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C/EBPβ contributes to hepatocyte growth factor-induced replication of rodent hepatocytes

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***Background/Aims*: Hepatocyte replication can be induced in vivo by hepatocyte growth factor (HGF), which might be used for gene therapy or to promote liver regeneration. However, the biochemical steps critical for this process are not clear. C/EBPβ and C/EBPα are liver-enriched transcription factors that induce and inhibit hepatocyte replication, respectively. Because of their role in hepatocyte replication, this study examined the effect of HGF upon C/EBP proteins in vivo.**

***Methods*: Rats were treated with HGF, and the effect upon C/EBPs was evaluated in liver extracts. Normal or C/EBPβ-deficient mice were treated with HGF, and the effect upon hepatocyte replication was determined.**

***Results*: HGF had no effect in rat liver upon C/EBPα or C/EBPβ mRNA, nuclear protein, or nuclear DNA binding activity. However, HGF increased phosphorylated p90-RSK and ERK to18- and 3-fold normal, respectively. These kinases phosphorylate C/EBPβ and increase its transcriptional activity. The percentage of hepatocytes that replicated in C/EBPβ-deficient mice after HGF administration was only 1.1%, which was lower than the value of 6.6% for hepatocytes from HGF-treated normal mice ($P = 0.005$).**

***Conclusions*: C/EBPβ contributes to the induction of hepatocyte replication in response to HGF in rodents, which is likely due to post-translational modifications.**

***Keywords*: CCAAT-enhancer binding protein; Hepatocyte growth factor; Liver regeneration; Gene therapy**

1. Introduction

Induction of hepatocyte replication could promote gene therapy with vectors that only transduce dividing cells, or cause liver regeneration. Hepatocyte growth factor (HGF) is a heterodimeric 90 kD protein [1] synthesized by mesenchymal cells that binds to the c-Met receptor and induces replication of hepatocytes [2]. A cumulative dose of 10 mg/kg of HGF given IV as 8 doses over 24 h induced replication in 11% of rat hepatocytes [3], while 5 mg/kg/day induced replication of 20% of mouse hepatocytes [4]. Lower doses of HGF induced hepatocyte replication at lower levels [5–10].

Several signaling pathways are activated by HGF, which can also induce cell scattering and tube formation in some cells. These include p21-Ras and its downstream mediator extracellular signal-regulated kinase [ERK; also known as mitogen activated protein kinase (MAPK)], p90-ribosomal S6 kinase (p90-RSK), phosphatidylinositol 3 kinase (PI3K), phospholipase Cγ, $pp60^{c\text{-}src}$, β1-integrin, and the Wnt pathway [1, 11–15]. However, the pathway(s) necessary for inducing hepatocyte replication in vivo are unclear. We previously reported that HGF increased activating protein 1 (AP-1) DNA binding activity, a transcription factor that induces genes involved in replication, and increased phosphorylation of Elk-1 [3], a reaction catalyzed by ERK or related proteins. However, other signal transduction molecules may contribute to HGF-induced hepatocyte replication.

The CCAAT-enhancer binding protein (C/EBP) family plays an important role in liver regeneration, as some members inhibit, but others promote, hepatocyte

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replication [16–18]. C/EBP proteins form homo- and heterodimers, which bind to DNA and activate responsive genes. The major 42 kD isoform of C/EBPα (C/EBPα-42 kD) inhibits replication in hepatocytes [19–22]. Furthermore, C/EBPα nuclear protein levels and DNA binding activity decrease 2- to 3-fold after 70% partial hepatectomy (PH) [23–25]. The major 35 kD isoform of C/EBPβ (C/EBPβ-35 kD) which is also known as liver activator protein (LAP) may potentiate liver regeneration, as its nuclear levels increase 2- to 5-fold after 70% PH [23–25]. Furthermore, mice with homozygous C/EBPβ deficiency have a 50% decrease in the peak hepatocyte replication rate after 70% PH [26] and C/EBPβ is required for primary hepatocytes to replicate in response to transforming growth factor α (TGFα) [27]. C/EBP proteins might therefore play a role in HGF-induced hepatocyte replication. The transcriptional activity of C/EBPβ can be affected by phosphorylation by several kinases. These include p90-RSK [27], ERK [28–30], protein kinase A (PKA) [31–32], protein kinase C (PKC) [32–33], calcium-calmodulin-dependent protein kinase (CaMKII) [34], casein kinase II [35], and cyclin-dependent kinase 2 (Cdk2) [30]. Since HGF activates some of these kinases, post-translational modifications of C/EBPβ could contribute to HGF-induced hepatocyte replication in vivo. In this study, we demonstrate that C/EBPβ contributes to HGF-induced hepatocyte replication in vivo, and that this likely involves post-translational modifications.

2. Materials and methods

2.1. Reagents

Reagents were from Sigma Chemical (St. Louis, MO) unless otherwise noted. Antibodies that recognized C/EBP proteins regardless of their phosphorylation were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-C/EBPα antibody 14AA (1:300 dilution for immunoblot) recognizes an internal region, and the anti-C/EBPβ antibody C-19 (1:500 dilution) recognizes the C-terminus. An antibody that recognizes human C/EBPβ phosphorylated at T235 (#3084) or rat C/EBPβ phosphorylated at T189 (1:1000 dilution) was from Cell Signaling Technology, Inc. (Beverly, MA). Other antibodies were from New England Biolabs, Inc (Beverly, MA). Anti-phospho-p90-RSK antibody #9344 (1:1000 dilution) recognizes p90-RSK1 phosphorylated at T360 and S364, or p90-RSK3 phosphorylated at homologous positions [36]. Anti-phospho-ERK antibody #9101 (1:1000 dilution) recognizes ERK1 or ERK2 phosphorylated at T202 and Y204. Anti-phospho-p38-MAPK antibody #9211 (1:1000 dilution) recognizes p38-MAPK phosphorylated at T180 and Y182. Anti-phospho-PKC antibody #9371 (1:1000 dilution) recognizes PKCα, βI, βII, and δ isoforms phosphorylated at the C-terminus corresponding to S660 of human PKCβII [37]. Anti-phospho-CREB antibody #9191 (1:1000 dilution) recognizes CREB phosphorylated at S133. Anti-CREB antibody #9192 (1:1000 dilution) recognizes CREB regardless of its phosphorylation. Recombinant human HGF was purified as previously described [3].

2.2. Animal procedures

Animals received humane care and the protocols complied with institutional guidelines. Male Sprague–Dawley (Harlan Sprague Dawley, Indianapolis IN) rats were treated with HGF as described [3]. The offspring of heterozygous C/EBPβ-deficient mice [38] were genotyped by PCR of tail DNA with mouse C/EBPβ primers (5′-AGAAGACGGTGGACAAGCT-3′ and 5′-GGGCAGCTGCTTGAA CAAGTT-3′), and a neomycin primer (5′-CTTGTTCAATGGCCGA TCCCA-3′) to generate a 215 nt C/EBPβ or a 350 nt 'knock-out' product. Homozygous normal or C/EBPβ-deficient mice from the same colony were injected intraperitoneal (IP) with phosphate buffered saline containing 0.27 mg/ml of HGF, 0.5 mg/ml dextran sulfate, and 1 mg/ml of bovine serum albumin (BSA) at 6.5 weeks after birth as detailed below. 5-bromo-2′-deoxyuridine (BrdU) was injected at 100 mg/kg/dose and immunostaining for BrdU-labeled cells was performed [3].

2.3. Northern blot analysis

RNA was prepared from rat livers using guanidinium [39] and Northern blot was performed using total RNA and randomly labeled probes for rat C/EBPα [40], rat C/EBPβ [41], rat C/EBPδ [41], and human 28S rRNA [42].

2.4. Immunoblot analysis

Fifteen micrograms of liver nuclear extracts [3] was electrophoresed on SDS-PAGE, and transferred to a Hybond ECL nitrocellulose membrane (Amersham, Cleveland, OH). For the Santa Cruz anti-C/EBP antibodies, the blocking solution was TBS-T (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) with 5% fat-free Carnation Dry Milk (TBS-milk). For other antibodies, the blocking solution was TBS-T with 5% BSA (TBS-BSA). Membranes were blocked for 1.5 h at RT, and incubated with antibody in blocking buffer overnight at 4 °C. Membranes were incubated with a horseradish peroxidase (HRP)-conjugated donkey-anti-rabbit IgG (Amersham Pharmacia Biotech, Inc., Piscataway NJ) and bands detected by chemiluminescence using Lumi-light Western Blotting Substrate (Boehringer Mannhem Corporation, Indianapolis IN). Protein levels were quantified on a densitometer.

2.5. Electrophoretic mobility shift assay (EMSA)

The top strand of the end-labeled double-stranded oligonucleotide albumin C/EBP probe was TCGACTGATTTTGTAATGGGGTCGA [43]. EMSA was performed with 2 μg of liver nuclear extract and 0.5 ng (10,000 cpm) of probe as described [3]. For supershift analyses, 1 μl of antibody was incubated with extract for 30 min at 4 °C, then the probe was added and samples incubated for 30 min at RT. Bands were quantified with a phosphorimager, and activity was normalized to the binding activity in samples from normal rats after subtraction of the signal present in a sample that did not receive protein.

2.6. MAPK activity assay

MAPK activity was measured as described in detail previously [3] using Elk1-GST (New England Biolabs) as a substrate, and determination of the amounts of radiolabeled Elk1 after incubation with [γ-^{32}P]-ATP and nuclear extract protein and electrophoresis on 8% SDS-PAGE.

2.7. Phospho-p90-RSK immunostaining

Frozen liver sections were fixed in 3% paraformaldehyde for 2 min at 4 °C. Sections were incubated overnight at 4 °C with a 1:6000 dilution of anti-phospho-p90-RSK antibody in TBS-BSA. Samples were incubated with an HRP-coupled donkey anti-rabbit IgG and developed with 3, 3′-diaminobenzidine as described previously [3].

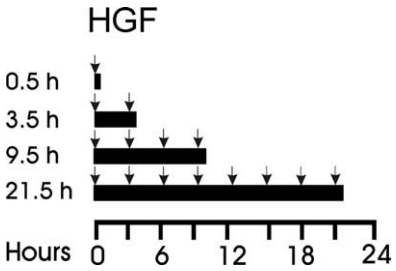

Fig. 1. Schematic diagram of the injection regimen for HGF into rats. Rats were injected IV with up to 8 doses of 1.25 mg/kg/dose of HGF every 3 h, as indicated by the vertical arrows. Livers were harvested 30 min after the preceding dose of HGF, as indicated by the time at which the horizontal bar ends. The collection time is indicated at the left in hours and refers to the time after the first dose of HGF.

3. Results

3.1. C/EBP RNA levels, nuclear protein levels, and nuclear DNA binding activity

The regimen for administration of HGF to rats was 1.25 mg/kg/dose every 3 h for up to 21 h (Fig. 1). This induced replication of 11% of hepatocytes at 24–33 h after the first dose of HGF [3]. Livers were collected at 0.5, 3.5, 9.5, or 21.5 h, which is 30 min after a preceding dose of HGF, and includes the time when the decision to replicate was made. HGF had no statistically significant effect upon mRNA levels for C/EBPα or C/EBPβ at any of these times (Fig. 2A), although there was a modest increase in mRNA levels for C/EBPβ mRNA levels at 0.5 h, and significant differences might have been found if more animals were evaluated. The effect of HGF upon nuclear C/EBP protein levels was examined next. The major C/EBPα proteins are the full-length C/EBPα-42 kD with transcriptional activity,

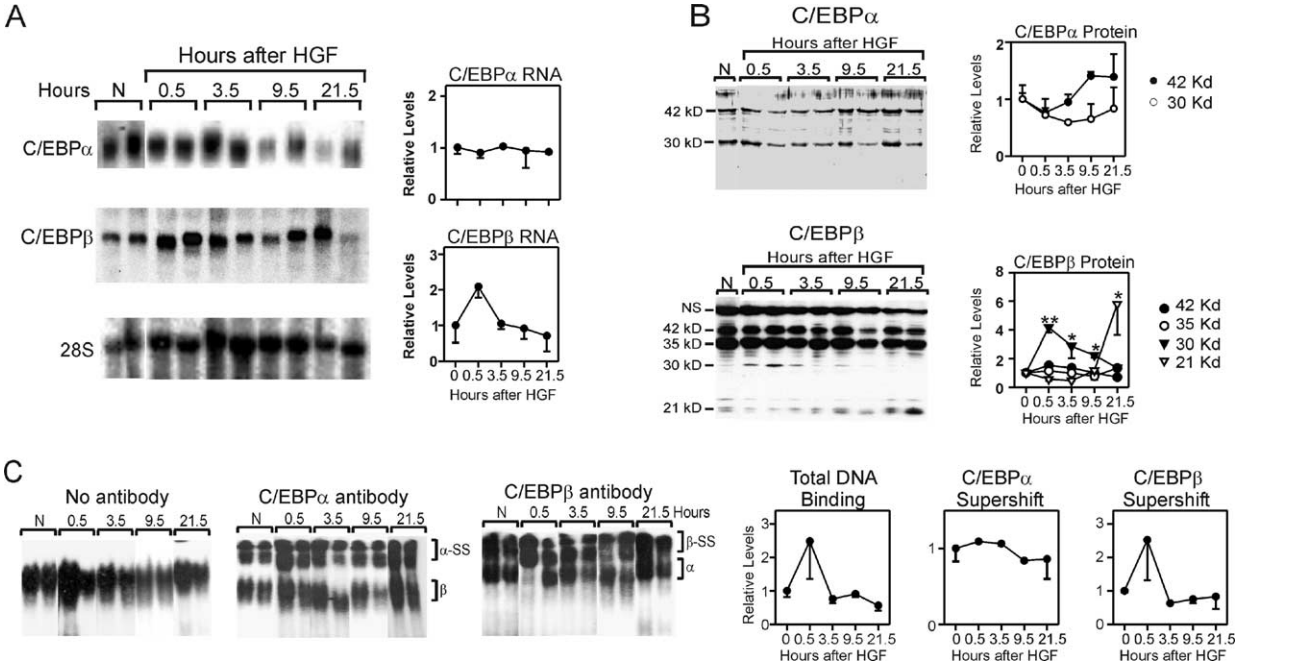

Fig. 2. Analysis of C/EBP RNA, nuclear protein, and nuclear DNA binding activity in rats after HGF. Samples were from normal untreated rats (N), or rats sacrificed at the indicated time in hours after the first dose of HGF, as diagramed in Fig. 1. Values in HGF-treated rats were compared with those in normal rats using the Student's *t*-test. Duplicates represent samples from different rats, and samples from two additional animals whose data are not shown here were analyzed at each time point. *indicates a *P*-value of 0.005–0.05, **indicates a *P*-value of 0.0005–0.005, and ***indicates a *P*-value <0.0005 when values in HGF-treated rats were compared with those in normal rats in this and subsequent figures. (A) RNA levels. Total liver RNA was used for northern blot. The cDNA probe is indicated at the left. The signal for the C/EBP RNA was normalized to the 28S rRNA signal obtained after reprobing the same blot, and the average signal relative to normal rats ± standard error of the mean (SEM) is shown. There were no significant differences. (B) Evaluation of C/EBP protein levels. Liver nuclear extract proteins were electrophoresed on 12% SDS-PAGE and immunoblot performed. The position of C/EBPα and C/EBPβ proteins of the indicated size in kD is indicated on the left of the top and bottom panels, respectively. A non-specific (NS) band at 60 kD is indicated. The levels of each isoform were quantified with a phosphorimager, and plotted as values relative to levels for the same isoform in normal rats. (C) Electrophoretic mobility shift assay (EMSA) for C/EBP proteins. EMSA was performed using liver nuclear extracts and a radiolabelled C/EBP probe without any antibody (left panel) or after supershift with an anti-C/EBPα (middle panel) or anti-C/EBPβ (right panel) antibodies. The approximate position of C/EBPα (α) and C/EBPβ (β) complexes, as well as the positions of the more slowly migrating supershifted complexes that appear after incubation with anti-C/EBPα (α-SS) or anti-C/EBPβ (β-SS) antibodies, are indicated. Protein: DNA complexes were quantitated with a phosphorimager, and the activity relative to that in normal liver was determined. None of the values were statistically different from normal.

and C/EBPα-30 kD, which binds DNA but lacks the N-terminal transcriptional activation domain [44]. There were no changes in the nuclear levels of any C/EBPα isoforms in response to HGF (Fig. 2B). Coumassie staining demonstrated that all samples had similar levels of protein (data not shown). The major C/EBPβ isoform is the transcriptionally active C/EBPβ-35 kD. C/EBPβ-21 kD is referred to as liver inhibitory protein (LIP) because it lacks the N-terminal transcriptional activation domain. In addition, a protein of 30 kD that lacks the N-terminus is induced by lipopolysaccharide [45]. Finally, C/EBPβ-42 kD [26] probably initiates translation at a position upstream from that used for C/EBPβ-35 kD [46]. HGF had no significant effect upon levels of C/EBPβ-42 kD or C/EBPβ-35 kD (Fig. 2B), but increased C/EBPβ-21 kD to 6-fold normal at 21.5 h ($P=0.02$ *vs.* levels in normal liver), and increased C/EBPβ-30 kD to 4- to 2-fold normal at 0.5–9.5 h ($P<0.03$). However, these isoforms still represented a small fraction of total C/EBPβ.

EMSA was performed using liver nuclear extracts and a C/EBP binding site. For rats that received HGF, the total C/EBP DNA binding activity, and the amount of complex that was supershifted with C/EBPβ antibodies, were 2.5 ± 1.1 and 2.5 ± 1.2-fold, respectively, that of normal at 0.5 h (Fig. 2C). Although neither of these values were statistically significant from those in normal rats, significant differences might have been found if more animals were analyzed. DNA binding activity was similar to normal at other times.

3.2. Effect of HGF upon kinases

The effect of HGF upon kinases that affect C/EBPβ activity was evaluated using immunoblot with antibodies specific for phosphorylation at a position that increases kinase activity. Controls were from rats that did not receive any injection, or PBS-treated rats. Fig. 3 shows activities in samples that were harvested at 20 min or earlier after one dose of HGF. HGF increased phosphorylated p90-RSK (Fig. 3A) and ERK (Fig. 3B), which peaked at 18- and 3-fold, respectively, levels in normal liver. These values were statistically higher than in livers from PBS-treated rats. In contrast, both HGF and PBS caused a modest reduction in phosphorylated p38-MAPK at some times (Fig. 3C). The increase in phosphorylated ERK in response to HGF was consistent with the 40% increase in phosphorylation of Elk-1 (Fig. 3D), a protein that is phosphorylated by ERK or p38-MAPK. HGF increased phosphorylated CREB in liver at 5 min over that observed with PBS, but no differences were detected at 10 or 20 min (Fig. 3E). Since CREB is efficiently phosphorylated by PKA [47] or CaMKII [48] this suggests that PKA or CaMKII may have been transiently activated by HGF. Levels of phosphorylated PKC (Fig. 3F) increased to 5- to 6-fold that of normal for rats that received HGF, but this may have been due to the stress of performing an IV injection, as these values were not statistically different from those observed in PBS-treated animals.

To assess if activation of ERK resulted in phosporylation of C/EBPβ, immunoblot was performed with an antibody that recognizes rat C/EBPβ phosphorylated at T189, a site of ERK phosphorylation (Fig. 3G). HGF resulted in levels of phosphorylated T189 that were 3.4-fold that in normal rats at 5 min, which was statistically higher than the value of 1.7-fold normal found in PBS-treated rats. These data are consistent with the hypothesis that ERK activation results in phosphorylation of C/EBPβ in rat liver.

Levels of phosphorylated kinases were also measured at 3.5 and 9.5 h after initiation of HGF, which represented samples obtained 30 min after the 2nd and 4th doses of HGF, respectively. HGF increased phosphorylated p90-RSK to levels that were $\geq$5.7-fold normal (Fig. 4A) and phosphorylated ERK to levels that were $\geq$1.5-fold normal (Fig. 4B) at 3.5 and 9.5 h. HGF decreased phosphorylated p38 MAPK levels to $\leq$51% of normal. This suggests that the response to HGF was not abrogated after repeated doses, although the magnitude was somewhat lower than after the first dose of HGF.

3.3. Immunostaining of liver for p90-RSK

Immunostaining was used to identify the cell types that contained phosphorylated p90-RSK, as it was possible that the increased phosphorylation of p90-RSK was due to HGF-induced changes in non-parenchymal cells (NPC). PBS-treated rats had little detectable phosphorylated p90-RSK in hepatocytes (Fig. 5A). In contrast, there were high levels of phosphorylated p90-RSK in the nuclei of hepatocytes at 10 min after HGF (Fig. 5B). We conclude that the increase in p90-RSK activity in liver nuclear extracts was primarily due to increased activity in hepatocytes.

3.4. C/EBPβ contributes to HGF-induced hepatocyte replication in mice

The importance of C/EBPβ in HGF-induced hepatocyte replication was examined in C/EBPβ-deficient mice using the protocol shown in Fig. 6A. Fig. 6B shows the average levels of replication in hepatocytes and NPC after administration of HGF, while representative examples of BrdU labeling are shown in Figs. 6C–J. Although HGF induced replication of 6.6 ± 1.7% of hepatocytes from normal mice, only 1.1 ± 0.3% of hepatocytes replicated in C/EBPβ-deficient mice after administration of HGF ($P=0.005$). However, some replication occurred in C/EBPβ-deficient mice in response to HGF, as hepatocyte replication was statistically higher than in C/EBPβ-deficient mice that received PBS (0.2 ± 0.02%; $P=0.01$). The percentage of replicating NPC was not statistically different between the groups. We conclude that C/EBPβ contributes to the effect of HGF upon hepatocyte replication, but is not necessary for replication to occur.

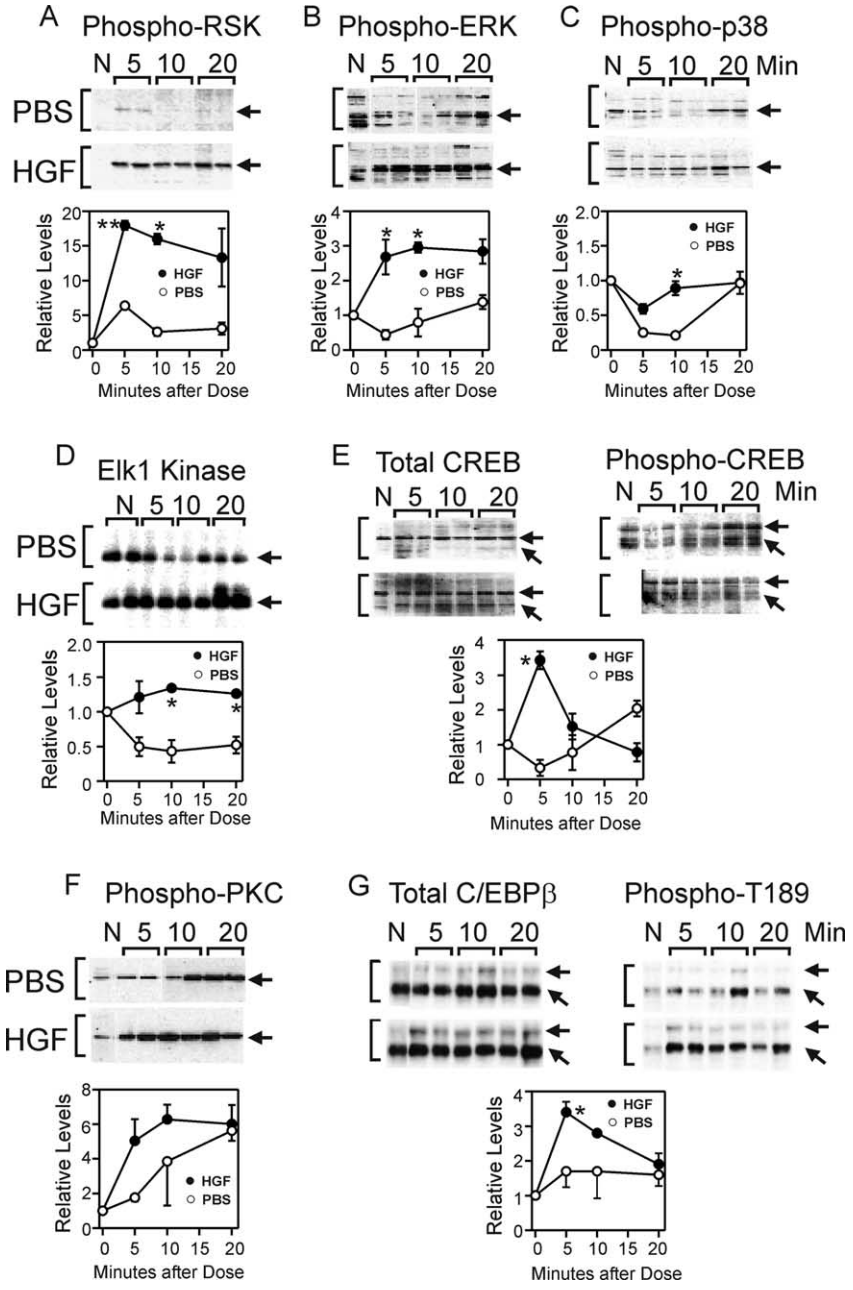

Fig. 3. Time course of activation of signal transduction proteins after HGF. Rats were treated with a single dose of 1.25 mg/kg of HGF (HGF), or with PBS (PBS), as indicated at the left. Livers were harvested 5–20 min later, as indicated above the lanes. Samples from normal rats that did not receive any injection are indicated (N). For all panels, the activity was normalized to that in normal liver, and plotted as the average ± SEM. Statistical comparisons were between samples from HGF-treated and PBS-treated rats at the same time after injection. (A) Phospho-p90-RSK. Immunoblot was performed with 15 μg of nuclear extract protein, 7.5% SDS-PAGE, and the anti-phospho-p90-RSK antibody. (B) Phospho-ERK. Immunoblot was performed on 10% SDS-PAGE with an antibody that recognizes the 48 kD phosphorylated ERK1 (arrow) and the 50 kD phosphorylated ERK2. Levels of both proteins were quantified together. (C) Phospho-p38 MAPK. Immunoblot was performed on 10% SDS-PAGE with an antibody that recognizes phosphorylated p38-MAPK. (D) Elk-1 kinase activity. The ability of extracts to phosphorylate GST-Elk 1 was determined as described in the methods section. The arrow indicates the 39 kD phosphorylated GST-Elk1. (E) Phosphorylated CREB. Immunoblot was performed on 10% SDS-PAGE with an antibody that recognizes CREB regardless of its phosphorylation state (left; Total CREB) or an antibody that recognizes phosphorylated CREB (right, Phospho-CREB). The horizontal arrow identifies the 50 kD CREB, while the slanted arrow indicates the 43 kD ATF-1. The ratio of phospho-CREB to total CREB was determined, and the amount of phospho-CREB relative to that found in normal rats calculated. (F) Phospho-PKC. Immunoblot was performed on 10% SDS-PAGE with an antibody that recognizes the 85 kD phosphorylated PKC. (G) Phospho-T189 C/EBPβ. Immunoblot was performed on 12% SDS-PAGE with an antibody that recognizes C/EBPβ regardless of its phosphorylation state (left, Total C/EBPβ) or an antibody that recognizes C/EBPβ that is phosphorylated at T189 (right, Phospho-T189). The horizontal and slanted arrows identify C/EBPβ-42 and -35 kD, respectively. The ratio of the phospho-C/EBPβ−35kD to the total C/EBPβ−35kD was determined, and the amount of phospho-C/EBPβ−35kD relative to that found in normal rats calculated.

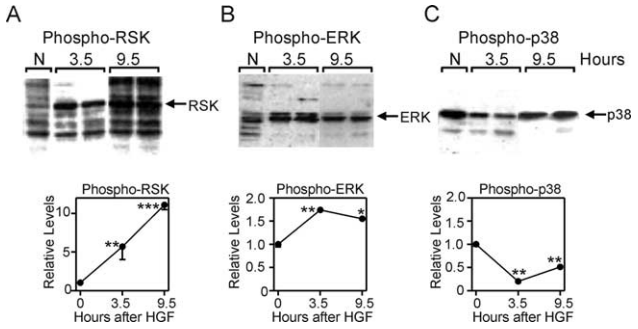

Fig. 4. Phosphorylation of signaling transduction proteins at 3.5 and 9.5 h after initiation of HGF. Immunoblot was performed on nuclear extracts that were prepared from rat livers that were harvested at 3.5 or 9.5 h after initiation of treatment with HGF. Phosphorylated proteins are identified as in Fig. 3, and statistical comparisons were between samples from HGF-treated and normal rats. (A) Phospho-p90-RSK. (B) Phospho-ERK. (C) Phospho-p38-MAPK.

4. Discussion

4.1. Post-translational changes in C/EBPβ may contribute to HGF-induced hepatocyte replication

This study evaluated the effect of HGF upon C/EBP proteins in livers of rodents, as these proteins play an important role in hepatocyte replication. The doses of HGF used were biologically relevant, as they induced replication of hepatocytes in rats [3] and mice (Fig. 6). HGF had no statistically significant effect upon RNA levels for C/EBPα or C/EBPβ (Fig. 2A), or C/EBPδ (data not shown), or upon nuclear levels of the major C/EBPα or C/EBPβ isoforms, although small differences might have been missed due to the small number of animals evaluated. HGF resulted in a 6-fold increase in C/EBPβ-21 kD at 21.5 h, a protein that is more effective at inducing hepatocyte replication than is

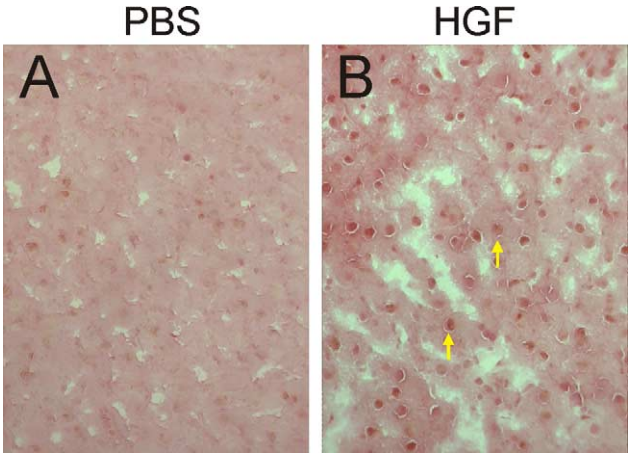

Fig. 5. Immunostaining of liver for phospho-RSK. Rats were injected with PBS or 1.25 mg/kg of HGF, and livers harvested 10 min later. Immunostaining was performed with an anti-phospho-p90-RSK antibody, and sections were counterstained with eosin. Arrows identify hepatocyte nuclei with phospho-p90-RSK. [This figure appears in colour on the web.]

C/EBPβ-35 kD after overexpression from an adenoviral vector in liver [49]. HGF also resulted in C/EBPβ-30 kD levels that were 2- to 4-fold normal at 0.5–9.5 h. An increase in these inhibitory isoforms might decrease the overall activity of C/EBPβ, although both remained a small fraction of total C/EBPβ. The increase in these isoforms could be due to post-translational cleavage [46] or differential use of translation initiation codons [50–51]. Levels of C/EBPδ and GADD153 (a protein that can heterodimerize with C/EBP proteins and inhibit their transcriptional activity) remained undetectable after administration of HGF (data not shown). Our finding that HGF had no statistically significant effect on the levels of C/EBP DNA binding activity differs from results in hepatoma cells, where HGF induced nuclear translocation of C/EBPβ that was dependent on PI3K and increased DNA binding activity [14]. The higher baseline nuclear C/EBPβ levels in rat hepatocytes in vivo than in the serum-starved hepatoma cells, or the small number of animals analyzed here, may make it difficult to observe a statistically significant increase in nuclear C/EBPβ in response to HGF.

Although HGF did not have a major effect on C/EBP RNA, nuclear protein levels, or nuclear DNA binding activity, it probably increased the transcriptional activity of rat C/EBPβ by inducing its phosphorylation. In this study, HGF increased activated ERK to levels that were up to 3-fold normal, and increased phospho-T189 C/EBPβ (a site phosphorylated by ERK) to 3.4-fold normal. Transfection of constitutively activated p21-Ras (which activates ERK) increased the transcriptional activity of human C/EBPβ by $>$5-fold by phosphorylation of the homologous position [28], while ERK phosphorylated the homologous position of chicken C/EBPβ [29]. Phosphorylation of rat C/EBPβ at T189 can also be performed by cyclin-dependent kinase 2 and is important for malignant transformation [30], which may involve activation of the *c-fos* promoter [52–53].

HGF also increased levels of activated p90-RSK to up to 18-fold that in normal rats. Phosphorylation of rat C/EBPβ at S105 is necessary for TGFα to induce replication in primary hepatocytes, and p90-RSK is pivotal in this process, as a dominant-negative p90-RSK mutant prevented TGFα-induced hepatocyte replication [27]. Thus, activation of p90-RSK likely increased the activity of C/EBPβ in rats that were treated with HGF, and contributed to hepatocyte replication. PKA can also phosphorylate rat C/EBPβ at S105 [31–32] and PKA activity may have been transiently increased, as phosphorylation of one of its major substrates (CREB) was increased at 5 min in this study (Fig. 3E). In this study, we did not directly demonstrate that HGF induced phosphorylation of C/EBPβ at S105 due to the lack of reagents that efficiently recognized this phosphorylated form (data not shown). However, the in vitro data demonstrating the role of p90-RSK in activating C/EBPβ transcriptional activity make it very likely that physiologically important phosphorylation occurred. Activation of ERK and p90-RSK may be related, as ERK can

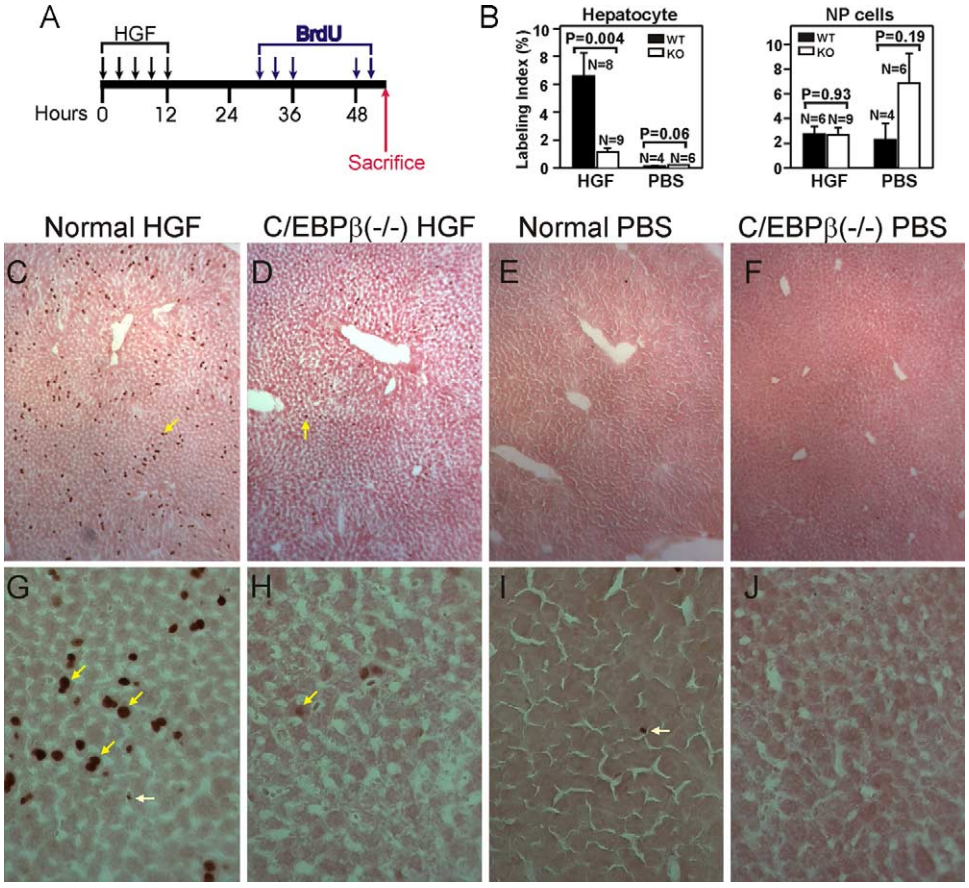

Fig. 6. Effect of HGF upon hepatocyte replication in normal and C/EBPβ-deficient mice. (A) Regimen for injection of HGF and BrdU labeling. Mice were injected with 5 doses of 5 mg/kg/dose of HGF given every 3 h beginning at 0 h, as indicated by black arrows. Control normal and C/EBPβ-deficient mice received PBS instead of HGF at the same times. BrdU was injected at 30, 33, 36, 48, and 51 h after the start of the protocol, as indicated by blue arrows. Animals were sacrificed at 52 h (red arrow). (B) Quantification of the labeling index in hepatocytes and non-parenchymal cells (NPC) of normal and C/EBPβ-deficient mice after injection of HGF or PBS. Normal (WT) or C/EBP-β-deficient (KO) mice were injected with HGF or PBS followed by BrdU according to the regimen in panel A. The percentage of replicating hepatocytes or non-parenchymal cells (NPC) was determined by immunostaining for BrdU-labeled nuclei, and average values ± SEM for the indicated number of mice determined. (C–J). Examples of BrdU immunostaining. Immunostaining of liver sections for BrdU-labeled cells was performed, and sections were counterstained with eosin for livers from HGF-treated normal (panels C and G), HGF-treated C/EBPβ-deficient (panels D and H), PBS-treated normal (panels E and I), and PBS-treated C/EBPβ-deficient (panels F and J) mice. Panels (C–F) and (G–J) were taken at 10× and 40× original magnification, respectively. Yellow and white arrows identify BrdU-labeled hepatocytes and NPC, respectively. (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article.)

phosphorylate p90-RSK [36] which is sufficient to increase p90-RSK kinase activity [54]. Activation of ERK and p90-RSK by HGF may also result in phosphorylation of other transcription factors that contribute to replication, such as Elk-1 and c-Fos [55–56].

4.2. C/EBPβ contributes to HGF-induced replication in vivo

The most compelling data that C/EBPβ is critical for HGF-induced hepatocyte replication is the fact that replication after administration of HGF to homozygous C/EBPβ-deficient mice was only 17% of that in normal mice after HGF. However, some replication still occurred, as HGF increased replication in C/EBPβ-deficient mice to 5.5-fold that in C/EBPβ-deficient mice that received PBS. This demonstrates that C/EBPβ contributes, but is not essential for, HGF-induced hepatocyte replication. This is in contrast to the requirement for C/EBPβ for replication in TGFα-treated primary hepatocytes [27]. This difference may be due to activation of other signal transduction pathways by HGF but not TGFα. Alternatively, replication in C/EBPβ-deficient hepatocytes after TGFα may be delayed, rather than abrogated. Induction of some replication with HGF in C/EBPβ-deficient mice is consistent with the fact that hepatocyte replication still occurs, albeit at reduced levels, after 70% PH in C/EBPβ-deficient mice [26]. These alternative pathways that induce replication in HGF-treated rodents could involve activation of AP-1, as reported previously [3]. We are also investigating if other signal transduction pathways that have not yet been

evaluated, such as PI3K, are induced in vivo by HGF in rodent hepatocytes.

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