Id1 Is Down-Regulated by Hepatocyte Growth Factor via ERK-Dependent and ERK-Independent Signaling Pathways, Leading to Increased Expression of p16\(^{\text{INK4a}}\) in Hepatoma Cells

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Abstract

Hepatocyte growth factor (HGF) inhibits the proliferation of several tumor cell lines and tumor growth in vivo. We showed previously that HGF induces cell cycle arrest at G1 in a human hepatoma cell line, HepG2, by up-regulating the expression of p16\(^{\text{INK4a}}\) through strong activation of extracellular signal-regulated kinase (ERK). However, although essential, the activation was not sufficient for the up-regulation of p16. In this study, we examined regulatory mechanisms of p16 expression through a transcription factor, Ets, which has been shown previously to bind to the promoter. The treatment of HepG2 cells with HGF induced ERK-dependent phosphorylation of Ets, which leads to its activation, before the up-regulation of p16, suggesting that another factor suppresses Ets activity. We found that HGF reduces the amount of Id1, which is a dominant-negative inhibitor of Ets, leading to a decrease in Ets associated with Id1. Id1 was down-regulated via transcriptional regulation not via the ubiquitin-proteasome-mediated pathway. Inhibition of the HGF-induced high-intensity ERK activity had a modest effect on the Id1 down-regulation, and inhibition of the phosphatidylinositol 3-kinase 3-kinase pathway had no effect, showing that Id1 is regulated by ERK-dependent and -independent pathways other than the phosphatidylinositol 3-kinase pathway. Exogenously expressed Id1 suppressed the up-regulation of p16 by HGF and the antiproliferative effect of HGF. Knockdown of Id1 significantly enhanced the activity of the p16 promoter coordinately with the activation of ERK. Our results indicated that down-regulation of Id1 plays a key role in the inhibitory effect of HGF on cell proliferation and provides a molecular basis for cancer therapy with HGF.

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Introduction

Cell proliferation is tightly regulated by extracellular factors such as growth factors and cytokines. To maintain tissues and organs and to avoid tumorigenesis, the extracellular factors regulate both stimulation and inhibition of cell proliferation. Hepatocyte growth factor (HGF) was originally described as a mesenchymal cell-derived mitogenic protein for primary hepatocytes (1, 2). HGF is a heparin-binding glycoprotein produced by stroma cells in a wide variety of tissues and plays a key role in tumor-stroma interactions. HGF is unique because it has opposing effects on the regulation of cell proliferation: whereas treatment with HGF stimulates the proliferation of some tumor cell lines, it inhibits the proliferation of several others (3-5). Elucidation of the cause of the differences in the effects of HGF on cell proliferation is essential for applying HGF to cancer therapy. Because the HGF signal is transduced through the receptor tyrosine kinase c-Met, the product of the c-met protooncogene, the opposing effects of HGF on cell proliferation are thought to be derived from differences in downstream signaling pathways (6, 7). The association of HGF with c-Met induces phosphorylation of some tyrosine residues on c-Met followed by the recruitment of various signal transducers and adaptors such as Grb2/Sos, Shc, and Gab1 (6, 8, 9), which leads to the activation of two major signaling pathways, the extracellular signal-regulated kinase (ERK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway.

We reported previously that HGF inhibits the proliferation of a hepatoma cell line, HepG2, by arresting the cell cycle at G1 (10, 11). The inhibition was suppressed by a low concentration of MEK inhibitor, which causes the high-intensity ERK activity to become weak; thus, the G1 arrest is dependent on the high-intensity ERK activity induced by HGF (10, 12). We also showed that the strong activation of ERK is required for up-regulation of a cyclin-dependent kinase (Cdk) inhibitor, p16\(^{\text{INK4a}}\) (13), which is also known as a tumor suppressor. p16 preferentially associates with Cdk4 and Cdk6 and inhibits cell proliferation by preventing binding to the regulatory subunit, cyclin D (14, 15). We showed that up-regulation of p16 with HGF treatment of the cells increases the association of p16 with Cdk4, leading to the redistribution of other Cdk inhibitors, p21\(^{\text{CIP1}}\) and p2\(^{\text{KIP1}}\), from Cdk4 to Cdk2. Because Cdk2 activity is responsible for progression of the cell cycle through the G1 phase in HepG2 cells, the repression of Cdk2 activity by the Cdk inhibitors results in G1 arrest (13). Therefore, up-regulation of p16 is crucial for the inhibitory effect of HGF on the proliferation of HepG2 cells.
The expression of p16 is regulated by various transcription factors and regulatory factors, such as JunB, Ets, Bmi1, and CBX7, in various cells (16-19). The activation of Ets, a transcription factor, is generally regulated through phosphorylation by ERK (20, 21). We reported previously that Ets1 and/or Ets2 actually associate with the minimal promoter of p16 (~247 promoter) in the presence of HGF (13), implying that activation of Ets through phosphorylation by the strong ERK activity regulates the G1 arrest through up-regulation of p16. However, we recently found that the strong ERK activity is essential but not sufficient for the up-regulation of p16 (22). Thus, the regulatory mechanisms responsible for the up-regulation of p16 in the context of HGF signaling remain elusive.

In this study, we first examined whether HGF treatment facilitates phosphorylation of Ets1 and Ets2 at the ERK-dependent phosphorylation sites. The HGF treatment induced the phosphorylation, indicating that direct regulation of Ets occurs through the phosphorylation by ERK in the context of HGF signaling. However, the phosphorylation did not coincide with the up-regulation of p16 by HGF, and the high-intensity ERK activity is not sufficient for the up-regulation (22), suggesting that an ERK-independent pathway participates in the up-regulation of p16 by regulating the activation of Ets. We thus examined the regulatory mechanisms of Ets activity using a negative regulator, inhibitor of DNA binding (Id), in the presence of HGF.

Id proteins belong to a family of helix-loop-helix proteins. Although they lack a DNA-binding domain, they associate with basic helix-loop-helix transcription factors and form nonfunctional heterodimers that have no ability to bind DNA, thereby acting as dominant-negative inhibitors of basic helix-loop-helix (23, 24). The most studied Id protein, Id1, is also reported to bind to a non-helix-loop-helix protein, Ets, and inhibit its DNA-binding ability (25). The expression of Id1 is increased in proliferating cells and decreased by the induction of cell differentiation, and ectopic expression of Id1 represses the differentiation of some kinds of cells (26-28), suggesting that Id1 is a key regulator for the proliferation and differentiation of cells. Id1 is also considered a target in cancer therapy (29-31), because it is expressed in a wide variety of primary human tumors, such as endothelial, breast, prostate, and cervical cancers, and squamous cell carcinoma (32-36), and its expression is involved in malignant cell behavior (36). Although accumulating evidence indicates important biological effects of Id1, signaling pathways and signaling molecules responsible for the expression of Id1 are not well defined.

We showed here that (a) treatment of HepG2 cells with HGF down-regulated Id1 expression at the mRNA and protein levels, leading to a decrease in the amount of Ets associated with Id1; (b) Id1 protein was rapidly degraded via the ubiquitin-proteasome-mediated pathway, but the rate of degradation was not affected by HGF treatment; (c) repression of the high-intensity ERK activity with a MEK inhibitor, PD98059, had only a modest effect on the down-regulation of Id1 induced by HGF; (d) inhibition of the PI3K pathway with the addition of a specific inhibitor, LY29004, had no effect on the down-regulation of Id1; (e) high-level expression of Id1 suppressed the up-regulation of p16 by HGF, activation of the ~247 promoter of p16 in the presence of HGF, and antiproliferative effect of HGF on HepG2 cells; and (f) knockdown of Id1 with short hairpin RNA (shRNA) and small interfering RNA (siRNA) was essential for full activation of the ~247 promoter in addition to the high-intensity ERK activity. Our findings indicated that down-regulation of Id1 induced by HGF through ERK-dependent and ERK-independent pathways other than the PI3K pathway contributes to the up-regulation of p16 and plays a key role in the inhibitory effect of HGF on tumor cell proliferation.

Results
HGF Treatment Down-Regulates Expression of Id1
We showed previously that HGF treatment activates the minimal promoter of p16 (~247 promoter) through the proximal Ets-binding site in the promoter, and the transcription factor Ets1 and/or Ets2 physically bind to the Ets-binding site, indicating that Ets is involved in the up-regulation of p16 with HGF treatment in HepG2 cells (13). Because Ets is activated through phosphorylation at the NH2 terminus by ERK (20, 21) and the activation of the ~247 promoter with HGF treatment requires high-intensity ERK activity (13), the ERK activity induced by HGF seemed to directly regulate Ets activity in the context of HGF signaling. However, our recent findings have shown that the high-intensity ERK activity is not sufficient for the up-regulation of p16 (22), suggesting that ERK-independent pathways also participate in the up-regulation of p16 by HGF through regulation of Ets activity. We thus examined the regulatory mechanisms of Ets activity with HGF treatment in a hepatoma cell line, HepG2.

First, we examined the phosphorylation of Ets1 and Ets2 at the ERK-dependent sites (Thr18 in Ets1 and Thr22 in Ets2), which facilitates Ets activity. The phosphorylation was accelerated as late as 3 h after HGF treatment (Fig. 1), consistent with the activity of ERK, which reaches maximum levels at 5 min after the treatment (10). However, the phosphorylation of Ets proteins greatly preceded the prominent increase in p16 mRNA, which is regulated by the Ets-binding site in the promoter, after 8 h of the treatment (13). Furthermore, the high-intensity ERK activity is not sufficient for the increased expression of p16 (22). Thus, another factor was assumed to be responsible for the regulation of Ets activity through an ERK-independent pathway.

The transcriptional activities of Ets family proteins are repressed by helix-loop-helix proteins, the Id family (24, 25). The most studied Id protein, Id1, is abundant in young cells, which have low-level expression of p16, whereas the amount of Id1 is low in senescent cells, which have high-level expression of p16 (18). Thus, Id1 seemed to be involved in the regulation of p16 expression in the context of HGF signaling, although signaling pathways and signaling molecules responsible for the expression of Id1 were not well defined. We examined the amount of Id1 mRNA by conducting a quantitative real-time PCR (qRT-PCR) analysis. A relatively high level of the transcript was found in HepG2 cells in the absence of HGF, and HGF treatment caused a significant decrease in the level by 48 h (Fig. 2A). The decrease of Id1 mRNA with HGF treatment in HepG2 cells was also confirmed with a qRT-PCR analysis (Fig. 2B). Immunoblotting showed that the decrease in
the transcript is accompanied by that in the protein (Fig. 2C). A time-course analysis showed that the amount of Id1 protein had decreased at 6 h after HGF treatment and decreased progressively until 48 h after the treatment (data not shown). The down-regulation of Id1 protein coincided with the up-regulation of p16 (data not shown) and led to decrease of Ets1 associated with Id1 (Fig. 2D), suggesting that the down-regulation of Id1 participates in the effect of HGF on the up-regulation of p16 through the regulation of Ets activity.

**Id1 Protein Is Rapidly and Constantly Degraded via the Ubiquitin-Proteasome-Mediated Pathway, but the Stability of the Protein Is Not Altered by HGF**

Recent evidence has shown that the amount of Id1 protein is regulated by ubiquitin-proteasome-mediated degradation (37). To examine whether the degradation of Id1 protein is involved in the down-regulation of Id1 with HGF treatment in HepG2 cells, we analyzed the stability of Id1 protein in the absence or presence of HGF. Experiments abrogating newly synthesized proteins using cycloheximide indicated that the degradation of Id1 is rapid ($t_{1/2}$ ~40 min) in the absence of HGF, and HGF does not have a significant effect on the rate of the degradation just after the treatment of the cells (Fig. 3). The proteasome inhibitor, MG132, abrogated the rapid degradation of Id1 protein (Fig. 3), suggesting that the degradation process is regulated by the ubiquitin-proteasome-mediated pathway. Because Id1 protein was rapidly degraded from 0 to 24 h and from 24 to 48 h after HGF treatment (Fig. 2C), we further examined the rate of degradation at 24 h after the treatment. The rate was not significantly altered in the absence or presence of HGF even at that time point (data not shown). These results indicated that Id1 protein is constantly degraded at a rapid rate by the ubiquitin-proteasome-mediated pathway in HepG2 cells, but HGF treatment does not affect the degradation rate. Because HGF treatment of the cells induced down-regulation of Id1 at the mRNA and protein levels (Fig. 2A-C), it was suggested that the amount of Id1 is regulated at the mRNA level in the presence of HGF.
Inhibition of the High-Intensity ERK Activity Alleviates, but not Completely Abrogates, the Down-Regulation of Id1

The high-intensity ERK activity is essential but not sufficient for the up-regulation of p16 with HGF treatment in HepG2 cells (22). Thus, the effect of ERK signaling on the regulation of Id1 expression was examined in the presence of HGF. The down-regulation of Id1 induced by HGF treatment was alleviated by pretreatment of HepG2 cells with a low concentration of the MEK inhibitor PD98059 (10) but was not completely abrogated (Fig. 4A), showing that the expression of Id1 is regulated through not only ERK-dependent but also ERK-independent pathways. The ERK-dependent pathway is essential but not sufficient for the up-regulation of p16 (22); thus, it was strongly suggested that Id1 is involved in the up-regulation of p16 in the context of HGF signaling. On the other hand, inhibition of the PI3K pathway, another pathway of HGF signaling, with the addition of a specific inhibitor, LY29004, had no effect on the down-regulation of Id1 protein and the up-regulation of p16 induced by HGF (Fig. 4B), consistent with the independence of the PI3K pathway from the effect of HGF on the inhibition of cell proliferation (10).

Down-Regulation of Id1 by HGF Is Essential for the Up-Regulation of p16

To elucidate the role of the down-regulation of Id1 in the context of HGF signaling, experiments involving high-level expression of p16 with HGF treatment in HepG2 cells (22) would be reasonable. However, the degradation of Id1 protein was so rapid ($t_{1/2} \sim 40$ min; Fig. 3) that it was difficult to express enough exogenous Id1 protein for evaluation of its role in p16 expression. Recent studies have shown the NH$_2$ terminus of some short-lived proteins, including Id1, to be responsible for their rapid degradation via the ubiquitin-proteasome pathway and that addition of a protein tag to the NH$_2$ terminus stabilizes the protein (37-40). We thus added a flag-tag to the NH$_2$ terminus of Id1 and introduced the flag-Id1 expression plasmid into HepG2 cells. The amount of exogenous protein was several times greater than that of endogenous Id1 and remained unchanged in the presence of HGF (Fig. 5A; data not shown). The high level of flag-Id1 repressed the up-regulation of endogenous p16 induced by HGF (Fig. 5A), showing that the down-regulation of Id1 is required for the up-regulation of p16 with HGF treatment in HepG2 cells. To further examine whether the high-level expression of flag-Id1 affects the activity of the p16 gene promoter, we introduced the flag-Id1 expression plasmid together with a reporter plasmid including the $-247$ promoter of p16, which was placed upstream of the luciferase gene (41). Our previous results showed that the $-247$ promoter includes an Ets-binding site, which is responsible for the HGF-dependent up-regulation of p16 (Fig. 5B, top; ref. 13). HGF treatment of HepG2 cells with a mock plasmid induced a 6-fold increase in the activity of the promoter (Fig. 5B). The high-level expression of flag-Id1 reduced the activity to 37%, showing that the down-regulation of Id1 is essential for activation of the p16 promoter in the context of HGF signaling. Also, the high-level expression of flag-Id1 alleviated the antiproliferative effect of HGF on HepG2 cells (Fig. 5C). These results support that the down-regulation of Id1 is required for the inhibition of cell proliferation through the up-regulation of p16 in the presence of HGF (13).

Knockdown of Id1 Significantly Enhances the Activity of the p16 Promoter

Id1 was expressed at a relatively high level in proliferating HepG2 cells in the absence of HGF (Fig. 2A-C), and down-regulation of Id1 was essential for the up-regulation of p16 induced by HGF (Fig. 5A and B). These results raised the possibility that Id1 plays a role in repressing activation of the p16 promoter in proliferating cells in the absence of HGF. To

**FIGURE 3.** Degradation of Id1 protein. Cells were seeded at a density of $2.5 \times 10^5$ per 35 mm dish. After 24 h of culture, the medium was replaced with fresh medium with cycloheximide (CHX; 50 μg/mL), cycloheximide plus HGF (50 ng/mL), or cycloheximide, HGF plus MG132 (20 μmol/L). Cell lysates were prepared at the indicated times and evaluated with immunoblotting for Id1. The effect of HGF was assessed using phospho-ERK. Tubulin was used as a loading control. Bottom, intensity of the Id1 band was quantitated with NIH ImageJ software and graphed. Data were normalized so that the intensity at 0 h was 100%. The log$_{10}$ of the percentage of each intensity was plotted versus each time point. Representative of three independent experiments.
examine this possibility, we adopted RNA interference mediated by the DNA-based expression of a shRNA targeted at Id1 and examined whether knockdown of Id1 leads to activation of the p16 promoter in the absence of HGF. Introduction of the Id1 shRNA-expressing construct into HepG2 cells effectively repressed the expression of Id1 and had no effect on Id3 (Fig. 6A), showing that the effect of Id1 shRNA is specific. Introduction of the Id1 shRNA into proliferating cells had only a weak effect on activation of the p16 promoter in the absence of HGF (data not shown; Fig. 6C and D also support this result). The weak effect of knockdown of Id1 seemed to reflect the requirement of the high-intensity ERK activity for activation of the p16 promoter in addition to the reduction of Id1, because the transcriptional activity of Ets is also regulated by direct phosphorylation by ERK (20, 21). To activate ERK with little or no effect on the amounts of Id1 and p16 in proliferating HepG2 cells, we used a HepG2 cell clone expressing a high level of epidermal growth factor (EGF) receptor (22). EGF treatment of the cell clone induced high-intensity ERK activity similar to that induced by HGF treatment (Fig. 6B), whereas EGF did not induce p16 expression and did not inhibit proliferation of the cell clone (22). Also, the expression level of Id1 was not altered significantly with EGF treatment of the cell clone (Fig. 6B). Whereas EGF treatment of the cell clone increased the activity of the −247 promoter 2-fold, shRNA-mediated knockdown of Id1 in addition to EGF treatment induced a 4-fold increase in the activity of the promoter (Fig. 6C). We also achieved a similar result with siRNA, which has a different sequence from the shRNA (Fig. 6D), showing that the effect of the knockdown of Id1 on the −247 promoter was specific and not caused by off-target gene silencing. Previous reports showed that knockdown of Id3 up-regulated p16

FIGURE 4. Effect of inhibitors of cell signaling on the expression of Id1. HepG2 cells were seeded at a density of 2 × 10^4 per 100 mm dish. After 24 h of culture, the medium was replaced with fresh medium, and following preincubation with or without PD98059 (10 μmol/L; A), or LY294002 (5 μmol/L; B) for 1 h, HGF (50 ng/mL) was added. Cell lysates were prepared at the indicated times and subjected to immunoblotting. Experiments were done twice with similar results and representative data are shown. Repeatedly confirmed with a time-course analysis that the amount of Id1 protein within 3 h after HGF treatment was not influenced by HGF (data not shown).

FIGURE 5. Effect of high-level expression of flag-Id1 on the up-regulation of p16, activation of the p16 promoter, and inhibition of proliferation of HepG2 cells induced by HGF. A. Detection of high-level expression of flag-Id1 and effect of the expression on the up-regulation of p16. Cells were transiently transfected with a vector encoding flag-Id1 or an empty vector (Mock). At 24 h after transfection, the medium was replaced with fresh medium with HGF, and cells were further cultured. Cell lysates were prepared at the indicated times and subjected to immunoblotting. Experiments were done twice with similar results and representative data are shown. B. Effect of high-level expression of flag-Id1 on activation of the p16 promoter. Cells were transiently transfected with vectors encoding the −247 promoter of the p16 gene fused to the luciferase gene (top) together with a vector encoding flag-Id1 or an empty vector (Mock). At 24 h after transfection, the medium was replaced with fresh medium with or without HGF, and cells were further cultured. Cell lysates were prepared at 48 h after HGF treatment and subjected to the luciferase assay. Luciferase activities were normalized as described in Materials and Methods. The average fold increase in luciferase activity compared with mock transfection is indicated. Mean ± SD of triplicate determinations from a representative experiment (B). *, P < 0.01, Student’s t test. Experiments were done twice with similar results and representative data are shown. C. Effect of high-level expression of flag-Id1 on the inhibition of proliferation of HepG2 cells induced by HGF. Cells were seeded at a density of 3.5 × 10^3 per 100 mm dish and transiently transfected as described in A. At 24 h after transfection, the medium was replaced with fresh medium with or without HGF, and cells were further cultured. Cell numbers were counted at 96 h. Mean ± SD of triplicate determinations. *, P < 0.01, Student’s t test.
in human prostate cancer cell lines (42), and knockout mice lacking both Id1 and Id3 exhibited increase in p16 (24, 43), suggesting that Id3 also has an ability to regulate the expression of p16. However, we found that the Id1 shRNA activated the p16 promoter, whereas the shRNA had no effect on the amount of Id3 (Fig. 6A), and HGF treatment of HepG2 cells did not significantly affect the amount of Id3 protein (Fig. 2C), showing that Id3 is not involved in the up-regulation of p16 with HGF treatment in HepG2 cells. It is also worth noting that introduction of the Id1 shRNA and siRNA into cells in the absence of EGF had little effect on activation of the p16 promoter, supporting the requirement of strong activation of ERK for the activation of the promoter (Fig. 6C and D). These results showed that down-regulation of Id1 induces strong activation of the p16 promoter including the Ets-binding site in coordination with the strong activation of ERK.

Discussion

In this study, we found that Id1 is rapidly down-regulated by HGF and uncovered a role of Id1 in the inhibitory effect of HGF on the proliferation of a hepatoma cell line, HepG2. In the absence of HGF, the cells express a relatively high level of Id1, which predominantly represses the expression of Ets, leading to low levels of p16 (Fig. 7A). HGF treatment of the cells induced down-regulation of Id1 and Id1 associated with Ets (Fig. 2A-D). The amount of Id1 was regulated not only by ERK-dependent but also by ERK-independent pathways other than the PI3K pathway (Fig. 4A and B). The down-regulation of Id1 was required for the up-regulation of p16 through an Ets-binding site in the p16 promoter (Fig. 5A and B) and the antiproliferative effect of HGF (Fig. 5C). Knockdown of Id1 significantly enhanced the activity of the p16 promoter coordinately with the strong activation of ERK (Fig. 6C and D). These results showed that down-regulation of Id1 via ERK-dependent and ERK-independent pathways other than the PI3K pathway is involved in the up-regulation of p16, which leads to the inhibition of cell proliferation of HepG2 cells, in the context of HGF signaling (Fig. 7B).

The amount of Id1 is regulated at the protein level via the ubiquitin-proteasome-mediated pathway in HeLa cells (37).
Down-Regulation of Id1 by HGF for Up-Regulation of p16

Treatment of HepG2 cells with a proteasome inhibitor, MG132, abrogated the degradation of Id1 protein (Fig. 3), suggesting that the ubiquitin-proteasome-mediated pathway is also involved in the regulation of Id1 protein in HepG2 cells. However, HGF treatment of the cells had subtle effects on the rate of degradation (Fig. 3), showing that the amount of Id1 is not regulated at the protein level in the context of HGF signaling. A recent report showed that Id1 protein undergoes nuclear cytoplasmic shuttling (44), which is also involved in regulating its degradation (37). Our preliminary data showed that the localization of Id1 protein is not altered by HGF treatment in HepG2 cells (data not shown), also supporting that the rate of degradation of Id1 protein is not affected by HGF treatment. The amount of Id1 mRNA was also reduced with HGF treatment (Fig. 2A); thus, the rapid and constant degradation of Id1 protein seems to enable Id1 expression to be regulated at the mRNA level.

We showed that the MEK inhibitor PD98059 partially abrogates the down-regulation of Id1 (Fig. 4A), and the PI3K inhibitor LY294002 has no effect on the amount of Id1 (Fig. 4B). Thus, ERK-dependent and ERK-independent pathways are involved in the down-regulation of Id1 induced by HGF, but the PI3K pathway is not. These results implied that the transcription factor responsible for the expression of Id1 in the context of HGF signaling is regulated by ERK-dependent and ERK-independent pathways other than the PI3K pathway. The expression of Id proteins (Id1, Id2, and Id3) was shown to be regulated by MYC oncoproteins (24). It is reasonable that MYC is involved in the down-regulation of Id1 with HGF treatment in HepG2 cells, because MYC is regulated independently of the Ras-ERK pathway, and its expression is responsible for the induction of hepatocellular carcinoma from which the HepG2 cell line is derived and for the neoplastic properties of hepatocellular carcinoma (45-48). We thus examined the amount of c-MYC protein in the absence or presence of HGF, but HGF showed no effect on the level of c-MYC protein in HepG2 cells (Fig. 2C). Also, an assay of the secreted alkaline phosphatase reporter under the control of a promoter regulated by MYC showed no effect of HGF on the activation of MYC (data not shown). These results suggested that MYC is not involved in the regulation of Id1 in HepG2 cells.

A 1.2-kb fragment of the human Id1 promoter was shown to contain two regions responsible for Id1 expression (49). The upstream region contains consensus sequences for Egr-1, YY-1 and CREB and plays a role in the serum responsiveness of the Id1 promoter in human breast cancer cells, suggesting that the expression of Id1 in the absence of HGF in HepG2 cells is regulated via this region. On the other hand, the downstream region, with which a large complex containing Sp-1 and NF-1 actually associates, is responsible for repression of the Id1 promoter (49), suggesting that this region is directly involved in the down-regulation of Id1 at the mRNA level with HGF treatment in HepG2 cells. It has been shown that the DNA-binding activity of Sp-1 is stimulated through phosphorylation by ERK (50, 51), supporting the idea that Sp-1 is involved in the down-regulation of Id1 induced by HGF through the ERK-dependent pathway. However, although phosphorylation of Sp-1 actually occurs with HGF treatment, HGF induces the phosphorylation of Sp-1 through the PI3K pathway, and the phosphorylation facilitates transcriptional activation of Sp-1 (52). Therefore, it is difficult to believe that the down-regulation of Id1 with HGF treatment in HepG2 cells is regulated via this region. On the other hand, the down-regulation of Id1 by HGF, however, cells expressing an activated H-ras gene, which induces strong activation of ERK, showed down-regulated expression of NF-1 (54), and the signaling pathways, in which NF-1 is implicated, are still ambiguous. Thus, the role of NF-1 in the down-regulation of Id1 by HGF remains to be elucidated.

HGF treatment inhibits cell proliferation in several tumor cell lines including HepG2 cells (3-5) but not in normal hepatocytes and other tumor cell lines (1, 10, 55-58). Elucidation of the cause of the difference in the effect of HGF on cell proliferation is essential for applying HGF to cancer therapy. We showed here that down-regulation of Id1 induced with HGF treatment in HepG2 cells is involved in the up-regulation of p16, which inhibits cell proliferation. Id1 is generally expressed at high levels in proliferating cells, and induction

FIGURE 7. A schematic model of the involvement of Id1 in the regulation of p16 expression in the context of HGF signaling in HepG2 hepatoma cells. Our previous data showed that HGF treatment activates the minimal promoter of p16 (−247 promoter) through the proximal Ets-binding site, the transcription factor Ets1 and/or Ets2 physically bind to the Ets-binding site, and activation of the −247 promoter with HGF treatment requires high-intensity ERK activity (13). In this study, we showed that Id1 is rapidly down-regulated by HGF and uncovered a role of Id1 in the inhibitory effect of HGF on the proliferation of HepG2 cells as follows. A, In the absence of HGF, the level of ERK activity is low and a relatively high level of Id1 is expressed, so the transcription factor Ets is kept inactive. This leads to low levels of p16, which enable the cells to proliferate normally. B, HGF treatment of the cells elicits strong activation of ERK, which phosphorylates Ets1 and Ets2 at the conserved threonine residue (Fig. 1). HGF treatment also induces down-regulation of Id1 at the mRNA level (Figs. 2A-C and 3) via ERK-dependent and ERK-independent pathways (Fig. 4). The down-regulation of Id1 decreases Ets associated with Id1 (Fig. 2D), leading to activation of the p16 promoter (Figs. 5 and 6) in coordination with the strong activation of ERK (Fig. 6). The up-regulation of p16 results in the inhibition of cell proliferation through cell cycle arrest at G1 via inactivation of CdK2.
of differentiation, which results in an arrest of cell proliferation, decreases Id1 levels (26-28). Also, ectopic expression of Id1 accelerates cell proliferation (59), and Id1 is involved in cellular transformation, which enables cells to progressively proliferate despite any signal for the inhibition of cell proliferation (24, 60, 61). These results indicate the importance of Id1 to the regulation of cell proliferation and suggest that the levels of Id1 are partly responsible for the difference in the effect of HGF on cell proliferation. Furthermore, recent reports showed the expression of Id1 to be related to the serum-independent proliferation of cancer cells (62), invasive behavior, and metastasis of tumors (29, 31, 33, 35), suggesting that Id1 expression is involved in not only the regulation of cell proliferation in vitro but also carcinogenesis and malignant cell behavior in vivo. Therefore, our results showing the rapid down-regulation of Id1 induced by HGF and the signaling pathways responsible for the down-regulation are expected to provide a molecular basis for cancer therapy.

Materials and Methods

Cell Culture

HepG2 cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C. After 24 h of culture, the medium was replaced with fresh medium with or without HGF (50 ng/mL), and the cells were further cultured.

Preparation of Cell Lysate

Cells were washed twice with ice-cold PBS containing 1% EDTA and 0.2 mmol/L Na₂VO₄ and lysed with lysis buffer [50 mmol/L Tris·HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Tween 20, 30 mmol/L tetrasodium pyrophosphate, 50 mmol/L NaF, 1 mmol/L Na₂VO₄, 5 μg/mL leupeptin, 1 μg/mL pepstatin A, and 1 mmol/L phenylmethylsulfonyl fluoride]. The cell lysates were cleared by centrifugation, and the protein concentration of the precleared lysate was correctly measured with the BCA protein assay reagent (Pierce).

Antibodies and Immunoblotting

Antibodies used for immunoblotting were obtained as follows; anti-Id1 (C-20), anti-Ets1 (C-20), anti-Ets2 (C-20), anti-p16 (H-156), anti-Cdk4 (H-22), and anti-Id3 (C-20) antibodies from Santa Cruz Biotechnology; anti-phospho-Ets1 (pThr 38) antibodies from Cell Signaling Technology; anti-phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204), anti-c-MYC, and anti-phospho-Akt (Ser473) antibodies from Cell Signaling Technology; anti-α-tubulin (T9026) from Sigma; and anti-rabbit and anti-mouse immunoglobulin conjugated to hors eradish peroxidase from Amersham Biosciences. Equal amounts of protein in the precleared cell lysates (20-70 μg total protein) were resolved by SDS-PAGE on a 12% gel after heat denaturation. Immunoblotting was done as described previously (11). The intensity of bands on the autoradiograms was quantitated by NIH ImageJ software.

qRT-PCR of Id1 mRNA

Total RNA was purified with Isogen (Nippon Gene) according to the manufacturer's instructions, and 5 μg RNA was used for cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen) with oligo(dT) primers. PCR amplification of the cDNA was done as described previously (13) using a pair of Id1-specific oligonucleotide primers (forward 5’-CCCATTCTGTITTCAGCCAGT-3’ and reverse 5’-ATCGGTCT-TGTTCCTCCTCA-3’) with 28 cycles of denaturing (94°C, 30 s), annealing (65°C, 30 s), and extension (72°C, 1 min). PCR products were resolved on a 1.5% agarose gel and visualized with ethidium bromide staining. Experiments conducted using various amounts of input template ensured that the assay was quantitative under these conditions. We also performed qRT-PCR using RNA and cDNA prepared independently of the samples described above. Id1-specific primers were described above, and p16-specific primers were described previously (11). PCR was done in 40 cycles on an ABI Prism 7500 using SYBR Green PCR Master Mix (Applied Biosystems). Each sample was run in triplicate and the average value was considered for analysis. Relative quantities ( Ct) were obtained by normalization relative to α-tubulin.

Construction of an Id1 Expression Plasmid

PCR amplification of Id1 cDNA for constructing an Id1 expression plasmid was done using a pair of oligonucleotide primers specific to the coding sequence of human Id1 (forward 5’-TAAGAGGCACCCGATCCGGACAGACGAGCAGCATGATGCTG-3’ and reverse 5’-GCTCTAGATCAGCGACACAAAGGTAGTGACC-3’) and cDNA as described above with 28 cycles of denaturing (94°C, 30 s), annealing (67°C, 30 s), and extension (72°C, 1.5 min). The PCR product was digested with NotI and XbaI and inserted into NotI and XbaI sites of pME-Flag.

shRNA- and siRNA-Mediated RNA Interference

A DNA oligonucleotide containing the sense target sequence of human Id1 (5’-TCTACACTGCACGAAATG-3’), a hairpin loop, and the antisense target sequence was synthesized, annealed, and inserted into a polIII-mediated siRNA expression plasmid vector, pSilencer 1.0-U6 (Ambion). The shRNA expression plasmid was transfected as described below. We also performed RNA interference experiments for Id1 with the use of a synthetic siRNA (Integrated DNA Technologies), which has a different sequence from the shRNA (5’-CGACAGGAACGGCGUGUACUCAGC-3’).

Transient Transfection

HepG2 cells were seeded at a density of 2.5 × 10⁵ in 6-well plates and cultured for 24 h. They were treated with 4 μg plasmid mixed with 11.2 μL jetPEI (Polyplus Transfection) for 24 h, and the medium was replaced with fresh medium. The cells were cultured further in the absence or presence of HGF (50 ng/mL).

Luciferase Reporter Assays

The p16 promoter construct containing the 5’ truncated promoter region of the p16 gene (bp -247 to +1 from the transcription initiation site) fused with the luciferase gene was kindly provided by Dr. E. Hara. The promoter construct was cotransfected with a standard amount of the pSV-β-galactosidase control plasmid (Promega). Transfected cells were stimulated with HGF (50 ng/mL) for 48 h, and cell extracts were prepared
with reporter lysis buffer (Promega). The luciferase and β-galactosidase assays were done in 20 μL cell extract with the Luciferase Assay System Kit (Promega) and β-Galactosidase Enzyme Assay System (Promega), respectively, on a microtiter plate multilabel counter (Amersham Pharmacia Biotech). Each luciferase activity was normalized to the corresponding β-galactosidase activity.

**Statistical Analysis**
The paired Student's t test was used to test for significance where indicated.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**References**
Molecular Cancer Research

Id1 Is Down-Regulated by Hepatocyte Growth Factor via ERK-Dependent and ERK-Independent Signaling Pathways, Leading to Increased Expression of p16INK4a in Hepatoma Cells

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