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Insulin-Like Growth Factor (IGF)-II Regulates CCAAT/Enhancer Binding Protein α Expression via Phosphatidyl-Inositol 3 Kinase in Human Hepatoblastoma Cell Lines

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Abstract To reveal growth factor and its signal pathway to CCAAT/enhancer binding protein alpha (C/EBPα) in hepatocyte differentiation, we used Huh-6 and HepG2, human hepatoblastoma (HBL) cell lines that maintain the expression of genes in hepatoblasts and remain at that stage of differentiation. Insulin-like growth factor (IGF)-II, hepatocyte growth factor (HGF), and dexamethasone (Dex) stimulated HBL cells for Northern blot analysis. Bromodeoxyuridine (BrdU) up-take assay and Western blot analysis on albumin was performed to unveil proliferation and differentiation activity of IGF-II. C/EBPα and phosphorylation of Akt were analyzed by Western blot analysis. LY294002 and wortmannin, specific inhibitors of PI3 kinase, and PD98059, a specific inhibitor of mitogen-activated protein (MAP) kinase, were used to examine the signaling pathway of C/EBPα upregulated by IGF-II. Luciferase assay was performed to study the promoter activity of C/EBPα. Actinomycin D was used to analyze half-life of C/EBPα mRNA. IGF-II up-regualted C/EBPα by Northern blot and Western blot while HGF and Dex did not by Northern blot. IGF-II promoted proliferation and differentiation by BrdU up-take assay and Western blot analysis on albumin. Akt phosphorylated by IGF-II, suggested that phosphatidyl-inositol (PI) 3 kinase mediated the signaling pathway of IGF-II. LY294002 and wortmannin suppressed expression of C/EBPα. IGF-II activated the promoter activity and prolonged half-life of mRNA, suggesting that IGF-II activated promoter and stabilized mRNA. LY294002 and wortmannin suppressed the promoter activity of C/EBPα while PD98059 did not, suggesting that activation of the promoter was mediated by PI3 kinase. J. Cell. Biochem. 102: 161–170, 2007.

Key words: insulin-like growth factor-II; Northern blot; LY294002; luciferase assay; mitogen-activated protein kinase; PD98059

CCAAT/enhancer binding protein alpha (C/EBPα) is a liver-enriched transcription factor, regulating the expression of liver-specific genes [Takiguchi, 1998]. In rodents, the expression of C/EBPα appears in hepatoblasts at 10.5 days of gestation and increases toward birth [Kuo et al., 1990; Shiojiri et al., 2004]. Hepatoblasts are immature hepatocytes in fetal liver with potentials of proliferation and differentiation into either hepatocytes or biliary epithelial cells (bipotentiality). Since the expression of C/EBPα is limited in hepatocytes during liver development, it is suggested that C/EBPα is involved in hepatocyte differentiation. Indeed, hepatocytes deficient in C/EBPα remain as hepatoblasts [Tomizawa et al., 1998]. This indicates that C/EBPα is indispensable in the differentiation of hepatoblasts into hepatocytes. It is therefore expected that knowledge of the regulation of C/EBPα will provide us with useful information on the mechanism of hepatocyte differentiation. However, neither growth factors nor signal pathways for the regulation of C/EBPα was known.

Insulin-like growth factor (IGF)-II influences both proliferation and differentiation, mediated by IGF-I receptor (IGF-IR). Once IGF-II binds to

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IGF-IR, the receptor autophosphorylates and activates phosphatidyl-inositol (PI) 3 kinase or mitogen-activated protein (MAP) kinase [LeRoith and Roberts, 2003]. Disruption of IGF-II results in dwarfism in mice, but the liver develops normally [DeChiara et al., 1991]. This is attributed to the compensation by IGF-I binding to IGF-IR that mediates IGF-II in fetus. In rodents, the expression of IGF-II appears in the mesenchyme of the septum transversum where hepatoblasts originate at 9.5 days of gestation, and diminishes at 11 days after birth [Brown et al., 1986; Streck and Pintar, 1992]. In human liver, the expression of IGF-II is more abundant in fetus than in adults, with the peak being from around birth until the age of 9 months [Li et al., 1996]. This indicates that IGF-II is deeply involved in hepatocyte differentiation, but its mechanism in promoting hepatocyte differentiation is not clear. Interestingly, the expression of IGF-II appears slightly earlier than C/EBPα in the proximity of hepatoblasts, suggesting that IGF-II might influence C/EBPα. However, there is as yet no evidence concerning any possible interaction between C/EBPα and IGF-II in fetal liver.

Hepatoblastoma (HBL) arises in the liver of patients under 2 years of age, often accompanied by Beckwith–Wiedemann syndrome (BWS) and mutation of β-catenin gene. Since BWS and β-catenin are involved in development, it is suggested that HBL occurs due to developmental disorder. Since tumor cells of HBL resemble hepatoblasts microscopically, it is speculated that HBL remains at the stage of hepatoblasts [Ishak and Glunz, 1967]. Hence, HBL is expected to maintain the expression of genes specific to hepatoblasts. On this basis, it is thought that HBL presents an ideal model for analyzing the mechanism of hepatocyte differentiation.

Consequently, we decided to address the possibility that IGF-II upregulates C/EBPα as well as to examine the signaling pathway to the promoter region of C/EBPα with HBL cell lines.

MATERIALS AND METHODS

Cell Culture

Huh-6 and HepG2 were purchased from RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's Minimum Essential Medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Trace Scientific, Melbourne, Australia) in 5% carbon dioxide at 37°C in a humidified chamber. Cells were spread onto 10-cm dishes (Asahi Techno Glass, Funabashi, Japan) for Northern blot analysis and Western blot analysis, 6-well plates for luciferase assay (Asahi Techno Glass), or 96-well flat bottom wells for proliferation assay (Asahi Techno Glass).

Stimulation of Growth Factors

After 24-h culture in 10-cm dishes (Asahi Techno Glass) at a density of 2×10^5 cells/dish, the medium was changed to serum-free DMEM. Twenty-four hours later, IGF-II (Wako Pure Chemicals, Osaka, Japan), hepatocyte growth factor (HGF) (Wako), or dexamethasone (Dex) (Wako) was added. When LY294002 (Wako) (50 μM) or wortmannin (Wako) (1 μM), specific inhibitors of PI3 kinase, PD98059 (20 μM), a specific inhibitor of MAP kinase (Wako), or Actinomycin D (1 μg/ml) (Wako), a specific inhibitor of mRNA transcription, was used, they were added 30 min prior to the addition of IGF-II.

Northern Blot Analysis

Total RNA (1 μg), isolated with Isogen (Nippon Gene CO., LTD., Tokyo, Japan), was subjected to electrophoresis in a denaturing formaldehyde-agarose gel and then transferred to a nylon filter. Hybridization was performed with DIG northern starter kit (Roche Diagnostics K.K., Tokyo, Japan) following the manufacturer's instructions. A 600-bp fragment of 3′-untranslated region of the C/EBPα (EcoRI-HindIII) from pB28E5.0 plasmid was used as a probe. Images were scanned and analyzed with ImageJ 1.34s (National Institutes of Health, Bethesda, MD). The intensity of 28s rRNA was used as internal control. The expression level of C/EBPα was calculated as the signal intensity of C/EBPα divided by that of 28s rRNA. The experiments were repeated three times. In some experiments, the filters were re-hybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Bromodeoxyuridine (BrdU) Up-Take Assay

Huh-6 or HepG2 were spread at a density of 1,000 cells per well. Following 24 h of culture under DMEM with 10% FBS, medium was changed to DMEM without FBS. After 24 h of culture under DMEM without FBS, IGF-II was

added to the medium. Seventy-two hours later, BrdU Cell Proliferation Assay (Exalpha Biological, Inc., Watertown, MA) was performed according the the manufacturer's instruction. Briefly, after 2 h incubation with BrdU, cells were fixed and washed with wash buffer supplemented in the kit, and incubated with a monoclonal anti-BrdU antibody for 1 h. After wash, peroxidase-labeled goat anti-mouse IgG was added to the cells. After wash, each well was added with tetra-methylbenzidine (TMB), a chromogenic substrate that converts a solution form colorless to a blue one. The absorbance was analyzed with a multiple plate reader at a wavelength of 490 nm with a BIO-RAD Model 550 microplate reader (Bio-RAD, Hercules, CA). The absorbance indicates viability of cells, representing cell number.

Western Blot Analysis

Twenty micrograms of protein isolated from cultured cells was subjected to sodium dodecyl sulphate polyacylamide gel electrophoresis, and transferred to a nylon filter. Primary antibodies were polyclonal rabbit anti-rat C/EBPα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal rabbit anti-phospho-Akt (S473) (Cell Signaling Technology, Danvers, MA), polyclonal rabbit anti-Akt (Cell Signaling Technology), polyclonal rabbit anti-human albumin (Cappel, Aurora, OH), and mouse monoclonal anti-α-tubulin antibody (Lab Vision, Fremont, CA). Second antibodies were HRP-linked anti-rabbit antibody (Amersham Bioscience, Tokyo, Japan) and HRP-linked anti-mouse antibody (Amersham Bioscience). Dilutions were 1:500 for primary antibodies, and 1:1,000 for second antibodies. The filters were reprobed with tubulin. The specific antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Bioscience).

Plasmid Construction

EcoRI-NruI (2.2-kb), 0.7-kb (NcoI-NruI), or 0.5-kb (PstI-NruI) fragments of human C/EBPα promoter from pG28E3.8 were blunted with a Klenow fragment and then subcloned into SmaI of pGL3-basic (Promega Corporation, Tokyo, Japan) (p2.2CEBPA/GL3, p0.7CEBPA/GL3, and p0.5CEBPA/GL3, respectively). A 0.29-kb (ApaI-NruI) fragment from pG28E3.8 was subcloned into ApaI-EcoRV of pBluscriptIISK(−) (p0.29CEBPA/Blue). A KpnI-SstI fragment from p0.29CEBPABlue was subcloned into KpnI-SstI of pGL3basic (p0.29CEBPA/GL3). A 70-bp fragment (-60 ± 10bp relative to the transcription start site) from pG28E3.8 was amplified with PCR, subcloned into BamH1-Sst1 of pBluescriptIISK(-) (p0.06CEBPA/Blue), followed by sequence confirmation. A SstI-BamHI fragment from p0.06CEBPABlue was subcloned into SstI-BglII of pGL3basic (p0.06CEBPA/GL3). pB28E5.0 and pB28E3.8 were kindly provided by Dr. Kleanthis G. Xanthopoulos (Anadys Pharmaceuticals, Inc., San Diego, CA).

Dual Luciferase Assay

After 24-h culture in six-well plates at a density of 3×10^4 cells/well, Huh-6 and HepG2 cells were transfected with 2 μg of reporter plasmid containing respective genomic fragments, pGL3-control (Promega) vector harboring the SV40 promoter-linked firefly luciferase gene, or pGL3-basic, together with 0.2 μg of a control vector, the renilla luciferase gene fusing the HSV-TK promoter (pRL-TK) (Promega) with Lipofectin (Invitrogen Japan K.K., Tokyo, Japan), followed by 24-h culture in DMEM with 10% charcoal stripped FBS. After 24h culture under serum-free condition, IGF-II (200 ng/ml) was added with or without LY294002 (50 μM), wortmannin (1μM), or PD98059 (20 μM), followed by 48 h culture. The transcriptional activity was measured with a dual luciferase reporter assay system (Promega) using AB-2200 (Atto Corporation, Tokyo, Japan).

Statistical Analysis

The expression levels of C/EBPα and cell proliferation were analyzed statistically with one-factor analysis of variance. Relative luciferase activities were analyzed by Wilcoxon signed–ranks test between the groups of cell lines with or without the stimulation of IGF-II. All statistical analysis was preformed with JMP5.0J (SAS Institute Japan, Tokyo, Japan). A P-value < 0.05 was accepted as statistically significance.

RESULTS

Huh-6 and HepG2 cells were used because of their strong expression of C/EBPα. The expression levels of C/EBPα were analyzed to address the possibility of IGF-II stimulating C/EBPα.

Northern blot analysis was performed 48 h after the addition of IGF-II. Image analysis of C/EBPα showed that its expression level increased as the concentration of IGF-II increased, with statistical significance being observed at 20 and 200 ng/ml with 2.2 and 2.6 times, respectively, in Huh-6 (Fig. 1A), and with 2.1 and 2.2 times, respectively, in HepG2 (Fig. 1B) ($P < 0.05$). This clearly demonstrated that IGF-II was directly involved in the upregulation of C/EBPα. Expression of GAPDH was not changed. After addition of IGF-II at 200 ng/ml, C/EBPα was upregulated to 2.4 times in Huh-6 and 2.0 times in HepG2 at 6 h with statistical significance ($P < 0.05$) (Fig. 1C).

Since HGF and Dex are involved in hepatocyte differentiation, they were added in medium to clarify whether they upregulated C/EBPα

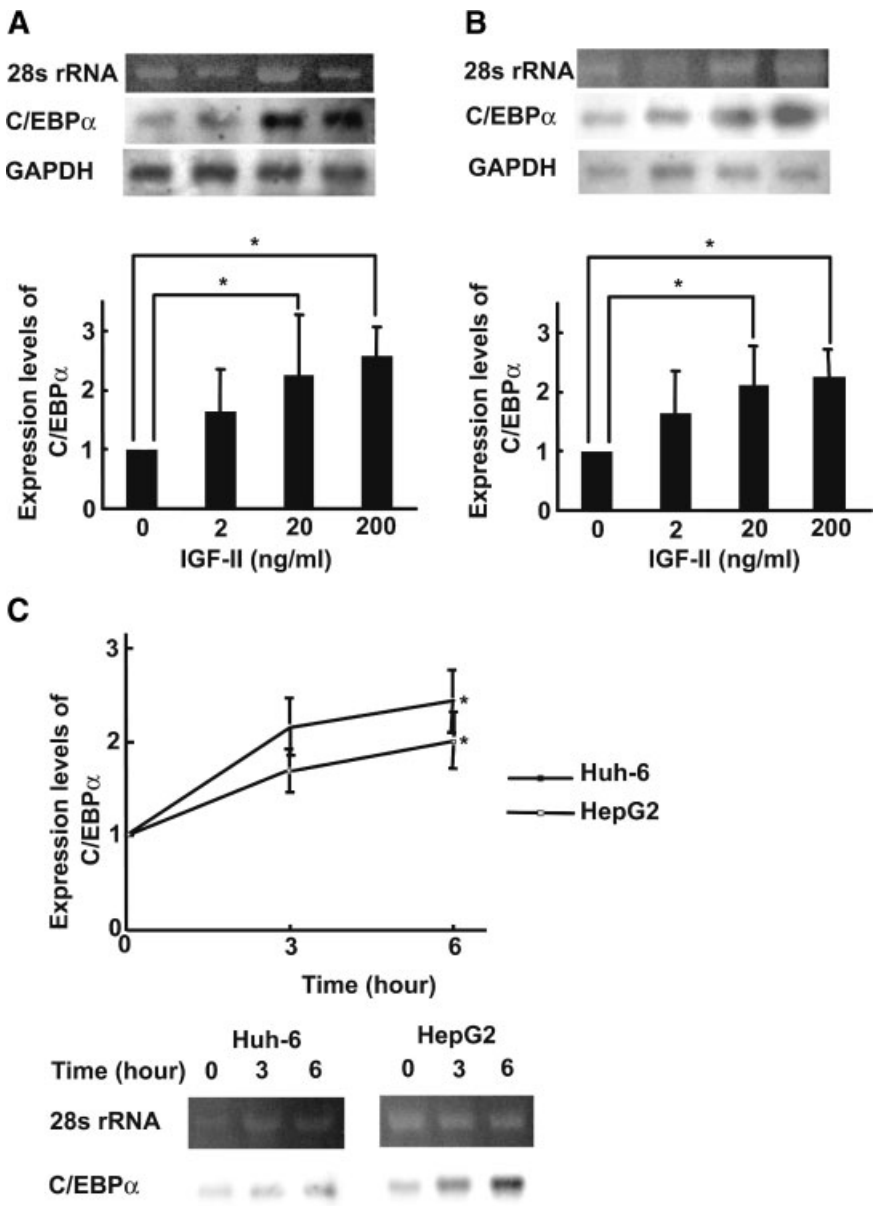

Fig. 1. IGF-II upregulated C/EBPα. Huh-6 (**A**) and HepG2 (**B**) cells were stimulated with IGF-II, and the expression of C/EBPα was analyzed by Northern blot analysis, with 28s rRNA as an internal control. The expression levels of C/EBPα increased as the Huh-6 and HepG2 cells were stimulated with IGF-II at densities of 20 and 200 ng/ml, with statistical significance. Expression of GAPDH was not changed (A, B). C/EBPα was upregulated toward 6 h after addition of IGF-II (200 ng/ml) (**C**). Experiments were repeated three times. *, $P < 0.05$; error bars,standard deviation.

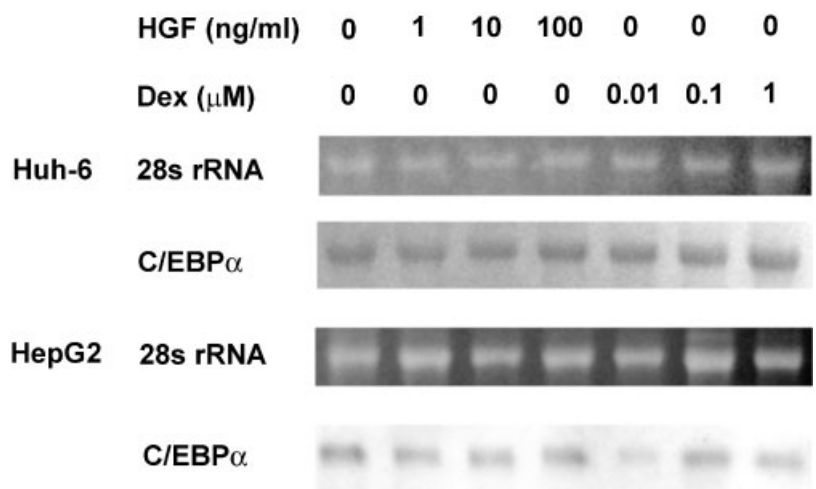

Fig. 2. C/EBPα not stimulated by HGF or dexamethasnone. Huh-6 and HepG2 cells were stimulated with hepatocyte growth factor (HGF) or dexamethasone (Dex), and expression of C/EBPα was analyzed by Northern blot analysis. No significant change was observed. Experiments were repeated three times.

in HBL cell lines. Northern blot analysis was performed 48 h after addtition of HGF or Dex. Image analysis showed no significant changes of expression of C/EBPα were observed (Fig. 2).

Since IGF-II is a strong mitogen, BrdU up-take assay was performed after addition of IGF-II in medium to examine cell poliferation. At 200 ng/ml, cells proliferated to 2.4 times (Huh-6) and 2.1 times (HepG2), respectively, with statistical significance ($P < 0.05$) (Fig. 3). It was clearly shown that IGF-II stimulated proliferation of Huh-6 and HepG2 cells.

Our data clearly showed that IGF-II upregulated C/EBPα. Western blot analysis was performed to examine the expression level of C/EBPα in protein level. Forty-two kilo dalton as well as 30 kD was upregulated (Fig. 4). C/EBPα was upregulated at RNA and protein level. Since Akt is a major down-stream molecule of PI3 kinase, phosphorylation of Akt was analyzed with Western blot analysis to address the possibility of activation of PI3 kinase by IGF-II. In Huh-6 and HepG2, Akt was significantly phosphorylated with IGF-II while expression levels of Akt were not changed (Fig. 4). It was clearly shown that IGF-II upregulated C/EBPα at protein level, and its signal was transmitted by PI3 kinase-Akt. C/EBPα is involved in differentiation. Western blot analysis was performed with albumin, a marker of mature hepatocytes. Albumin was upregulated by IGF-II to show that IGF-II promoted differentiation of Huh-6 and HepG2 cells (Fig. 4).

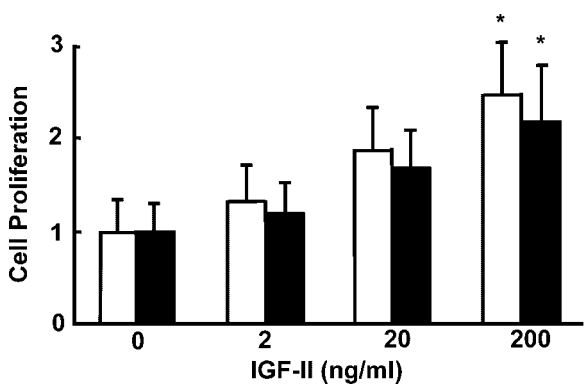

Fig. 3. Cell proliferation stimulated by IGF-II. Huh-6 (open bars) and HepG2 (black bars) cells were stimulated with IGF-II, and cell proliferation was analyzed by BrdU up-take assay. Cells proliferated to 2.4 times (Huh-6) and 2.1 times (HepG2) at 200 ng/ml with statistical significance. n = 3; *, $P < 0.05$; error bars,standard deviation.

Western blot analysis clearly showed that PI3 kinase/Akt pathway was activated by IGF-II. Inhibitors were used to confirm that signaling pathway of IGF-II was PI3 kinase, not MAP kinase. Since LY294002 and wortmannin specifically inhibit PI3 kinase, and PD98059 does MAP kinase, they were added 30 min prior to IGF-II stimulation to clarify the signal pathway from IGF-II. Without IGF-II, LY294002 and wortmannin suppressed the expression of C/EBPα to 5.1 and 23.2%, respectively, ($P < 0.05$) in Huh-6, and 20.8 and 27.6%, respectively, ($P < 0.05$) in HepG2, whereas with IGF-II, LY294002 and wortmannin suppressed the expression level of C/EBPα to 25.9 and 38.8%, respectively, ($P < 0.05$) in Huh-6 and 3.2 and

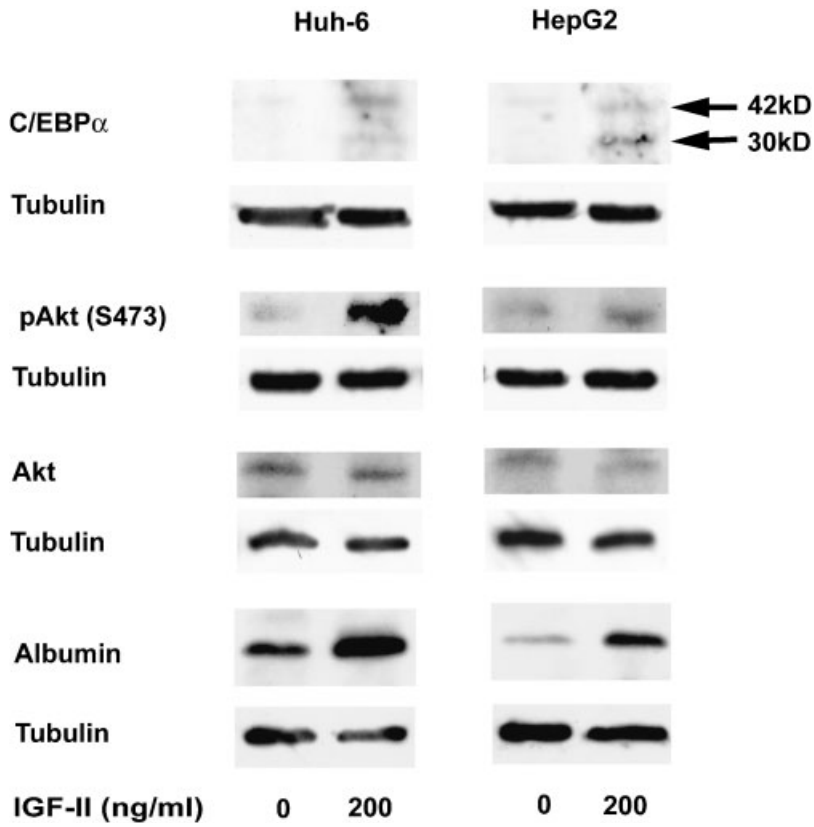

Fig. 4. Akt phosphorylated, and C/EBPα and albumin upregulated by IGF-II. Huh-6 and HepG2 cells were stimulated with IGF-II, and protein was isolated for Western blot analysis. IGF-II upregulated C/EBPα in 42 and 30 kD significantly as well as albumin in both cell lines. Akt was significantly phosphorylated by IGF-II in both cell lines while expression of Akt was not changed. The filters were reprobed with tubulin. pAkt: phosphorylated Akt at Ser 473.

50.8% ($P < 0.05$), respectively, in HepG2 (Fig. 5). When PD98059 was added, the expression levels of C/EBPα were 80.3% in Huh-6 and 105% in HepG2 (without IGF-II), and 95.6% in Huh-6 and 90.3% in HepG2 (with IGF-II) (Fig. 5). Expression of GAPDH was not changed. It appears that PD98059 did not suppress or stimulate the expression of C/EBPα. It was clearly shown that the signal from IGF-II to upregulate C/EBPα was inhibited by LY294002 and wortmannin but not by PD98059.

From the data above, it was expected that IGF-II activated the promoter region of C/EBPα, and the activation was inhibited by LY294002 and wortmannin. To clarify the promoter activity of C/EBPα stimulated by IGF-II, reporter plasmids with 2.2-, 0.7-, 0.5-, 0.29-, and 0.06-kb lengths of C/EBPα promoter were constructed (Fig. 6A). Huh-6 cells and HepG2 cells were transfected with the reporter plasmids, stimulated with IGF-II with or without LY294002, wortmannin, or PD98059, and subjected to luciferase assay. The relative luciferase activity was strongest with the 2.2-kb fragment of the C/EBPα promoter region and lowest with the 0.06-kb fragment. It appeared that IGF-II stimulated activity of 2.2- and 0.7-kb fragments of the C/EBPα promoter region. When LY294002 or wortmannin was added, the relative luciferase activity tended to be lower than that without LY294002 or wortmannin. Only the 2.2-kb fragment had statistical significance with the addition of LY294002 without IGF-II (49.2%) in Huh-6 and (50.6%) in HepG2 or with IGF-II (48.9%) in Huh-6 and (48.7%) in HepG2 as compared to that without the addition of IGF-II or LY294002 ($P < 0.05$) (Fig. 6B). 2.2-kb fragment had statistical significance with addition of wortmannin without IGF-II (50.3%) in Huh-6 and (51.5%) in HepG2 or with IGF-II (50.5%) in Huh-6 and (47.6%) in HepG2 (Fig. 6B). It appears that LY294002 and wortmannin partly inhibited the activation of C/EBPα promoter region by IGF-II.

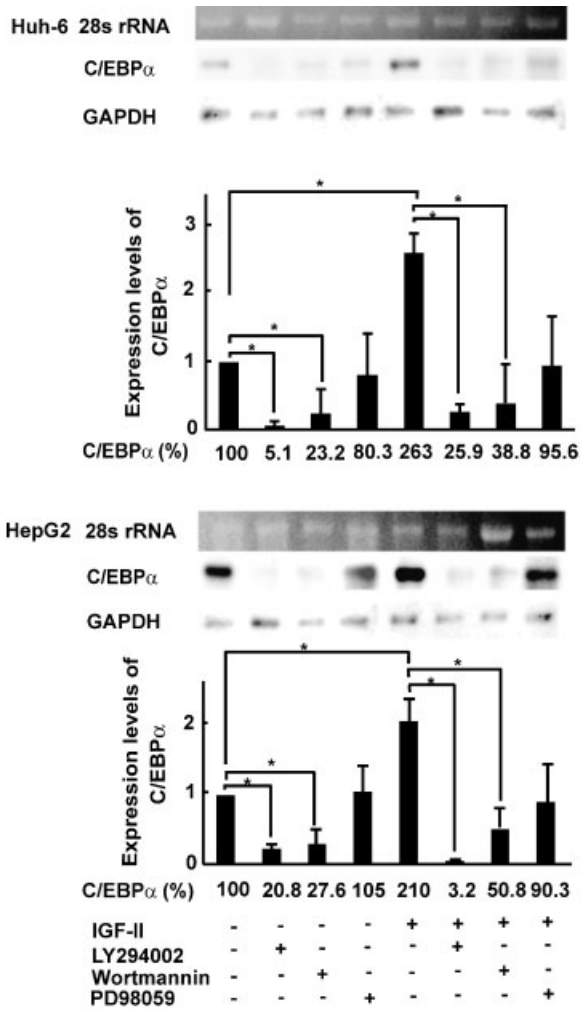

Fig. 5. LY294002 and wortmannin downregulated the expression of C/EBPα. Huh-6 and HepG2 cells were stimulated with IGF-II (200 ng/ml) with or without LY294002 (50 μM), wortmannin (1μM), or PD98059 (20 μM), followed by Northern blot analysis. The expression levels of C/EBPα were suppressed by LY294002 and wortmannin with or without the stimulation of IGF-II ($P < 0.05$). PD98059 did not suppress the expression levels of C/EBPα. Expression of GAPDH was not changed. n = 3; *, $P < 0.05$; error bars,standard deviation.

C/EBPα could also be regulated at post-transcriptional level, the role of IGF-II on mRNA stabilization was investigated. Actinomycin D was used to induce transcription arrest to analyze half-life of mRNA. In the initial experiments, the expression levels of C/EBPα was zero 6 h after addition of Actinomycin D in HepG2 while that was 33% at 3 h. RNA was, then, isolated 4 h after addition of Actinomycin D, and expression of C/EBPα was zero. The expression level of C/EBPα was shown in Figure 7. Half-life of C/EBPα was 1.5 h in Acrtinomycin D treated and 3.0 h in IGF-II and Actinomycin D treated Huh-6, and 2 h in Actinomycin D treated and 3.0 h in IGF-II and Actinomycin treated HepG2. It appears that IGF-II prolonged half-life of C/EBPα mRNA.

DISCUSSION

C/EBPα is the major player at the late stage of hepatocyte differentiation. However, growth factors known to be involved in the upregulation of C/EBPα in hepatocytes have still to be found. No experimental model, however, exists for maturation of human hepatocytes. We, therefore, used human HBL cell lines. IGF-II is more abundantly expressed in liver before and around birth than in adult, suggesting that it has a role in hepatocyte differentiation [Brown et al., 1986]. Although IGF-II may regulate the expression of C/EBPα, there is no direct evidence that alteration of the expression of C/EBPα occurs by IGF-II. The present study clearly showed that IGF-II upregulated C/EBPα, promoting differentiation as evidenced by upregulation of albumin. This is supported by the fact that IGF-II promotes the differentiation of ES cells into hepatocytes [Teratani et al., 2005]. C/EBPα is upregulated by HGF in immature hepatocytes isolated from fetal mouse liver [Suzuki et al., 2003]. In human HBL cells, HGF did not upregulate C/EBPα. Although the mechanism is not clear, it may be due to the difference of cells. Dex is important in hepatocyte differentiation since it promotes transdifferentiation of pancreatic acinar cells into hepatocyes [Shen et al., 2000]. Our results showed no change of expression levels of C/EBPα stimulated by Dex. It was clearly shown that IGF-II is important in hepatocyte differentiation, upregulating C/EBPα in human HBL cells. In fact, this result was contradictory to the expectation of a downregulation of C/EBPα by IGF-II, based on the fact that C/EBPα is a tumor suppressor gene while IGF-II is a potent mitogen, as shown in Figure 3. PI3k kinase/Akt pathway dephosphorylates C/EBPα on Ser193, and blocks its growth inhibitory activity [Wang et al., 2004]. This dephosphorylated C/EBPα promotes cell proliferation interacts with retinoblastoma (Rb), and reduces E2F-Rb repressors and acceleration of proliferation [Wang and Timchenko, 2005]. Since PI3 kinase/Akt pathway was activated in our experiments, C/EBPα might be dephosphorylated by IGF-II, leading

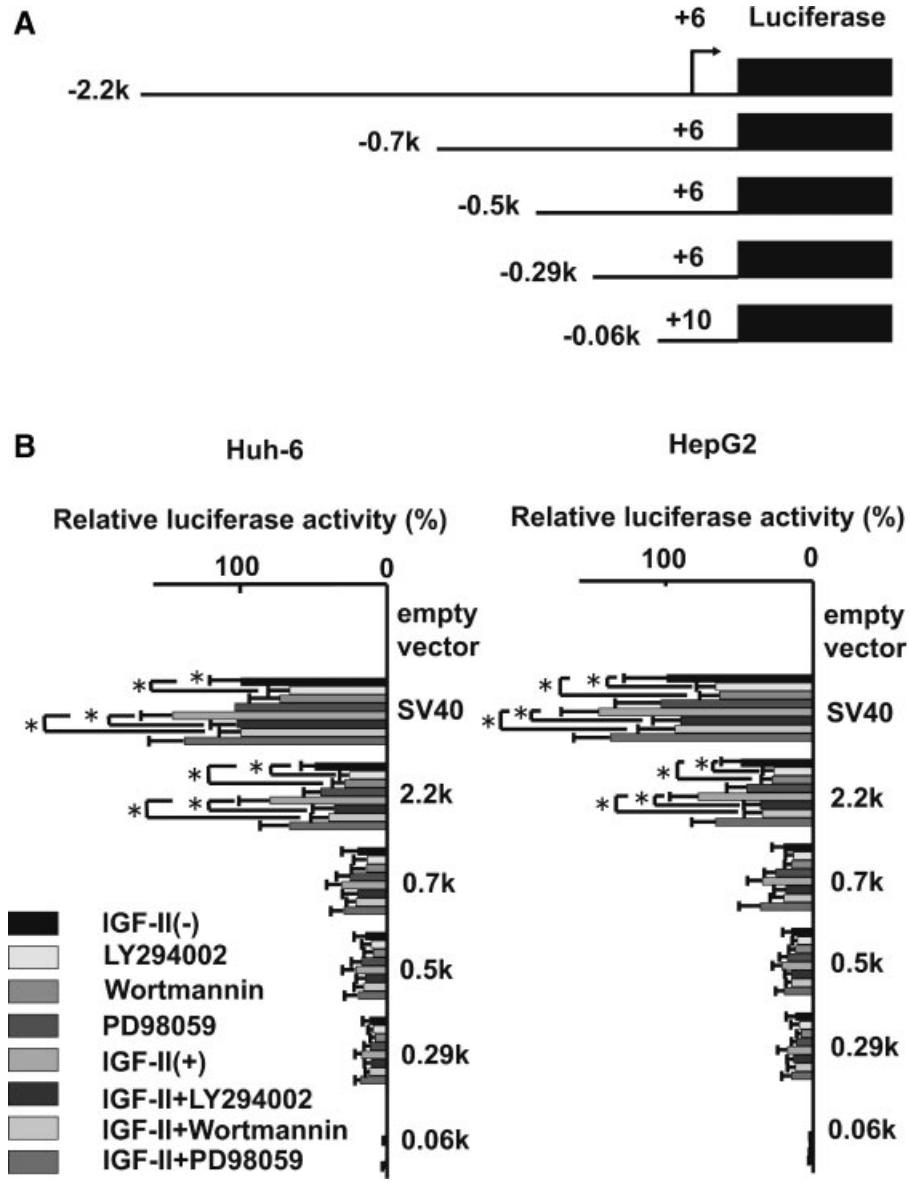

Fig. 6. LY294002 and wortmannin suppressed the transcriptional activity of C/EBPα promoter. 2.2- , 0.7- , 0.5- , 0.29- , and 0.06-kb lengths of the promoter regions of C/EBPα that were subcloned into reporter plasmids with luciferase gene (**A**). Huh-6 and HepG2 cells were transfected with 2 μg of each reporter plasmid as well as HSV-TK (**B**). The cells were stimulated with IGF-II with or without LY294002, wortmannin or PD98059. Relative luciferase activities were calculated as luciferase activity divided by that of pGL3 control without stimulation of IGF-II, LY294002, wortmannin or PD98059. It appeared that IGF-II stimulated activity of 2.2- and 0.7-kb fragments of C/EBPα promoter. LY294002 and wortmannin suppressed the relative luciferase activities of 2.2-kb length of C/EBPα promoter with or without the stimulation of IGF-II ($P < 0.05$). Empty vector: pGL3 basic; SV40: pGL3 control; n = 3; *, $P < 0.05$; error bars: standard deviation.

to proliferation. It is speculated that IGF-II promoted proliferation and differentiation for HBL cells consisting of immature hepatocytes. This is reasonable because immature hepatocytes proliferate to form liver lobule, differentiating into mature hepatocytes.

IGF-II binds IGF-IR, followed by activation of PI3 kinase or MAP kinase pathway for proliferation and differentiation [LeRoith and Roberts, 2003]. Our Western blot analysis clearly showed that Akt was phosphorylated with IGF-II stimulation, suggesting that PI3 kinase was activated by IGF-II. This is in accordance with our results that LY294002 and wortmannin abolished the expression of C/EBPα, suggesting that PI3 kinase regulates

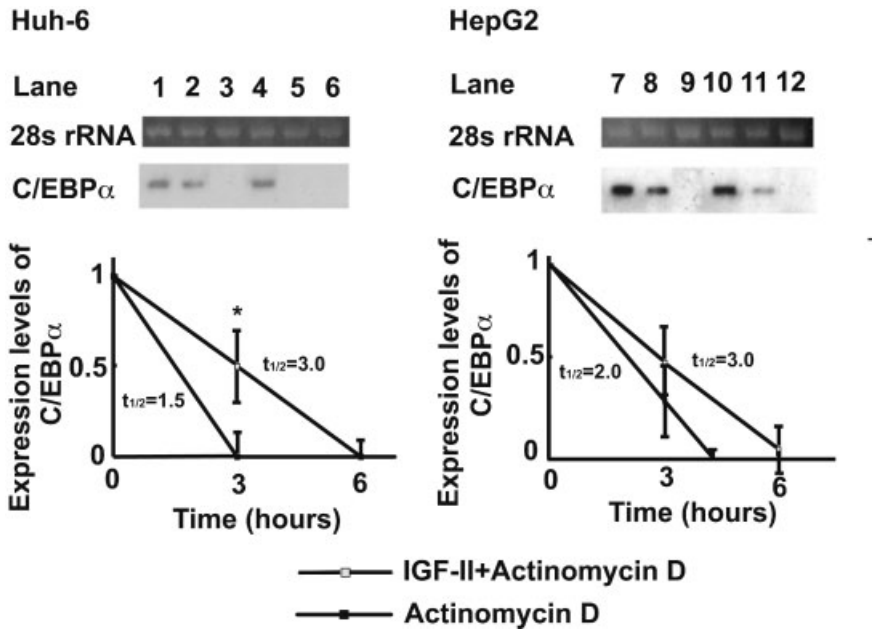

Fig. 7. IGF-II increased C/EBPα mRNA stability. RNA was isolated 0, 3, and 6 h after addition of Actinomycin D (1 μg/ml) with IGF-II (200 ng/ml) in Huh-6 (**lanes 1**, **2**, and **3**) and in HepG2 (**lanes 7**, **8**, and **9**). RNA was isolated 0, 3, and 6 h after addition of Actinomycin D (1μg/ml) from Huh-6 (**lanes 4**, **5**, and **6**), and 0, 3, and 4 h from HepG2 (**lanes 10**, **11**, and **12**). Our initial experiments showed that expression level of C/EBPα was 0 at 6 h in Actinomycin D treated HepG2 while that was 33% at 3 h. Expression of C/EBPα was investigated at 4 h in Actinomycin D treated HepG2. Half-life calculations are depicted as the time at which there was 50% C/EBPα mRNA remaining under the respective treatments. Half-life was prolonged in IGF-II treated both cell lines. $T_{1/2}$: half-life; $n = 3$; *, $P < 0.05$; error bars: standard deviation.

the expression of C/EBPα. These observations, taken together, confirm that PI3 kinase plays a major role in hepatocyte differentiation. This is supported by the fact that LY294002 significantly suppresses the differentiation of embryonic stem cells into cardiomyocytes [Klinz et al., 1999]. We propose that PI3 kinase plays a key role in the regulation of the activity of C/EBPα in hepatocytes.

Since Huh-6 and HepG2 produce IGF-I and IGF-II, and have IGF-IR, both growth factors may cross-talk at IGF-IR [Lund et al., 2004; Hopfner et al., 2006]. For example, liver of mice deficient in IGF-II develops normally, compensated by IGF-I [DeChiara et al., 1991]. LY294002 and wortmannin, therefore, might work on both IGF-II and IGF-I pathway since downstream from IGF-IR may be the same. In the present study, we focused on IGF-II since it is important for prenatal development while IGF-I is for pos-natal [DeChiara et al., 1991].

Once the signal pathway was unveiled, the next question was the promoter activity of C/EBPα. Our luciferase assay showed that IGF-II appeared to stimulate the promoter of C/EBPα. We, further, analyzed half-life of C/EBPα mRNA, to observe that it was prolonged in Huh-6 and HepG2 cells. It was hypothesized that IGF-II stimulated the promoter activity of C/EBPα as well as stabilized its mRNA. Our data clearly showed that both 42 and 30 kD of C/EBPα was upregulated, suggesting that they autorgulated the promoter activity of C/EBPα [Timchenko et al., 1995]. The promoter activity of C/EBPα was suppressed by LY294002 and wortmannin while PD98059 did not, suggesting that PI3 kinase mediated activation of the promoter of C/EBPα by IGF-II.

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