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Up-Regulation of p21^{CIP1} Expression Mediated by ERK-Dependent and -Independent Pathways Contributes to Hepatocyte Growth Factor-Induced Inhibition of HepG2 Hepatoma Cell Proliferation

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Abstract Strong activation of the ERK signal is required for hepatocyte growth factor (HGF) to inhibit proliferation of the human hepatocellular carcinoma cell line HepG2. However, it is still to be elucidated whether the activation alone is sufficient to induce the inhibitory effect. In this study, we constructed HepG2 cell clones expressing a high level of epidermal growth factor receptor (EGFR), and examined the effect of the strong activation of ERK on the proliferation of the cell clones. EGF treatment of the cell clones induced strong activation of ERK similar to HGF treatment, but did not inhibit cell proliferation. HGF treatment of the cell clones up-regulated the expression of a Cdk inhibitor p16^{INK4a}, which has previously been shown to be required to inhibit the proliferation of HepG2 cells, but EGF treatment did not. Furthermore, EGF treatment of the cell clones did not induce the up-regulation of another Cdk inhibitor p21^{CIP1}, whereas HGF treatment did. Knockdown of p21 by siRNA restored the proliferation of HepG2 cells inhibited by HGF, and restored Cdk2 activity suppressed in HGF-treated HepG2 cells. These results suggest that strong activation of ERK alone is not sufficient, and some other pathway(s), which is activated through the HGF receptor but not through EGFR, is also required to induce the up-regulation of p16 and p21 expression, and also suggest that in addition to the up-regulated expression of p16, that of p21 contributes to the suppression of Cdk2 activity leading to the inhibition of proliferation of HGF-treated HepG2 cells. J. Cell. Biochem. 104: 176–188, 2008.

Key words: HGF; HepG2; ERK; Cdk2; p21^{CIP1}; p16^{INK4a}; EGF receptor; c-Met

Hepatocyte growth factor (HGF) is a mesenchymal cell-derived glycoprotein that is mitogenic for hepatocytes in primary cultures as well as other cell types [Miyazawa et al., 1989; Nakamura et al., 1989; Rubin et al., 1991]. But it also inhibits proliferation of several types of tumor cell lines [Higashio et al., 1990; Tajima et al., 1991; Halaban et al., 1992; Shiota et al., 1992]. This inhibitory effect of HGF was observed in vivo in mouse models. For instance, when HGF transfectants of Fao hepatoma cells were transplanted into nude mice, the tumor formation of the transfectant was suppressed compared with the parental cells [Shiota et al., 1992], and c-*myc*-induced hepatocarcinogenesis was inhibited by HGF in a transgenic mouse model coexpressing c-*myc* and HGF [Santoni-Rugiu et al., 1996]. The opposing effects of HGF on the proliferation of cells are transduced through the activation of the same high-affinity receptor, the c-*met* proto-oncogene product (the c-Met receptor) [Bottaro et al., 1991; Naldini et al., 1991; Komada et al., 1992; Weidner et al., 1993]. Thus, the different downstream signalings of the c-Met receptor determine the opposing effects of HGF.

We previously investigated the intracellular signaling pathway involved in the anti-proliferative effect of HGF on the human hepatocellular carcinoma cell line HepG2 cells. HGF induced strong activation of ERK in HepG2 cells. A reduction of this strong

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activation to a weak activation by a low concentration (10 μM) of a MEK inhibitor PD98059 restored the proliferation of HepG2 cells inhibited by HGF. Thus, HGF-induced inhibition of HepG2 cell proliferation requires strong activation of the ERK pathway [Tsukada et al., 2001]. Our subsequent studies revealed one of the molecular pathways that link the strong activation of ERK to the inhibition. The activated ERK pathway induces the activation of a member of the Ets family of transcription factors, which up-regulates the expression of $p16^{INK4a}$, a cyclin-dependent kinase (Cdk) inhibitor. The p16 protein forms a complex with Cdk4, leading to the redistribution of other Cdk inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ from Cdk4 to Cdk2. The association of p21 and p27 with Cdk2 represses Cdk2 activity, resulting in a hypophosphorylated form of pRb. The hypophosphorylated pRb eventually causes cell cycle arrest at G1 [Tsukada et al., 2004; Han et al., 2005]. In addition to the enhanced expression of p16, the strong activation of the ERK pathway by HGF changes the expression of other regulatory molecules, including p21, cyclin A, and E2F1 [Tsukada et al., 2004], which may be responsible for the cell cycle arrest of HepG2 cells. Thus, the high intensity ERK signal induced by HGF plays a crucial role in the HGF-induced inhibition of proliferation of HepG2 cells.

The c-Met receptor is a receptor tyrosine kinase (RTK). The association of HGF with the c-Met receptor induces activation of the tyrosine kinase, and several tyrosine residues within the cytoplasmic region are highly phosphorylated. The phosphorylation of two residues (Tyr^{1349} and Tyr^{1356}) in the C-terminal region mediates the binding of signaling proteins, which stimulates intracellular signaling pathways [Ponzetto et al., 1994; Zhu et al., 1994]. In HepG2 cells, the strong activation of ERK is induced through one of these pathways. Although strong activation of ERK is required to inhibit the proliferation of HepG2 cells, it is still to be elucidated whether the activation alone is sufficient to induce the inhibitory effect, or some other signaling pathway(s), that is specifically induced by activation of the c-Met receptor, is also required.

More than 50 RTKs are encoded by the human genome [Blume-Jensen and Hunter, 2001]. Upon ligand binding, these RTKs are activated, and mediate the activation of downstream signaling pathways, including the ERK pathway [Schlessinger, 2000]. Thus, the inhibitory effect on the proliferation of HepG2 cells could be induced by the strong activation of ERK mediated by some of these RTKs. In this study, we examined the epidermal growth factor receptor (EGFR)-mediated signaling system in HepG2 cells. HepG2 cells expressed a low level of EGFR, which did not induce strong ERK activation upon EGF stimulation. Thus, we constructed HepG2 cell clones that expressed a high level of EGFR. EGF treatment of the cell clones induced the strong activation of ERK, but did not inhibit cell proliferation. While HGF treatment of the cell clones enhanced the expression of p16 and p21, EGF treatment did not. Furthermore, knockdown of p21 by siRNA restored the proliferation of HepG2 cells inhibited by HGF and the Cdk2 activity suppressed in HGF-treated cells. These results suggest that the strong activation of ERK alone is not sufficient, and some other pathway(s), which is activated through the c-Met receptor but not through EGFR, is also required to enhance the expression of p16 and p21, and suggest that in addition to the enhanced expression of p16, that of p21 contributes to the suppression of Cdk2 activity leading to the inhibition of proliferation of HGF-treated HepG2 cells.

MATERIALS AND METHODS

Reagents

Reagents were obtained as follows: anti-ERK2 antibody from Upstate Biotechnology, Inc.; anti-EGFR antibody from MBL; anti- phosphotyrosine antibody (PY20) from Transduction Laboratories; anti-p16 (H-156), anti-p21 (C-19), and anti-Cdk2 antibodies from Santa Cruz Biotechnology, Inc.; anti-α-tubulin antibody from Sigma; horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulins from Amersham Biosciences; recombinant human HGF from the Research Center of Mitsubishi Chemical Corp.; and EGF from PeproTech EC Ltd.

Cell Culture and Cell Proliferation Assay

HepG2 cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Iwaki) 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. For the cell proliferation assay, cells were seeded at a density of 5×10^4 cells/

well in 12-well plates and cultured with DMEM containing 10% FBS. After 24 h, cells were subsequently treated with HGF (50 ng/ml) or EGF (100 ng/ml) and cultured for 4 days. The medium was replaced with fresh medium containing HGF or EGF at 2 days. The cells were harvested after trypsinization, and the number of cells was counted using a hemocytometer.

Preparation of Cell Extracts and Immunoprecipitation

Cells were washed twice with ice-cold phosphate-buffered saline before being lysed. For the immunoblot analysis of ERK2, p16, p21, and α-tubulin, cells were lysed with ice-cold lysis buffer-1 (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton-X-100, 1 mM Na_3VO_4, 50 mM NaF, and 30 mM tetra-sodium pyrophosphate) containing 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. For the immunoprecipitation of EGFR, cells were lysed with ice-cold lysis buffer-2 (137 mM NaCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 2.68 mM KCl, 14.7 mM KH_2PO_4, 2 mM Na_3VO_4, 5 mM EDTA, 1% NP-40, and 0.5% deoxycholic acid) containing the same protease inhibitors. For the immunoprecipitation of Cdk2, cells were lysed with ice-cold lysis buffer-3 (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20, 1 mM Na_3NO_4, 50 mM NaF, and 30 mM tetra-sodium pyrophosphate) containing the same protease inhibitors. The lysates were cleared by centrifugation at 15,000 rpm for 15 min at 4°C, and the protein concentration in the lysates was measured using the BCA protein assay reagent (Pierce). For immunoprecipitation, equal amounts of cell lysate were incubated with 2 μg of antibody overnight at 4°C and with 20 μl of a 50% slurry of protein A-Sepharose (Amersham Biosciences) for 1 h 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 sample buffer (25 mM Tris–HCl, pH 6.8, 5% glycerol, 1% SDS, 0.05% 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 overnight at 4°C and then with horseradish peroxide-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were visualized with an enhanced chemiluminescence western blotting detection system (ECL, Amersham Biosciences).

Isolation of HepG2 Cell Clones Overexpressing EGFR

A Flp-In™ System was obtained from Invitrogen. HepG2 cells were transfected with pFRT/LacZeo using FuGENE6 transfection reagent, and cultured for 48 h. The cells were then cultured in selective medium containing Zeocin (250 μg/ml). Cells resistant to Zeocin were selected and expanded. A cell clone was used as a Flp-In host cell clone. The host cell clone was cotransfected with the expression plasmid encoding human EGFR and the Flp-expression plasmid pOG44 using JetPEI transfection reagent (Polyplus-transfection), and cultured for 48 h. The cells were then cultured in selective medium containing hygromycin B (350 μg/ml).

RNA Interference

p21-targeting siRNA was synthesized according to published data [Shats et al., 2004]. Its sense sequence (5′-GACCATATGGACCTGTCAC-3′) corresponds to nucleotide 325–343 of the human p21 coding sequence. A DNA oligonucleotide containing the sense target sequence, a hairpin loop, and the anti-sense target sequence was synthesized, annealed, and inserted into a polymerase III-mediated siRNA expression plasmid vector, pSilencer 1.0-U6 (Ambion, Inc.). The cells were seeded at a density of 3×10^5 cells/well in six-well plates and cultured with DMEM containing 10% FBS for 24 h. The medium was replaced with fresh medium and cells were treated with 4 μg of the expression plasmid mixed with 11.2 μl of jetPEI for 24 h. The medium was replaced with fresh medium, and cells were further cultured in the absence and presence of HGF (50 ng/ml). For the cell proliferation assay, the transfected cells were reseeded at a density of 5×10^4 cells/well in 12-well plates and cultured with DMEM containing 10% FBS for 24 h. The medium was replaced with fresh medium, and cells were further cultured in the absence or presence of HGF (50 ng/ml).

In Vitro Kinase Assay

Cells were washed twice with ice-cold phosphate-buffered saline and lysed with

ice-cold lysis buffer-3 containing the protease inhibitor mixture. The lysates were cleared by centrifugation, and the protein concentration in the lysates was determined using the BCA protein assay reagent. The protein (250–500 μg) from the supernatant was incubated with 2 μg of an anti-Cdk2 antibody overnight at 4°C and with 20 μl of a 50% slurry of protein A-Sepharose for 1 h at 4°C. The immune complexes were precipitated and washed twice with lysis buffer-3 and twice with kinase buffer (20 mM HEPES-NaOH, pH 7.5, 1 mM dithiothreitol, 10 μM Na_3VO_4, 2 mM β-glycerophosphate, 100 μM EGTA, 100 μM pheylmethylsulfonyl fluoride, 0.1 μg/ml leupeptin, and 12 mM $MgCl_2$). The precipitates were incubated with 30 μl of kinase buffer containing 0.2 μg of full-length pRb (QED Bioscience, Inc.), 24 μM ATP, and 0.22 MBq of [γ-^{32}P] ATP at 30°C for 30 min. Reactions were stopped by addition of 7.5 μl of 5× sample buffer. The mixture was heated at 98°C for 5 min. The proteins were separated by SDS–PAGE on a 7.5% gel. Phosphorylation of the substrate was visualized by autoradiography. The band intensity on the autoradiogram was quantitated by NIH Image-J software.

RESULTS

EGF Treatment Does Not Induce the Inhibition of Proliferation and Activation of ERK in HepG2 Cells

EGFR is a RTK, and upon ligand binding, EGFR is activated and induces the activation of the ERK pathway [Yamauchi et al., 1997]. Thus, like c-Met, EGFR may mediate the inhibition of proliferation of HepG2 cells. To test this, HepG2 cells were treated with EGF and the cell numbers were counted. In parallel, HepG2 cells were treated with HGF as a positive control. Marked inhibition of proliferation was observed in cells treated with HGF for 4 days, as described previously [Tsukada et al., 2001]. In contrast, EGF treatment did not affect the proliferation of HepG2 cells (Fig. 1A).

A high intensity ERK signal plays a crucial role in the HGF-induced inhibition of proliferation of HepG2 cells [Tsukada et al., 2001]. We thus examined the activation of ERK in EGF-treated HepG2 cells. Since HeLa cells express high levels of EGFR, and are extensively used to analyze the intracellular signaling mediated through EGFR, we used HeLa cells as positive control cells to show activation of ERK upon ligand treatment. Activation of the ERK pathway was assessed by monitoring the phosphorylation of ERK2 by immunoblot analysis. Strong activation of ERK2 was observed in HepG2 cells treated with HGF, but not EGF. The treatment of HeLa cells with EGF induced strong activation of ERK2 (Fig. 1B). These results suggest that HepG2 cells express a lower level of EGFR than HeLa cells, which does not mediate the strong activation of ERK. Alternatively, it is possible that the downstream signaling of EGFR does not induce activation of ERK in HepG2 cells. Then, we compared the expression level of EGFR in HepG2 cells with that in HeLa cells. The cell lysates were immunoprecipitated with an anti-EGFR antibody and subjected to an immunoblot analysis with the same antibody. In addition, the phosphorylation status of EGFR upon stimulation with EGF was analyzed by immunoblotting of the proteins that were immunoprecipitated with the anti-EGFR antibody, with an anti-phosphotyrosine antibody. In this analysis, a threefold amount of lysate of HepG2 cells over that of HeLa cells was immunoprecipitated and loaded on the gel. The phosphorylation level of EGFR was 28-fold lower and expression level of EGFR was 18-fold lower in HepG2 cells than in HeLa cells (Fig. 1C). These results suggest that the low level of phosphorylated EGFR is not sufficient to induce the activation of the ERK pathway in HepG2 cells and the inhibition of cell proliferation.

Overexpression of EGFR Mediates Activation of ERK, But Does Not Induce the Inhibition of Proliferation of HepG2 Cells

Since it is likely that the low-level expression of EGFR causes the insufficient activation of the ERK pathway in HepG2 cells treated with EGF, overexpression of EGFR could mediate activation of ERK, leading to the inhibition of proliferation of HepG2 cells. We thus constructed HepG2 cell clones overexpressing EGFR. To obtain cell clones equally expressing EGFR, the Flp-In system was used. The plasmid pFRT/lacZeo was transfected into HepG2 cells. Cell clones resistant to Zeocin were selected and expanded. One clone, which showed HGF-induced inhibition of cell proliferation similarly to parental HepG2 cells, was selected and used as a Flp-In host cell line. The expression plasmid encoding human EGFR (pcDNA5/FRT-hEGFR) was cotransfected with Flp-expression plasmid

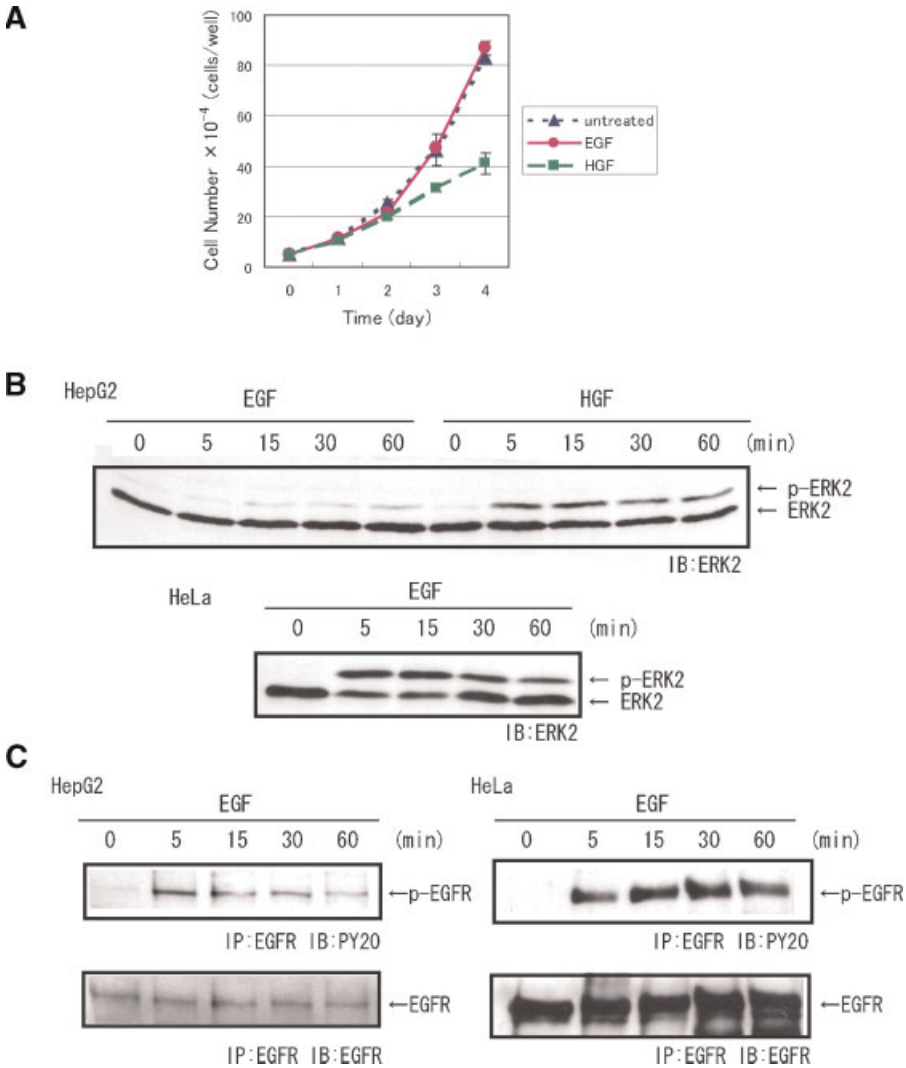

Fig. 1. The proliferation of HepG2 cells treated with EGF, and phosphorylation of ERK2 and of EGFR in the cells. **A**: The proliferation of HepG2 cells treated with EGF or HGF. Cells were seeded at a density of 5×10^4 cells/well (12-well plates) in DMEM with 10% FBS (DMEM/FBS). After 24 h, the medium was replaced with fresh medium, and cells were cultured in the presence of EGF (100 ng/ml) or HGF (50 ng/ml) or in the absence of growth factors (untreated). At the indicated intervals, cell numbers were counted. Each value represents the mean ± SD of triplicate determinations from a representative experiment. Similar results were obtained in at least two independent experiments. **B**: The phosphorylation of ERK2 in HepG2 cells and HeLa cells treated with EGF or HGF. Cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 24 h, the medium was replaced with fresh medium, and cells were cultured for 2 days. Then, cells were stimulated with EGF (100 ng/ml) or HGF (50 ng/ml), and cell lysates were prepared at the indicated times. Proteins (30 μg) from the cell lysates were separated by SDS–10% PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed with an anti-ERK2 antibody. Phosphorylation of ERK2 proteins is indicated by a shift to a slower electrophoretic mobility. Similar results were obtained in at least two independent experiments. **C**: The phosphorylation of EGFR in HepG2 cells and HeLa cells treated with EGF. Cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 24 h, the medium was replaced with fresh medium, and cells were cultured for 2 days. Then, cells were stimulated with EGF (100 ng/ml). Cell lysates were prepared at the indicated times, and immunoprecipitated (IP) with an anti-EGFR antibody. Proteins (3 mg) in the immunoprecipitates from lysates of HepG2 cells or proteins (1 mg) in the immunoprecipitates from lysates of HeLa cells were separated by SDS–10% PAGE. Immunoblot analysis (IB) was performed with an anti-phosphotyrosine or anti-EGFR antibody. Similar results were obtained in at least two independent experiments. The intensity of the bands was quantified using NIH Image-J software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pOG44 into the host cell line. Cell clones resistant to hygromycin B were selected and expanded. They were subjected to an analysis of the expression of receptor proteins by immunoblotting. Two clones (EGFR01 and 02) were selected for further analysis. Amounts of receptor proteins were 67- and 57-fold higher in EGFR01 and 02 cells, respectively, compared to

parental HepG2 cells (Fig. 2A). A low level of phosphorylated EGFR was observed in untreated cells, and EGF treatment strongly enhanced the phosphorylation of EGFR in EGFR01 and 02 cells (Fig. 2A).

Next, we examined the phosphorylation status of ERK2. EGF treatment for 5 min induced the phosphorylation of ERK2 in EGFR01 and 02 cells at a similar level to that in HGF-treated cells (Fig. 2B). A time course analysis of the phosphorylation showed that the sustained phosphorylation observed in parental HepG2 cells treated with HGF (Fig. 2B, bottom panel), was also observed in EGFR01 and 02 cells treated with EGF (Fig. 2B, top and middle panels). These results suggest that overexpression of EGFR mediates the activation of ERK in EGF-treated HepG2 cells, similar to that in HGF-treated cells.

To examine whether the activation of ERK leads to inhibition of the proliferation of EGFR01 and 02 cells, these cells were treated with EGF or HGF for 4 days, and cell numbers were counted. The proliferation was inhibited by HGF treatment, but not by EGF treatment (Fig. 2C). These results suggest that the activation of ERK alone is not sufficient to induce the inhibition of proliferation of HepG2 cells, and some other pathway(s), which is specifically induced by activation of the c-Met receptor, is also required.

Expression of $p16^{INK4a}$ and $p21^{CIP1}$ Is Not Enhanced in HepG2 Cell Clones Overexpressing EGFR Treated With EGF

Enhanced expression of p16 is required for the inhibition of proliferation of HepG2 cells treated with HGF [Han et al., 2005]. The strong activation of ERK is required for the enhanced expression of p16, because a MEK inhibitor, PD98059, which suppresses activation of ERK, inhibits the enhanced expression of p16 [Han et al., 2005]. We therefore examined whether p16 expression is enhanced in HepG2 cell clones overexpressing EGFR treated with EGF. EGF treatment did not enhance the expression in parental HepG2 cells, in that an elevated level of p16 was observed on HGF treatment (Fig. 3A). Similarly, the expression of p16 was not enhanced by EGF treatment of EGFR01 and 02 cells (Fig. 3B and data not shown). These results suggest that the activation of ERK alone is not sufficient to enhance p16 expression, and that the proliferation of EGFR01 and 02 cells is not inhibited by EGF treatment due to the unenhanced expression of p16.

In addition to the expression of p16, the treatment of HepG2 cells with HGF enhances the expression of another Cdk inhibitor, p21. Strong activation of ERK is required for the enhanced expression of p21, because the MEK inhibitor PD98059 inhibits the enhancement [Tsukada et al., 2004]. The p16 protein forms a complex with Cdk4, leading to the redistribution of p21 and $p27^{KIP1}$ from Cdk4 to Cdk2. The redistribution plays a crucial role in inhibiting the proliferation of HepG2 cells [Han et al., 2005]. Thus, it is likely that the enhanced expression of p21 is responsible for the inhibition of proliferation of HepG2 cells. We examined whether p21 expression is enhanced in HepG2 cells overexpressing EGFR treated with EGF. EGF treatment did not enhance p21 expression in EGFR01 and 02 cells (Fig. 3B and data not shown) or in the parental HepG2 cells (Fig. 3A). These results suggest that activation of ERK alone is not sufficient to enhance p21 expression.

Enhanced Expression of p21 Contributes to the Inhibition of Proliferation of HepG2 Cells Treated With HGF

The strong activation of ERK by EGF in HepG2 cells overexpressing EGFR did not enhance p21 expression, and cell proliferation was not inhibited, suggesting that the enhanced expression of p21 plays a crucial role in the inhibition of proliferation. We thus examined whether the enhanced expression of p21 is required for the inhibition of proliferation of HGF-treated HepG2 cells, by the siRNA method. The siRNA for p21 was synthesized according to published data [Shats et al., 2004], and inserted into an expression vector. HepG2 cells were transiently transfected with the expression vector or an empty vector, and treated with HGF. Introduction of the siRNA reduced the expression of p21 at 24 h after HGF treatment, when the expression is strongly enhanced, whereas introduction of the empty vector did not affect the level of p21 (Fig. 4A). The level of α-tubulin protein was not affected by the siRNA (Fig. 4A), and the introduction of siRNA targeting another gene did not affect the level of p21 (data not shown), confirming that the reduction in p21 expression was specific.

We examined the effect of the siRNA on the inhibited proliferation of HepG2 cells treated

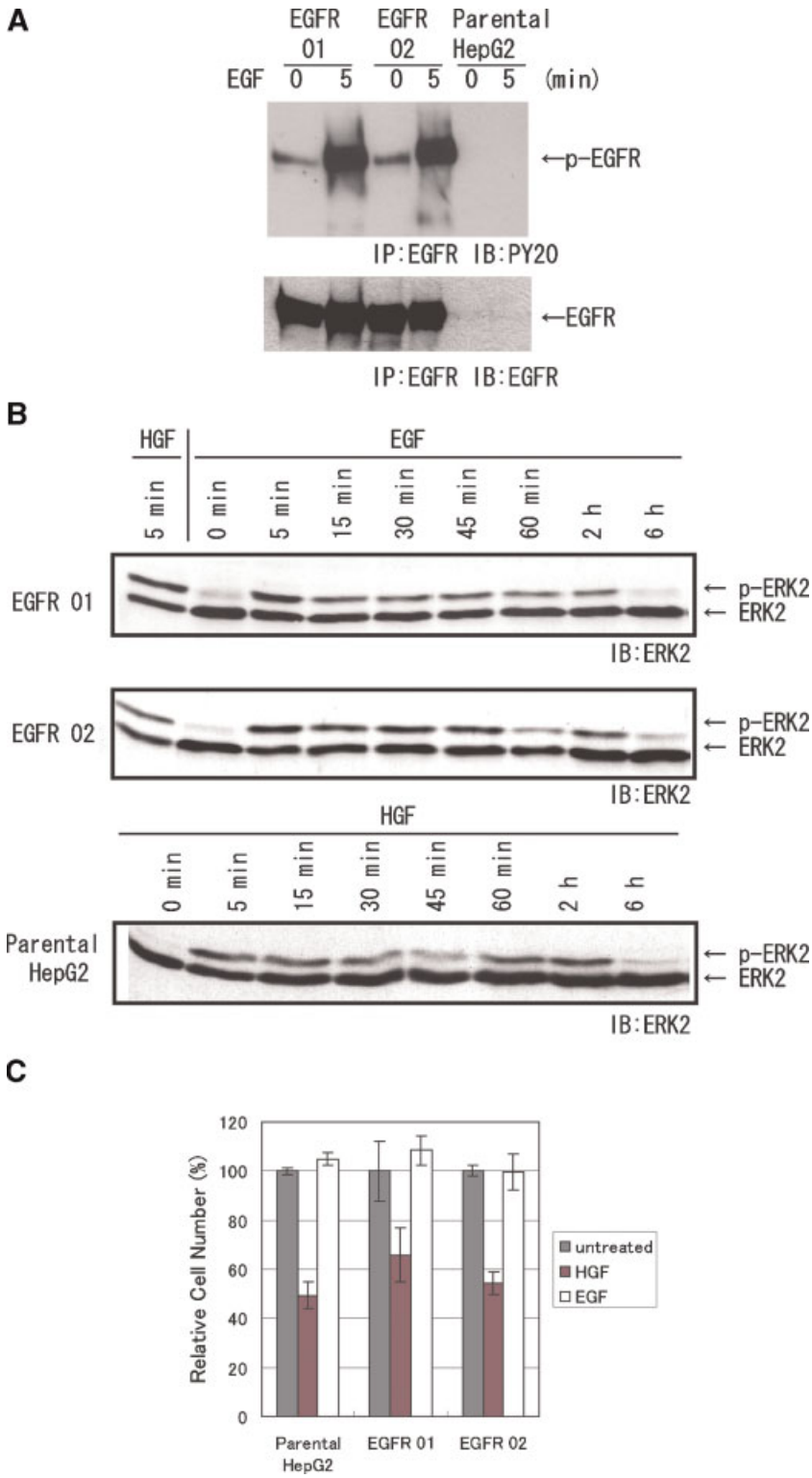

Fig. 2. The phosphorylation of EGFR and of ERK2 in and proliferation of HepG2 cells overexpressing EGFR treated with EGF. **A**: The phosphorylation of EGFR. HepG2 cells overexpressing EGFR (EGFR01 and 02) and parental HepG2 cells were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. The cell culture, stimulation with EGF, immunoprecipitation and immunoblot analysis were performed as described in Figure 1C. Similar results were obtained in at least two independent experiments. The intensity of the bands was quantified using NIH Image-J software. **B**: The phosphorylation of ERK2 in EGFR01 and 02 cells treated with EGF or HGF. Phosphorylation of ERK2 was assayed as described in Figure 1B. Similar results were obtained in at least two independent experiments. **C**: The proliferation of EGFR01 and 02 cells treated with EGF or HGF. Cells were cultured in the presence or absence of growth factors, as described in Figure 1A. After 4 days, cell numbers were counted. Each value represents the mean ± SD of triplicate determinations from a representative experiment. Similar results were obtained in at least two independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

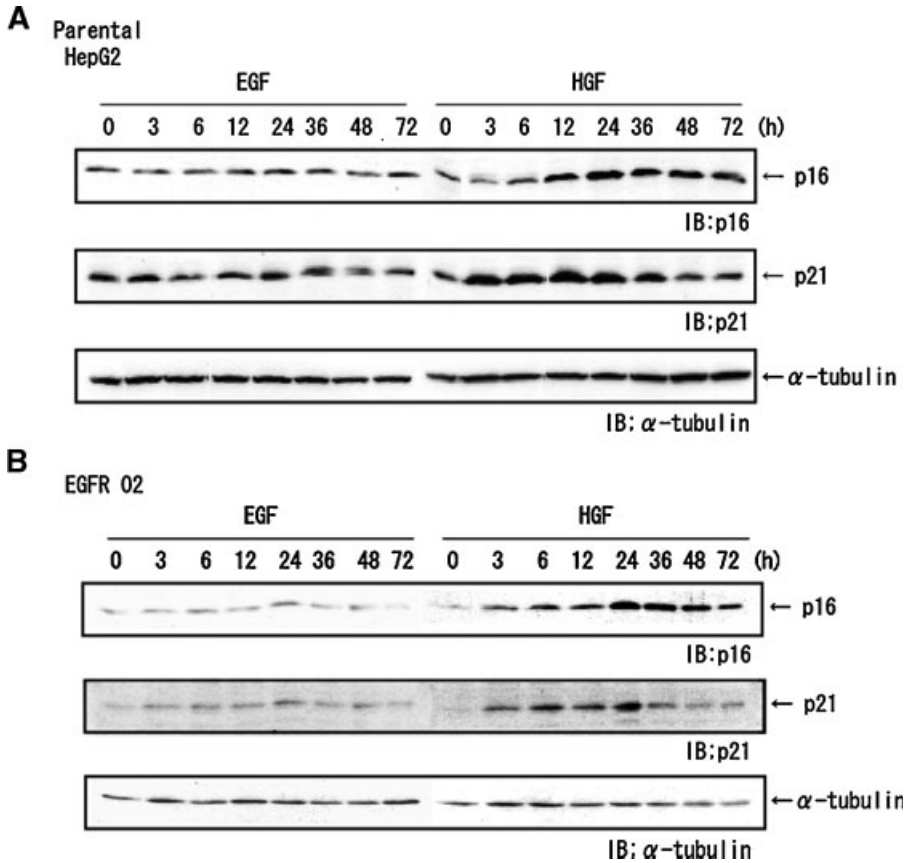

Fig. 3. The expression of p16 and p21 in HepG2 cells overexpressing EGFR treated with EGF. Parental HepG2 cells (**A**) and EGFR02 cells (**B**) were seeded at a density of 5×10^5 cells/100-mm dish in DMEM/FBS. After 24 h, the medium was replaced with fresh medium, and cells were cultured for 2 days. Then, cells were cultured in the presence of EGF (100 ng/ml) or HGF (50 ng/ml), and cell lysates were prepared at the indicated times. Proteins (30 μg) from the cell lysates were separated by SDS–10% PAGE. Immunoblot analysis was performed with an anti-p16, anti-p21, or anti-α-tubulin antibody. α-Tubulin was used as a loading control. Similar results were obtained in at least two independent experiments.

with HGF. HepG2 cells were transiently transfected with the expression vector encoding p21 siRNA or the empty vector, and were left untreated or treated with HGF. The number of cells was counted at 4 days after HGF treatment. The number of the cells transfected with the empty vector was reduced to about 40% by HGF treatment, compared to the untreated cells. Introduction of the siRNA for p21 restored the number of HGF-treated cells to about 70% (Fig. 4B). These results suggest that the enhanced expression of p21 contributes to the inhibition by HGF of HepG2 cell proliferation.

Enhanced Expression of p21 Contributes to the Suppression of Cdk2 Activity in HGF-Treated HepG2 Cell

The redistribution of p21 from Cdk4 to Cdk2 by p16 is likely to be responsible for the suppression of Cdk2 activity, which eventually leads to the HGF-induced inhibition of proliferation of HepG2 cells [Han et al., 2005]. We thus examined the effect of p21 siRNA on the suppression of Cdk2 activity in HGF-treated HepG2 cells. HepG2 cells were transiently transfected with the expression vector encoding p21 siRNA or the empty vector, and were left untreated or treated with HGF. Cells were harvested at 24 and 48 h after HGF treatment, and cell lysate was immunoprecipitated with an anti-Cdk2 antibody. Immune complex kinase assays were performed using full-length Rb as a substrate. The level of Cdk2 activity was very high in empty vector-transfected cells in the absence of HGF, and HGF treatment reduced the activity to 70% at 24 h and to 5% at 48 h. Protein levels of Cdk2 were not significantly changed by this treatment. Knockdown of p21 in HGF-treated cells using siRNA restored the Cdk2 activity to 90% at 24 h and to 60% at 48 h

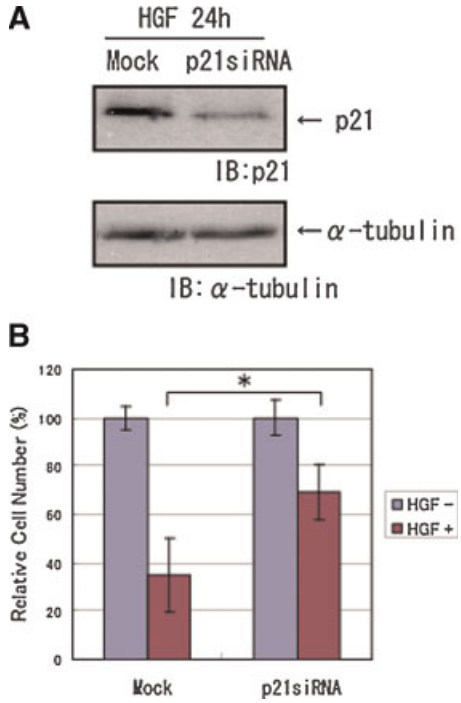

Fig. 4. Effect of p21 siRNA on the proliferation of HepG2 cells inhibited by HGF. Cells were transiently transfected with the expression vector encoding p21 siRNA or the empty vector (Mock). **A**: At 24 h after transfection, the medium was replaced with fresh medium, and cells were cultured in the presence of HGF (50 ng/ml). Cell lysates were prepared at 24 h. Proteins (30 μg) from the cell lysates were subjected to immunoblot analysis with the anti-p21 or anti-α-tubulin antibody. Similar results were obtained in at least two independent experiments. **B**: At 24 h after transfection, cells were reseeded and cultured in DMEM/FBS. After 24 h, the medium was replaced with fresh medium, and cells were cultured in the presence or absence of HGF (50 ng/ml). After 4 days, cell numbers were counted. Each value represents the mean ± SD of triplicate determinations from a representative experiment. Similar results were obtained in at least two independent experiments. $*P < 0.05$ by Student's *t*-test. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 5). These results suggest that the enhanced expression of p21 contributes to the suppression of Cdk2 activity, which leads to the inhibition of proliferation of HepG2 cells treated with HGF.

DISCUSSION

Strong activation of ERK is required for HGF to inhibit the proliferation of HepG2 cells [Tsukada et al., 2001]. In this study, we constructed HepG2 cell clones overexpressing EGFR, in which strong activation of ERK was induced by EGF treatment (Fig. 2B). However, the proliferation of the cell clones was not inhibited by EGF (Fig. 2C). The inhibitory effect of HGF on cell proliferation is caused by cell cycle arrest at G1, which results from the retinoblastoma tumor suppressor gene product pRb being maintained in its active hypophosphorylated form [Tsukada et al., 2001]. In HepG2 cells, the level of Cdk2 activity is high, which seems to be responsible for the hyperphosphorylation of pRb. The treatment of HepG2 cells with HGF markedly reduces the level of Cdk2 activity, resulting in the hypophosphorylated form of pRb [Tsukada et al., 2004]. The expression of p16 is up-regulated in HGF-treated HepG2 cells. The p16 forms a complex with Cdk4, leading to the redistribution of p21 and p27 from Cdk4 to Cdk2. The association of p21 and p27 with Cdk2 represses Cdk2 activity [Han et al., 2005]. The expression of p21 is also up-regulated in HGF-treated HepG2 cells [Shima et al., 1998; Tsukada et al., 2004]. Strong activation of ERK by HGF is required for the elevated expression of p16 and p21 [Tsukada et al., 2004; Han et al., 2005]. We demonstrated in this study that the expression of both p16 and p21 is not enhanced in EGF-treated HepG2 cells overexpressing EGFR (Fig. 3). Elevated p16 expression is required for the HGF-induced reduction of Cdk2 activity in HepG2 cells and inhibition of cell proliferation [Han et al., 2005]. We also demonstrated that the knockdown of p21 expression by siRNA restored the proliferation of HepG2 cells inhibited by HGF and Cdk2 activity suppressed by HGF (Figs. 4 and 5). Based on these results, we suggest that strong activation of ERK alone is not sufficient, and some other signaling pathway(s), which is specifically activated by HGF, is also required to enhance the expression of p16 and p21, which leads to the reduction of Cdk2 activity in the HGF-treated HepG2 cells and inhibition of proliferation.

The time course analysis showed that the amount of p21 protein increased at 3 h after HGF treatment and gradually decreased after 36 h (Fig. 3), indicating that the up-regulation of p21 expression precedes the inhibition of cell proliferation induced by HGF. The up-regulated p21 protein accumulates as a complex with cyclin D-Cdk4 [Han et al., 2005], which may act as a low affinity reservoir for p21. Redistribution of p21 from Cdk4 to Cdk2 is induced by the increase in p16 expression, which causes the inactivation of Cdk2 [Han et al., 2005]. The Cdk2 activity starts to decline

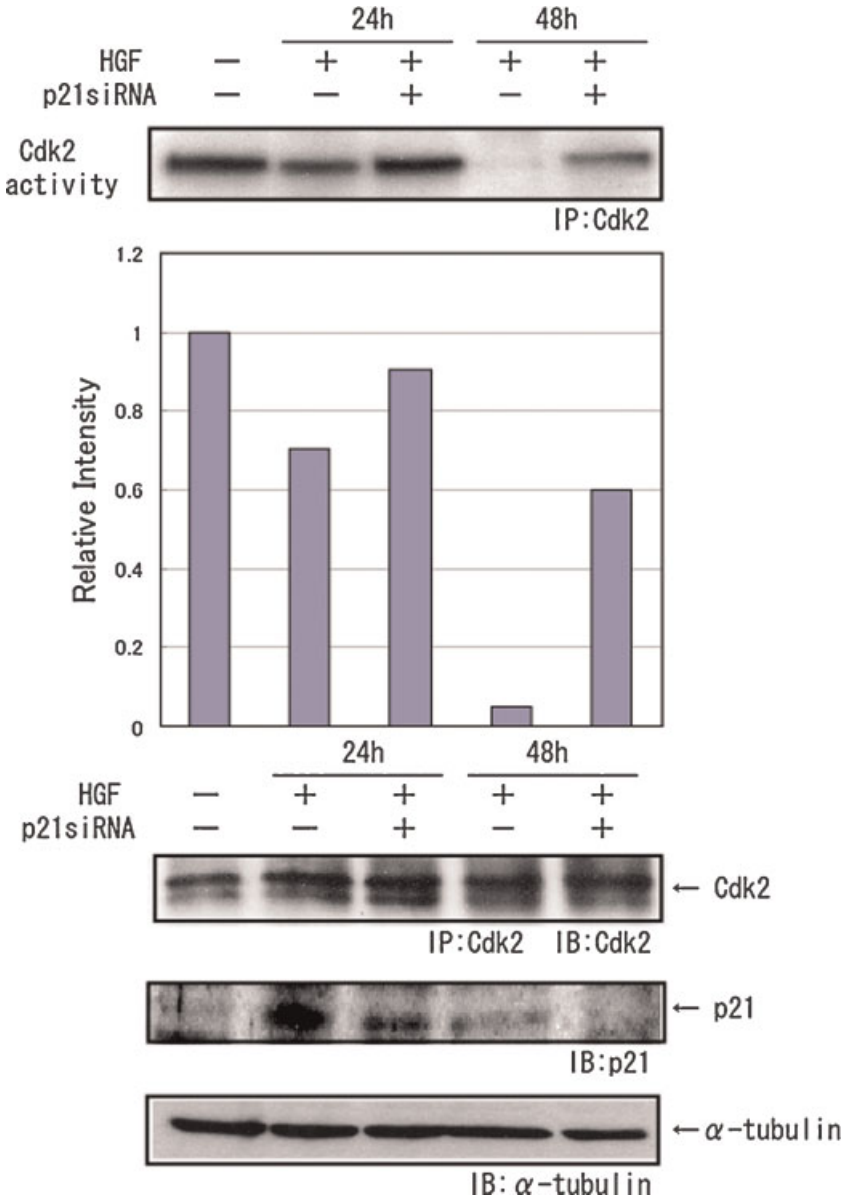

Fig. 5. Effect of p21 siRNA on the reduction of Cdk2 activity in HepG2 cells treated with HGF. Cells were transiently transfected with the expression vector encoding p21 siRNA (siRNA +) or the empty vector (siRNA −). At 24 h after transfection, the medium was replaced with fresh medium, and cells were cultured in the presence (HGF +) or absence (HGF −) of HGF (50 ng/ml). Cell lysates were prepared at 24 and 48 h, and were immunoprecipitated with an anti-Cdk2 antibody. Immune complex kinase assays were performed using pRb as a substrate (**top panel**). The intensity of the bands was quantified using NIH Image-J software and graphed (**second panel**). The amounts of Cdk2 in the immunoprecipitates were assessed by immunoblot analysis (**third panel**). Proteins (30 μg) in the immunoprecipitates were separated by SDS–10% PAGE. Immunoblot analysis was performed with the anti-Cdl2 antibody. The amounts of p21 and α-tubulin in the lysates were assessed by immunoblot analysis as described in Figure 3 (**fourth** and **bottom panels**, respectively). Similar results were obtained in at least two independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

at 24 h, and is drastically reduced at 48 h after HGF treatment (Fig. 5). This reduction in Cdk2 activity causes the inhibition of cell proliferation, which is observed at 72 h after HGF treatment. Although the up-regulated level of p21 at 48 h seems to be relatively low, it is still higher than the basal level. This higher level of p21 expression at 48 h is required for the reduction of Cdk2 activity by HGF, since siRNA-mediated knockdown of p21, which resulted in the basal level of p21 expression, restored the Cdk2 activity at 48 h (Fig. 5). The highest level

of p21 expression around 24 h may be required for the higher level of p21 expression at 48 h, because the repression of p21 expression at 24 h by p21 siRNA resulted in the basal level of p21 expression at 48 h (Fig. 5). Thus, the up-regulation of p21 expression by HGF is likely to be essential for the reduction of Cdk2 activity and inhibition of HepG2 cell proliferation.

Besides the ERK pathway, it has been shown that the PI3K pathway is involved in cell proliferation [Rahimi et al., 1996; Gille and Downward, 1999]. Thus, we previously tested the effect of a PI3K-specific inhibitor (LY294002) on the HGF-induced inhibition of HepG2 cell proliferation [Tsukada et al., 2001]. The inhibition of proliferation by HGF was not affected by LY294002, suggesting that the PI3K pathway is not necessary for the HGF-induced inhibition of HepG2 cell proliferation. The Stat3 pathway has been shown to be activated in HGF-treated MDCK cells [Boccaccio et al., 1998]. Our preliminary study showed that a Stat3-specific inhibitor did not affect the HGF-induced inhibition of HepG2 cell proliferation (data not shown), suggesting that the Stat3 pathway does not participate in the inhibition. Further examination is needed to identify the ERK-independent pathway involved in the HGF-induced inhibition of HepG2 cell proliferation.

The expression of p16 is enhanced at the transcriptional level in HGF-treated HepG2 cells [Han et al., 2005]. Previous studies using reporter assays for the promoter of the p16 gene and its mutants revealed that the proximal Ets-binding site in the promoter is required for the enhancement of p16 expression in HGF-treated HepG2 cells [Han et al., 2005]. In addition, a chromatin immunoprecipitation assay showed that Ets1 and/or Ets2 are physically associated with the Ets-binding site in HGF-treated HepG2 cells. These results suggested that Ets1 and/or Ets2 are responsible for the enhancement of p16 expression [Han et al., 2005]. Furthermore, the reporter assays also showed that the MEK inhibitor PD98059 reduces the enhanced promoter activity almost to the basal level, suggesting that the enhanced p16 expression through the Ets-binding site requires strong activation of ERK [Han et al., 2005]. In addition to the positive regulation [McCarthy et al., 1997; Slupsky et al., 1998], Ets activity is negatively regulated by the helix-loop-helix protein Id1 [Benezra et al., 1990; Ohtani et al., 2001]. Id1 has been shown to suppress the expression of p16 [Ohtani et al., 2001]. Moreover, the expression of Id1 has been shown to be regulated by an ERK-independent signaling pathway [Chen et al., 2006]. Our preliminary study using the RT-PCR method showed that Id1 mRNA is highly expressed in HepG2 cells (unpublished result), suggesting that a high level of Id1 suppresses the expression of p16 in HepG2 cells, and HGF treatment reduces the level of Id1. Thus, it seems possible that the level of Id1 is reduced through a signaling pathway other than the ERK pathway in the HGF-treated HepG2 cells but not in the EGF-treated HepG2 cells overexpressing EGFR, and that this reduction, together with the activation of Ets1 and/or Ets2, leads to the enhancement of p16 expression. Further examination of the regulatory mechanism of the activation of Ets is required to understand the signaling pathways involved in the enhancement of p16 expression.

Similar to the up-regulation of p16 expression, the up-regulation of p21 expression seems to be mediated by ERK-dependent and -independent signaling pathways downstream of the c-Met receptor in HGF-treated HepG2 cells. However, the pattern of up-regulation differed between p16 and p21: while the increase in p21 expression first occurred at 3 h after the treatment with HGF, the expression of p16 first increased at 12 h [Tsukada et al., 2004; Han et al., 2005]. Thus, signaling systems leading to the up-regulation of p21 and p16 expression are probably different. Two regulatory mechanisms of p21 expression have been reported: up-regulation at the transcriptional level and down-regulation through ubiquitin-mediated proteolysis [Gartel and Tyner, 1999; Bornstein et al., 2003]. Our analysis of p21 mRNA using the RT-PCR method suggested that the regulation of p21 expression occurs mainly at the transcriptional level in HGF-treated HepG2 cells (data not shown). Multiple transcription factor binding sites within the p21 promoter have been reported, and potential regulatory transcription factors include p53, the Ets family, the C/EBP family, the Stat family, and the Sp family [Gartel and Tyner, 1999; Lowe, 1999; Park et al., 2000; Liu and Huang, 2006]. Examinations of these transcription factors are required to understand the molecular mechanism of the transcriptional activation of p21.

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