells by TGF-β1 is not mediated through the ERK signaling pathway. Because the Smad pathway is known to be involved in proliferation inhibition of various cells by TGF-β1 (*22*), it may also mediate the proliferation inhibition of HepG2 cells.

Cell-Cycle Distribution of HepG2 Cells after Treatment with HGF or TGF-β1—We previously showed that HGF treatment of HepG2 cells maintained the hypophosphorylated form of pRb, suggesting that the HGF-induced proliferation inhibition of HepG2 cells is caused by cell cycle arrest at G1 (*20*). To prove the cell cycle arrest at G1, we analyzed the cell cycle distribution of HepG2 cells using flow cytometry. HGF treatment increased the number of cells in G1 phase and decreased that in S and G2/M phases. Both the increase and the decrease were also observed in HepG2 cells treated with TGF-β1 (Fig. 2, A and B). These results indicate that the HGF-induced proliferation inhibition of HepG2 cells is caused by cell cycle arrest at G1, similar to the TGF-β1–induced inhibition.

To determine whether the change in the cell cycle distribution of HepG2 cells treated with HGF is mediated through a high-intensity ERK signal, we tested the effect of the MEK inhibitor PD98059 (10 μM), which restores the proliferation of HepG2 cells inhibited by HGF. The increase in G1 phase cells and the decrease in S and G2/M phase cells were prevented by pretreatment with 10 μM PD98059 (Fig. 2, C and D), suggesting that a high-intensity ERK signal induced by HGF leads to the cell cycle arrest at G1 in HepG2 cells.

Phosphorylation Status of pRb at Ser780, Ser795 and Ser807/811 in HepG2 Cells Treated with HGF or TGF-β1—There are more than 10 phosphorylation sites on pRb. In cycling cells, pRb alternates between a hypophosphorylated form in early G1 and a hyperphosphorylated form after passage through the restriction point in late G1 (*23*). Phosphorylation at several serine residues in pRb, which leads to a hyperphosphorylated form of pRb, plays a crucial role in the G1-to-S phase transition of cell cycle. Phosphorylation at serine 780 causes the release of E2F proteins and activation of transcription of E2F-regulated genes important for DNA synthesis and cell cycle progression (*24*). Phosphorylation at serine 795 is required to inactivate the cell cycle arresting function of pRb (*25*). Phosphorylation at serines 807 and 811 disrupts c-abl binding (*26*). Thus, we examined the phosphorylation status of these sites of pRb in HepG2 cells treated with HGF or TGF-β1. Cell lysate was immunoprecipitated with an anti-pRb antibody, and the immunoprecipitates were immunoblotted with an antibody specific to pRb phosphorylated at Ser780, 795 or 807/811. Phosphorylation at these serine residues was detected in

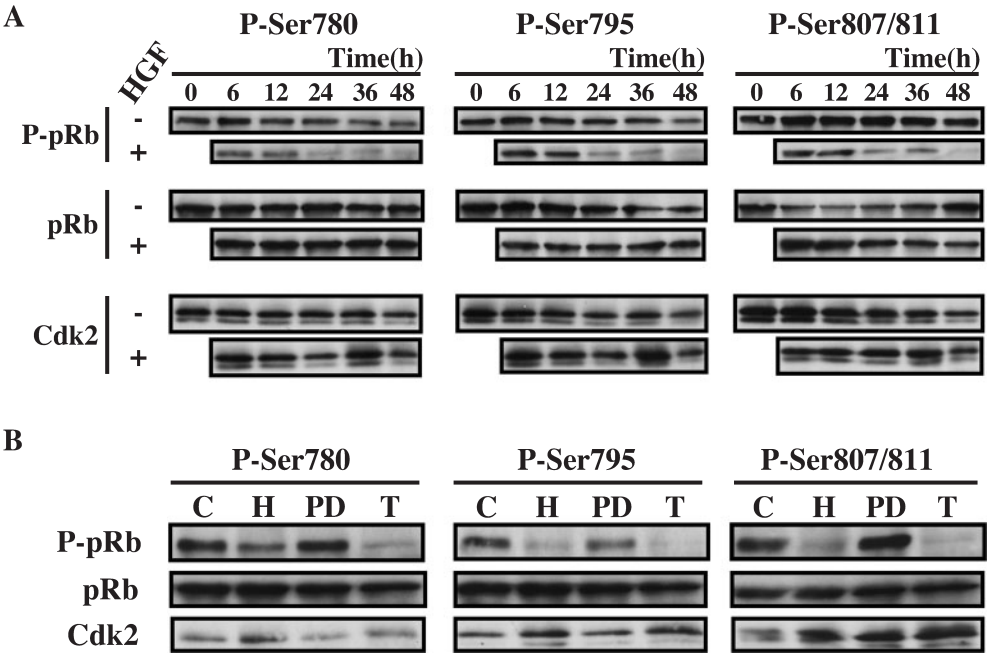

Fig. 5. **Immune complex kinase assay of Cdk2 in HepG2 cells treated with HGF.** A, effect of HGF on the activity of Cdk2 in HepG2 cells. Cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 2 days of culture, the medium was replaced with fresh medium and HGF (50 ng/ml) was added. Cells were then cultured in the presence or absence of HGF. At the indicated times, the cells were collected, and immune complex kinase assays were performed with an anti-Cdk2 antibody using Rb-C fusion protein as a substrate. The proteins were separated by SDS–10% PAGE and transferred to nitrocellulose membrane. The phosphorylation of pRb was examined by immunoblot analysis using antibodies specific to pRb phosphorylated at Ser780, 795, and 807/811 (upper panels). For a protein-loading control, the same blot was reprobed with anti–COOH-terminal pRb antibody (middle panels). Phosphorylation of this substrate by Cdk2 did not lead to mobility shift on the SDS-gel. Immunoprecipitated proteins were confirmed by immunoblot analysis using anti-Cdk2 antibody (bottom panels). B, effect of PD98059 on Cdk2 activity in HepG2 cells treated with HGF. Cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 48 h, the medium was replaced with fresh medium; and following preincubation with or without PD98059 (10 μM) for 1 h, HGF (50 ng/ml) or TGF-β1 (2 ng/ml) was added. Cells were then cultured in the presence of HGF (H), HGF and PD98059 (PD), or TGF-β1 (T), or in the absence of all three (C). After 2 days of culture, the cells were collected, and immune complex kinase assays were performed with an anti-Cdk2 antibody using Rb-C fusion protein as a substrate. The proteins were separated by SDS–10% PAGE and transferred to nitrocellulose membrane. The phosphorylation state of pRb was determined by immunoblot analysis using antibodies specific to pRb phosphorylated at Ser780, 795, and 807/811 (upper panels). For a protein-loading control, the same blot was reprobed with anti–COOH-terminal pRb antibody (middle panels). Immunoprecipitated proteins were confirmed by immunoblot analysis using anti-Cdk2 antibody (bottom panels).

proliferating HepG2 cells in the presence of serum. The bands detected with antibodies specific to pRb phosphorylated at Ser780, 795 and 807/811 corresponded to the hyperphosphorylated pRb. Treatment of the cells with HGF or TGF-β1 suppressed the phosphorylation at all these serine residues (Fig. 3). The decrease in the levels of phosphorylation at Ser780, 795 and 807/811 coincided with the decrease in the ratio of hyperphosphorylated pRb to hypophosphorylated pRb. The results suggest that the suppression by HGF or TGF-β1 of the phosphorylation of pRb crucial for G1 to S phase transition participates in the cell cycle arrest at G1 in HepG2 cells.

Cyclin A/Cdk2 Complexes are Responsible for the Phosphorylation of pRb in HepG2 Cells—Cdk2, Cdk4 and Cdk6 are known to catalyze the phosphorylation of pRb during the G1 phase (*27*, *28*). To identify the kinase responsible for the phosphorylation of pRb in HepG2 cells, cell lysate was immunoprecipitated with an anti-Cdk2, anti-Cdk4 or anti-Cdk6 antibody, and immune complex kinase assays were performed using pRb as a substrate. Phosphorylation of pRb was assessed based on a shift to a slower electrophoretic mobility. The immunoprecipitate with the anti-Cdk2 antibody phosphorylated pRb, whereas that with the anti-Cdk4 or anti-Cdk6 antibody did not. The phosphorylation of pRb by Cdk2 was not detected in the immunoprecipitate from HepG2 cells treated with HGF (Fig. 4A). We used NP-40 as detergent to prepare the cell lysate for the kinase assays. However, Tween-20 is usually used as detergent to prepare cell lysate for the kinase assay of Cdk4 activity (*29*). Therefore, we prepared cell lysate of HepG2 cells using Tween-20 as described by Matsushime *et al.* (*29*), and compared it with cell lysate prepared using NP-40. In the same report, the Cdk4 activity was assessed by incorporation of ^{32}P into pRb, and this system was therefore adopted here for immune complex kinase assays. The Cdk4 activity was detected in the lysate prepared using Tween-20 and was higher than that in the lysate prepared using NP-40. But it was much lower than the Cdk2 activity (data not shown). These results suggest that Cdk2 is mainly responsible for the phosphorylation of pRb in HepG2 cells, and that HGF treatment suppresses the activity of Cdk2.

Next, to identify the cyclin regulating Cdk2 activity in HepG2 cells, cell lysate was immunoprecipitated with an anti–cyclin A, anti–cyclin D2 or anti–cyclin E antibody, and immune complex kinase assays were performed using pRb as a substrate. The immunoprecipitate with the anti–cyclin A antibody phosphorylated pRb, whereas that with the anti–cyclin D2 or anti–cyclin E antibody did not, suggesting that cyclin A is a regulatory subunit for Cdk2 that phosphorylates pRb in HepG2 cells. The phosphorylation of pRb was not detected in the immunoprecipitate from HepG2 cells treated with HGF (Fig. 4B). These results suggest that cyclin A/Cdk2 complexes are responsible for the phosphorylation of pRb in HepG2

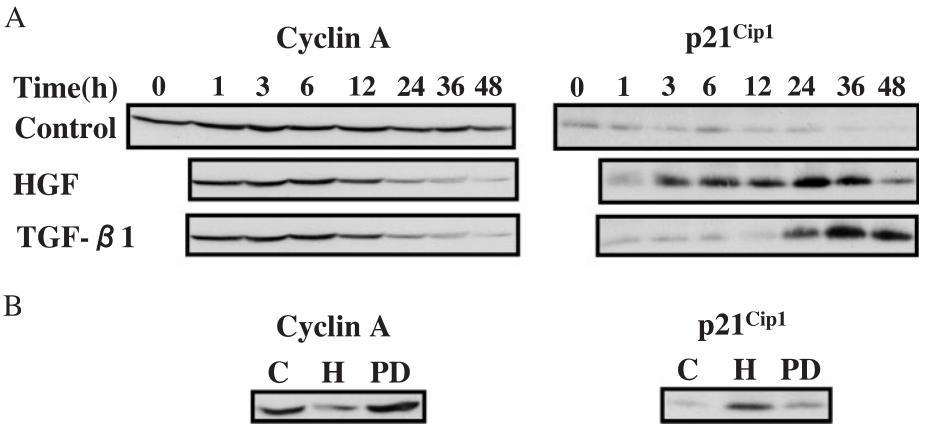

Fig. 6. **Effect of HGF or TGF-β1 on the expression of cyclin A and p21^{Cip1} in HepG2 cells.** A, cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 2 days of culture, the medium was replaced with fresh medium and HGF (50 ng/ml) or TGF-β1 (2 ng/ml) was added. Cells were then cultured in the presence or absence of HGF or TGF-β1, and their lysates were prepared at the indicated times. Proteins (50 μg) from the cell lysates were separated by SDS–10% PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with an anti–cyclin A antibody. Immunoprecipitation with proteins (1 mg) from the cell lysates was performed using anti-p21^{Cip1} antibody. The proteins were separated by SDS–12.5% PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with an anti-p21^{Cip1} antibody. B, effect of PD98059 on the expression of cyclin A and p21^{Cip1} in HepG2 cells treated with HGF. Cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 48 h, the medium was replaced with fresh medium; and following preincubation with or without PD98059 (10 μM) for 1 h, HGF (50 ng/ml) was added. Cells were then cultured with or without HGF. After 48 h of culture, their lysates were prepared. Proteins (50 μg) from the cell lysates were separated by SDS–10% PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with an anti–cyclin A antibody. Immunoprecipitation with proteins (1 mg) from the cell lysates was performed using anti-p21^{Cip1} antibody. The proteins were separated by SDS–12.5% PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with an anti-p21^{Cip1} antibody.

cells, and that HGF treatment suppresses the activity of the complexes. The expression of cyclin A was decreased, whereas the expression of cyclin D2 and E was increased in HepG2 cells treated with HGF (Fig. 4B).

Ser780, Ser795 and Ser807/811 of pRb were phosphorylated in proliferating HepG2 cells. Thus, we examined whether Cdk2 is responsible for the phosphorylation of pRb at these serine residues. Cell lysate was immunoprecipitated with the anti-Cdk2 antibody, and immune complex kinase assays were performed using a pRb-C fusion protein as a substrate. Phosphorylation was assessed by immunoblotting with an antibody specific to pRb phosphorylated at Ser780, 795 or 807/811. The immunoprecipitate with the anti-Cdk2 antibody efficiently phosphorylated pRb at these sites. On the other hand, the level of phosphorylation was decreased in the immunoprecipitate from cells treated with HGF (Fig. 5A). This decrease first appeared at 24 h after treatment and continued until at least 48 h. The level of phosphorylation was restored to the control level by pretreatment with the MEK inhibitor PD98059 at the concentration (10 μM) needed to restore the cell proliferation inhibited by HGF (Fig. 5B). These results suggest that the phosphorylation of pRb at Ser780, Ser795 and Ser807/811 is catalyzed by Cdk2 in HepG2 cells, and that HGF treatment inhibits the activity of Cdk2 through a high-intensity ERK signal. The phosphorylation of pRb at these sites was also decreased with the immunoprecipitate from cells treated with TGF-β1 (Fig. 5B).

Effect of HGF or TGF-β1 on the Expression of Cyclin A and p21^{Cip1} in HepG2 Cells—The activity of Cdk2 is regulated by activating or inhibitory phosphorylation events, by cyclins and by Cdk inhibitors (*23*). These regulations might be affected in HepG2 cells treated with HGF. Cdk2 contains two inhibitory phosphorylation sites at Thr14 and Tyr15, and one activating phosphorylation site at Thr160. The phosphorylation of Cdk2 at Tyr15 and Thr160 was examined by immunoblotting with an antibody specific to Cdk2 phosphorylated at Tyr15 or Thr160. HGF treatment did not affect the phosphorylation at Tyr15 and Thr160 (data not shown). Cyclin A appears to be a regulatory subunit of Cdk2 in HepG2 cells. As shown in Fig. 4B, HGF treatment decreased the level of cyclin A at 48 h. Thus, a time course analysis of the expression of cyclin A was performed in HepG2 cells treated with HGF. The decrease in cyclin A expression first appeared at 24 h after treatment and continued until at least 48 h. A similar decrease was observed in HepG2 cells treated with TGF-β1 (Fig. 6A). The expression of cyclin A returned to the control level on pretreatment with the MEK inhibitor PD98059 (10 μM) (Fig. 6B), suggesting that the decrease in the expression level of cyclin A is mediated through a high-intensity ERK signal.

Next, the expression level of Cdk inhibitors was analyzed by immunoblotting. A rapid increase in the level of p21^{Cip1} was detected in HepG2 cells treated with HGF. The increase first appeared at 3 h after treatment and continued until 36 h. TGF-β1 treatment of HepG2 cells also increased the level of p21^{Cip1}. But the increase was first observed at 24 h after treatment (Fig. 6A). The expression of p21^{Cip1} increased by HGF treatment returned to the control level on pretreatment with the MEK inhibitor PD98059 (10 μM) (Fig. 6B), suggesting that the increase occurs through a high-intensity ERK signal. The expression level of p27^{Kip1} did not change in HepG2 cells treated with HGF (data not shown).

Effect of HGF or TGF-β1 on the Expression of E2F-1 in HepG2 Cells—The transition from G1 to S phase is controlled by various regulatory molecules, in addition to the phosphorylation state of pRb. Members of the E2F family are transcription factors which regulate the expression of genes important for DNA synthesis and cell cycle progression (*30*, *31*). We examined the expression of one member, E2F-1, in HepG2 cells treated with HGF by immunoblotting analysis. HGF treatment decreased the level of E2F-1. The decrease first appeared at 24 h after

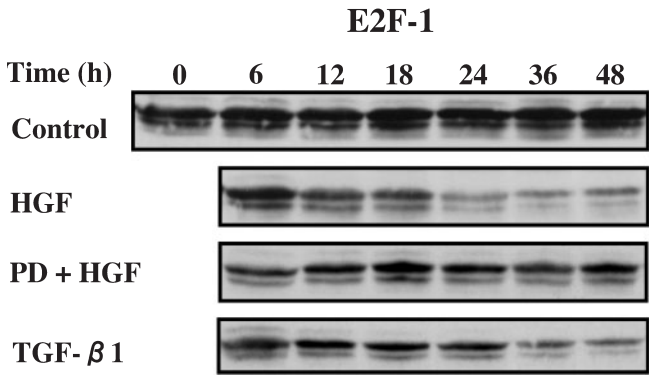

Fig. 7. **Effect of HGF or TGF-β1 on the expression of E2F-1 in HepG2 cells.** Cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 48 h, the medium was replaced with fresh medium; and following preincubation with or without PD98059 (10 μM) for 1 h, HGF (50 ng/ml) or TGF-β1 (2 ng/ml) was added. Cells were then cultured in the presence of HGF, HGF and PD98059 or TGF-β1, or in the absence of all of three, and their lysates were prepared at the indicated times. Proteins (50 μg) from the cell lysates were separated by SDS–8% PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with an anti–E2F-1 antibody.

the treatment and continued until at least 48 h. The expression of E2F-1 decreased by HGF treatment returned to the control level on pretreatment with the MEK inhibitor PD98059 (10 μM), suggesting that a high-intensity ERK signal leads to the decrease. TGF-β1 treatment also decreased the expression level of E2F-1 at 36 h after treatment (Fig. 7).

DISCUSSION

Our previous analysis of the phosphorylation status of pRb suggested that the HGF-induced proliferation inhibition of HepG2 cells is caused by cell cycle arrest at G1 (*20*). In this study, we analyzed the cell cycle distribution of HepG2 cells by flow cytometry, and found that HGF treatment increased the number of cells in G1 phase and decreased that in S and G2/M phases. Similar changes were found in HepG2 cells treated with TGF-β1, which is known to induce cell cycle arrest at G1 in HepG2 cells (*21*). These findings provide conclusive evidence that the HGF-induced proliferation inhibition of HepG2 cells results from cell cycle arrest at G1.

pRb plays a central role in the control of the cell cycle. pRb seems to act as a check during the G1 phase of cell cycle. Activity of pRb is regulated by phosphorylation. In cycling cells, pRb alternates between a hypophosphorylated form present in the early G1 phase and a hyperphosphorylated form after passage through the restriction point in late G1 phase (*23*, *28*). Our previous analysis showed that the hyperphosphorylated pRb was predominant in proliferating HepG2 cells in the presence of serum, and that HGF treatment decreased the hyperphosphorylated pRb and increased hypophosphorylated pRb. Furthermore, the hyperphosphorylated pRb was restored by pretreatment with the MEK inhibitor PD98059 (10 μM), which suppressed the effect of HGF on the proliferation of HepG2 cells (*20*). In the present study, we demonstrated that the increased distribution of cells in G1 phase and the decreased distribution of cells in S and G2/M phases by HGF treatment returned to the control level on pretreatment with 10 μM PD98059. Thus, the phosphorylation status of pRb regulated by HGF probably plays a crucial role in the cell cycle arrest at G1 in HepG2 cells. Phosphorylation at Ser780, Ser795 and Ser807/811 is required to inactivate pRb during the G1 to S phase transition (*24–26*). These sites were phosphorylated in proliferating HepG2 cells in the presence of serum. The phosphorylation of pRb was suppressed in HepG2 cells treated with HGF. Thus, the suppression by HGF of the phosphorylation of pRb at the sites crucial for the G1 to S phase transition appears to contribute to the cell cycle arrest at G1. The suppression may result from inactivation of the kinase activity which catalyzes the phosphorylation of pRb. Alternatively, it is possible that dephosphorylation of pRb is induced in HepG2 cells treated with HGF.

The phosphorylation of pRb at Ser780, Ser795 and Ser807/811 is catalyzed by cyclin/Cdk complexes, which are active during the G1 phase (*32*). In this study, *in vitro* immune complex kinase assays showed that Ser780, Ser795 and Ser807/811 were phosphorylated by the immunoprecipitate from HepG2 cells with the anti-Cdk2 antibody, and that HGF treatment of HepG2 cells suppressed the phosphorylation. These results suggest that Cdk2 is responsible for the phosphorylation of the serine residues crucial for the G1 to S phase transition in HepG2 cells and that the activity of Cdk2 is suppressed in HepG2 cells treated with HGF. These results also suggest that the suppression of the phosphorylation of pRb in HepG2 cells treated with HGF results from inactivation of the kinase activity, but not from induction of dephosphorylation of pRb. It is generally accepted that the cyclin D–dependent activation of Cdk4 and Cdk6 in cycling cells is constitutive throughout the cell cycle, while Cdk2 is activated by cyclin E in late G1 at/near the G1 restriction point and by cyclin A at S phase (*21*, *28*). Moreover, cyclin D/Cdk4/6 were previously shown to preferentially phosphorylate Ser780 and Ser795 on pRb (*24*, *25*). However, the phosphorylation of pRb was not detected in the immunoprecipitate of HepG2 cells with an anti-Cdk4, anti-Cdk6, anti–cyclin D2 or anti-cyclin E antibody in immune complex kinase assays assessed by mobility shift of the phosphorylated pRb on SDS-gel, and it was detected in the immunoprecipitate with anti-Cdk2 and anti–cyclin A antibodies. Thus, cyclin A/Cdk2 may be highly activated in proliferating HepG2 cells, in which pRb is phosphorylated at Ser780, Ser795 and Ser807/811. However, we can not exclude the possibility that cyclin D/Cdk4/Cdk6 and cyclin E/Cdk2 may also be activated, because a low level of the Cdk4 activity was detected in more sensitive immune complex kinase assays.

The activity of Cdk2 is regulated by activating or inhibitory phosphorylation events, by cyclins and by Cdk inhibitors (*23*). HGF treatment did not change the state of phosphorylation in Cdk2, but decreased the expression level of cyclin A and increased the expression level of a Cdk inhibitor $p21^{Cip1}$. The increase in $p21^{Cip1}$ expression first appeared in HepG2 cells at 3 h after the treatment with HGF. A rapid increase in $p21^{Cip1}$ expression was also observed by another group (*17*). However, the suppres-

sion of Cdk2 activity was first detected in HepG2 cells at 24 h after treatment with HGF. Thus, the increase in $p21^{Cip1}$ expression alone may not be enough to suppress the Cdk2 activity in HepG2 cells. The decrease in cyclin A expression first appeared at 24 h after treatment with HGF when the suppression of Cdk2 activity was first detected. These results suggest that the Cdk2 activity is regulated by the level of cyclin A in HepG2 cells, and that HGF treatment lowers this level, leading to the suppression of Cdk2 activity. In addition to the decrease in cyclin A expression, the increase in $p21^{Cip1}$ expression may also contribute to the suppression of Cdk2 activity.

The level of E2F-1 was decreased in HepG2 cells treated with HGF. E2F-1 is a member of the E2F family of transcription factors, which stimulates the expression of genes important for DNA synthesis and cell cycle progression (*30*, *31*). E2F-1 functions as a transcription repressor by binding to the hypophosphorylated form of pRb (*33*), which was predominant in HepG2 cells treated with HGF. The decrease in E2F-1 expression and increase in hypophosphorylated pRb were first detected in HepG2 cells at 24 h after HGF treatment. Thus, the activity of E2F-1 may be suppressed not only by binding to pRb but also by the decrease in expression.

Phosphorylation of pRb at Ser780, Ser795 and Ser807/811 by Cdk2 was suppressed in HepG2 cells treated with TGF-β1, similar to HGF treatment. Furthermore, TGF-β1 decreased the expression of cyclin A and E2F-1 and increased the expression of $p21^{Cip1}$, although the timing of the decrease in E2F-1 and the increase in $p21^{Cip1}$ differed from that after HGF treatment. Both HGF and TGF-β1 induce cell cycle arrest at G1 in HepG2 cells. Down-regulation of Cdk2 activity which suppressed the phosphorylation of pRb was observed in the HGF-treated and TGF-β1–treated HepG2 cells. Thus, common mechanisms may function in both the HGF-induced and TGF-β1–induced cell cycle arrest at G1 in HepG2 cells. The ERK pathway and the Smad pathway probably mediate the HGF signaling and TGF-β1 signaling in the proliferation inhibition of HepG2 cells, respectively. Further studies are required to clarify the mechanisms linking these pathways to the regulatory molecules that were suggested in this study to lead to the cell cycle arrest at G1 in the HGF-treated and TGF-β1–treated HepG2 cells.

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