Biochemical and Biophysical Research Communications 435 (2013) 120–125

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Up-regulation of cyclin-E_1 via proline-mTOR pathway is responsible for HGF-mediated G_1/S progression in the primary culture of rat hepatocytes

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ARTICLE INFO

Article history:
Received 29 March 2013
Available online 22 April 2013

Keywords:
Hepatocyte growth factor
Proline
Collagen synthesis
Mammalian target of rapamycin
Cyclin-E_1
Hepatocytes

ABSTRACT

Hepatocyte growth factor (HGF) is a key ligand that elicits G_1/S progression of epithelial cells, including hepatocytes. Proline is also required for DNA synthesis that is induced by growth factors in primary culture of hepatocytes. However, it remains unknown how proline contributes to the G_1/S progression of hepatocytes. The primary culture of rat hepatocytes using HGF plus proline can be a conceptual model for elucidating the molecular linkage of amino acids and growth factors during G_1/S progression. Using this *in vitro* model, we provide evidence that not only induction of cyclin-D_1 by HGF but also up-regulation of cyclin-E_1 by proline is required for hepatocytes to enter the S-phase. Proline-enhanced cyclin-E_1 induction, without changing its mRNA level, is associated with the activation of mammalian target of rapamycin (mTOR)-dependent pathways. Indeed, proline enhanced the ribosomal protein S6 phosphorylations (i.e., mTOR target), concomitantly with an increase in cyclin-E_1. Inversely, mTOR-inhibitor, rapamycin suppressed the proline-mediated induction of cyclin-E_1. As a result, DNA synthesis of hepatocytes, which was induced by HGF in the presence of proline, was largely abolished by mTOR-inhibitor treatment. Such a co-mitogenic effect of proline was also dependent on collagen synthesis: collagen synthesis inhibitors, such as *cis*-OH-proline, diminished the proline-induced cyclin-E_1, and then the G_1/S progression of hepatocytes was also suppressed. Overall, proline-mediated mTOR activation and collagen synthesis were found critical for HGF-induced DNA synthesis, partly via the sufficient accumulation of cyclin-E_1. This is the first report to demonstrate the molecular bridge between amino acids and growth factors that drive mitogenic outcomes.

1. Introduction

The liver has little regenerative capacity under a healthy condition, but once injured, hepatocyte proliferation occurs especially in hepatitis, cirrhosis as well as other conditions. Thus, liver regeneration is recognized as a spectacular example of controlled tissue growth. For example, most hepatocytes enter the cell cycle (i.e., G_0/G_1 priming and G_1/S progression) soon after 70%-hepatectomy [1]. Growth regulation of hepatocytes is also reproduced *in vitro* models [2]. In a primary culture, hepatocytes isolated from the normal liver maintain in the quiescent state of the cell cycle, but begin to proliferate in response to mitogenic factors, such as epidermal growth factor (EGF) [3] or hepatocyte growth factor (HGF) [4,5]. Among growth factors, HGF is the most potent mitogen, as its name indicates, for mature hepatocytes.

HGF was discovered from serum of 70%-hepatectomized rats [4]. Over the past 30 years, we and other groups have accumulated evidence that HGF is a hepatotorophic factor that induces liver regeneration under various pathological conditions [6–8] via stimulating the G_1/S progression of hepatocytes. HGF exerts a variety of biological activities through c-Met/HGF receptor [9,10]. Importantly, hepatocyte G_1/S progression, caused by 70%-hepatectomy, was diminished in mice, where hepatecyte-specific *c-met* deletion was achieved via the Cre-LoxP system [11], convincing that HGF is required for liver regeneration as a G_1/S progression factor. With regard to this, HGF is known to enhance cyclin-D1 expression (early G1 cyclin), possibly via the rapid activation of β-catenin, a key transcriptional factor for G_1/S progression [12,13].

In addition to growth factors, amino acids are required for the enhancement of cell cycle entry in hepatocytes. We found in 1984 that proline is essential for EGF-induced DNA synthesis in the primary culture of rat hepatocytes [14]. Indeed, the mitogenic effect of EGF was very poor in proline-deficient medium, such as DMEM, but the addition of proline reversed the lowered rate of DNA synthesis [14]. Proline is required not only for G_1/S progression [14,15] but also for metabolic reactions in cultured hepatocytes [16,17]. Proline is one of the known components of collagen triple helix, and a reduction in collagen synthesis leads

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.04.052

to a reduction in EGF-induced DNA synthesis [14], suggesting a role(s) for collagen in G_1/S progression. In addition, proline enhances the mammalian target of rapamycin (mTOR) system [18], which may be linked to cellular growth and proliferation, as discussed later.

HGF is a key regulator for G_1/S progression, and proline is also essential in this process. Thus, proline plus HGF-combined primary culture system could be a conceptual model for elucidating the crosstalk between amino acids and growth factors. However, it remains unknown how proline contributes to the mitogenic actions of growth factors. Using this model, we provide evidence that not only HGF-induced cyclin-D_1 but also proline-increased cyclin-E_1 (i.e., late G_1 cyclin) is required for the induction of G_1/S progression. Of note, the accumulation of cyclin-E_1 by proline depends on both the mTOR pathway and collagen *de novo* synthesis. We will discuss the possible cascade that is related to these molecular events.

2. Materials and methods

2.1. Materials

Recombinant human HGF was purified from the medium of CHO cells transfected with human HGF cDNA [5,6]. Recombinant EGF was purchased from Invitrogen (Carlsbad, CA). Proline, leucine and 2,2'-bipyridine were purchased from Nacalai (Kyoto, Japan). Glycine and cis-4-hydroxy-proline (*cis*-OH-prorine) were obtained from Sigma (St. Louis, MO). Rapamycin and LY294002 were from Cell Signaling Technology (Beverly, MA). The following reagents were used as the primary antibodies: anti-c-Met, anti-phosphotyrosine (pY99), anti-cyclin-D_1, anti-cyclin-E_1, anti-cdk2, anti-cdk4 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Erk, anti-GAPDH (Millipore, Billerica, MA), anti-phospho-ERK (T202/Y204), anti-phospho-Akt (Thr308), anti-Akt, anti-phospho-S6 (Ser235/236), anti-phospho-S6 (Ser240/244) (Cell Signaling Technology), and anti-β-actin (Sigma).

2.2. Isolation of rat hepatocytes

Male Sprague–Dawley (SD) rats were purchased from Japan SLC (Shizuoka, Japan). Hepatocytes were prepared from 8–15 week old SD rats by *in situ* perfusion of the liver with collagenase (Wako, Osaka, Japan), as reported [14,19]. The cells were cultured on plastic dishes precoated with Cellmatrix type I-A collagen (Nitta gelatin, Osaka, Japan) at a cell density of $3 \times 10^4/cm^2$ in DMEM (Nacalai) supplemented with 5% fetal bovine serum. After 3–4 h incubation, the medium was replaced with the serum-free DMEM.

2.3. [^{3}H]-thymidine incorporation assay

HGF or EGF was added to cultures of hepatocytes with or without proline, the culture was sustained for 20 h, and then pulse-labeled with 2.5 μCi/ml of [^{3}H]-thymidine for 8 h. The cells were washed twice with cold PBS and immersed in 10% trichloroacetic acid. After solubilization in 1 N NaOH, radioactivity of [^{3}H]-thymidine incorporated into DNA was measured using a β-counter [14,19].

2.4. Inhibitor treatments

The isolated hepatocytes were incubated in serum-free medium overnight. To determine the involvement of downstream signaling, rapamycin (30 nM) and LY294002 (5 μM) were added as mTOR- and PI3K-inhibitor, respectively, at the same time as HGF and/or proline treatments. To prohibit collagen synthesis, chemical inhibitors, such as *cis*-OH-prorine and 2,2'-bipyridine, were added to the medium soon after the treatments with proline at different concentrations.

2.5. Immunoprecipitation and Western blotting

Hepatocytes were washed twice with PBS and lysed with lysis buffer [50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 50 mM NaF, 0.5 mM PMSF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, and protease inhibitor cocktail (complete, Roche Applied Science, Indianapolis, IN)]. The lysates were pre-adsorbed with protein G-Sepharose (GE Healthcare, Little Chalfont, UK) and then incubated with antibody overnight at 4 °C. The beads were washed and dissolved in SDS–PAGE sample buffer. Cell lysates or immunoprecipitants were resolved by SDS–PAGE under reducing conditions, transferred to PVDF, and immunoblotted with primary antibodies, as reported [19].

2.6. Real-time PCR

The cyclin-E1 mRNA levels of rat hepatocytes were determined by a real-time PCR. See Supplemental manuscript for the detailed methods.

3. Results

3.1. Essential roles of proline for HGF-mediated DNA synthesis

Proline is required for the DNA synthesis of rat hepatocytes in the presence of EGF [14], but it remains unclear whether proline is required for HGF-induced G_1/S progression. Thus, we examined the role of proline in an HGF-supplemented culture system. As expected, HGF failed to induce DNA synthesis when hepatocytes were cultured in a proline-deficient medium (*i.e.*, DMEM) (Fig. 1A). Proline alone also did not stimulate the mitogenic action, as reported [14]. After the addition of proline (30 μg/ml) in DMEM, HGF successfully induced DNA synthesis: the level of incorporated [^{3}H]-thymidine by HGF was 5.6-fold higher than that by EGF. Such a mitogenic activity of HGF was dependent on the dose of proline (Fig. 1B): 10 μg/ml of proline was significant for HGF-induced DNA synthesis, and 1000 μg/ml of proline dramatically enhanced the HGF-mediated G_1/S progression, hence convincing the essential role of proline in growth factor-primed mitogenic actions. Using HGF as the potent mitogen in the DMEM-based culture system, we attempted to elucidate the mechanisms whereby proline elicits G_1/S progression, focusing on c-Met- and mTOR-related downstream events.

3.2. Up-regulation of cyclin-E1 by proline in rat hepatocytes

We first examined whether proline would enhance HGF-mediated c-Met tyrosine phosphorylation, which is the earliest event in mitogenesis [8,19]. However, proline did not promote the c-Met activation (Supplemental data, Fig. S1A). In this model, HGF enhanced the c-Met downstream pathways, such as AKT- and ERK-phosphorylation within 30 min post-challenge, while proline did not alter the HGF-mediated initial downstream signals (Fig. S1B), hence ruling out the possibility that proline promotes the c-Met activation itself.

We next examined the effect of proline on cyclin-related proteins. The key novel finding was that proline alone enhanced the expression of cyclin-E_1 (Fig. 2A), which is a late cyclin required for driving the hepatocyte cell cycle [20]. In contrast, HGF alone induced cyclin-D_1, known to be an early G_1 cyclin [21], without changing cyclin-E_1 accumulation. In both processes, basal levels

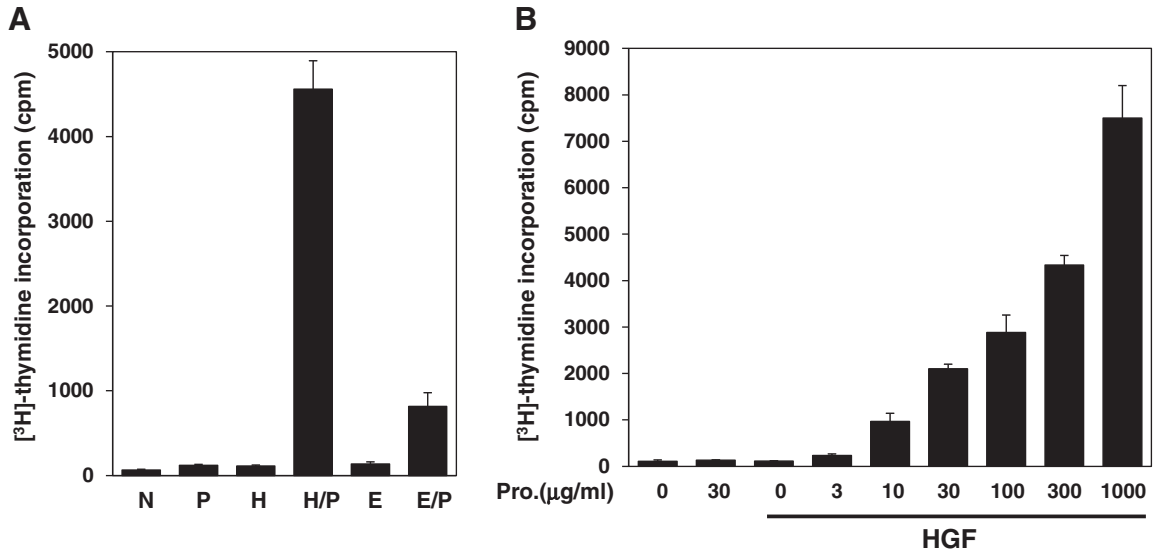

Fig. 1. Proline is required for HGF-induced DNA synthesis in adult rat hepatocytes. (A) Hepatocytes were cultured in DMEM for 24 h, and then 10 ng/ml HGF or 20 ng/ml EGF was added with or without 30 μg/ml proline. DNA synthesis was determined by [^{3}H]-thymidine incorporation. (B) Dose-dependent effect of proline on stimulation of DNA synthesis in hepatocytes. Hepatocytes were cultured in DMEM supplemented with 10 ng/ml HGF and proline in the range of 0–1000 μg/ml. Each value and vertical bar represents the mean ± S.D. of three wells. N: non-treated control, P: proline, H: HGF, E: EGF.

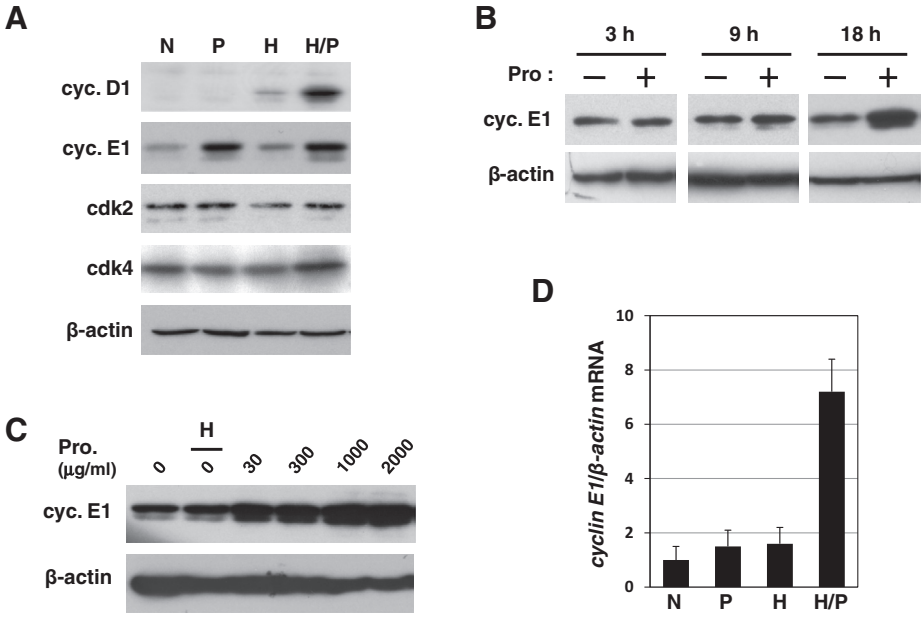

Fig. 2. Cyclin-E_1 accumulation is dependent on the presence of proline. (A) Western blot analysis of cell cycle proteins. Hepatocytes were cultured in DMEM with or without 30 μg/ml proline and 10 ng/ml HGF, and total cell lysates were harvested after 24 h. (B) Time-course analysis of cyclin-E_1 expression. (C) Dose-dependent effects of proline on cyclin-E1 expression. (D) Quantitative analysis of *cyclin* E_1 mRNA levels after 24 h culture of hepatocytes. The results were standardized with β-*actin* mRNA. N: non-treated control, P: proline, H: HGF.

of cdk2 and cdk4 were not altered by any treatments (Fig. 2A). HGF increased the cyclin-D_1 levels, as reported [12]. Proline did not induce cyclin-D_1, but markedly enhanced HGF-induced cyclin-D_1 expression (Fig. 2A), as discussed later.

We attempted to establish the inductive effect of proline on cyclin-E_1. Proline, but not HGF, increased the accumulation levels of cyclin-E_1 in a time-dependent manner (Fig. 2B). Moreover, proline dose-dependently increased the protein levels of cyclin-E_1 (Fig. 2C). We next examined the production patterns in each event: proline alone did not modify the cyclin-E_1 mRNA levels (Fig. 2D). In contrast, proline apparently increased cyclin-E_1 mRNA levels when combined with HGF, and this was associated with an increase in cyclin-D_1 (Fig. 2A). The cyclin-D_1-cdk4 complex is critical for transcriptional activation of cyclin-E_1 via Rb-E2F cascade [22]. Such a transcriptional system may explain the cyclin-E_1 mRNA up-regulation by proline and HGF (Fig. 2D), but this change did not modify the cycline-E_1 levels (Fig. 2A). A possible explanation is that excess cyclin-E_1 mRNA and its protein are often unstable and auto-cleavable [23,24]. Thus, we focused on the post-transcriptional mechanism of proline-induced cyclin-E_1.

3.3. Involvement of mTOR activation in proline-mediated cyclin-E_1 induction

Emerging evidence indicates that amino acids (including proline) enhance mTOR cascades in support of cellular homeostasis [25]. The PI3K-mTOR pathway is critical for DNA synthesis in the culture of the hepatocytes [26]. Thus, we focused on the possible contribution of mTOR. As expected, proline alone was shown to enhance mTOR activation, as evidenced by rpS6 Ser-235/236 phosphorylation (Fig. 3A). Likewise, HGF alone, or proline plus HGF, also increased the mTOR activation at similar levels. When rapamycin, an mTOR-inhibitor, was added to a proline-containing culture, proline-induced cyclin-E_1 was largely abolished (Fig. 3B). Likewise, rapamycin diminished the increase in cyclin-E_1, caused by HGF plus proline, suggesting that the neo-induction of cyclin-E_1 by proline depends the on mTOR pathway, rather than on a transcriptional mechanism, even in the presence of HGF. PI3K is known as an intermediate pathway during amino acid-primed mTOR activation, and LY294002, an inhibitor of PI3K, also diminished the proline-induced induction of cyclin-E_1 (Fig. 3B). As a result, rapamycin suppressed the inducible effect of proline on HGF-mediated DNA synthesis (Fig. 3C), partly via an inhibition of cyclin-E_1 up-regulation.

3.4. Specific roles of proline-induced cyclin-E_1 up-regulation upon exposure to amino acids

Not only proline but also leucine and glycine are known to enhance mTOR activation in several types of cells [27,28]. Thus, we addressed whether other mTOR-activating amino acids may enhance the HGF-mediated mitogenic response. Leucine did not alter the DNA synthesis in a primary culture of hepatocytes, neither with nor without HGF (Supplemental data, Fig. S2A). This phenomenon was also seen in the glycine-added culture system. In contrast to proline, leucine and glycine did not alter the basal levels of cylin-E_1 (Fig. S2B), suggesting the "specific" contribution of proline to HGF-mediated G_1/S progression.

3.5. Linkage of collagen and cyclin-E1 accumulation in proline-mediated DNA synthesis

The fact that proline (but not other amino acids) could elicit HGF-induced DNA synthesis encouraged us to examine proline-specific molecular events. Hepatocytes produce several types of collagens through an enzymatic conversion of proline to hydroxyproline [29]. Interestingly, proline-induced DNA synthesis depends on collagen synthesis [14,30]. Indeed, *cis*-OH-proline, an inhibitor for collagen triple helix stabilization, repressed the mitogenic response to HGF plus proline (Fig. 4A). Notably, proline-induced cyclin-E_1 up-regulation was largely diminished by *cis*-OH-proline (Fig. 4B). Such an inhibitory effect was also seen in hepatcytes treated with 2,2'-bipyridine, an iron chelator that inhibits proline hydroxylation.

4. Discussion

Several lines of evidence indicate that cyclin-E_1 play a key role in liver regeneration *in vivo* [31,32] and *in vitro* [33]. Indeed, the mitogenic response of hepatocytes to 70%-hepatectomy was impaired in cyclin-E_1-deficient mice [31], while forced induction of this cyclin leads to the enhanced regeneration [32]. HGF induced cyclin-D_1, as reported [12,34], but did not enhance cyclin-E_1 expression. Thus, one of the most important findings was that proline can increase both cyclin-D_1 and cyclin-E_1 under HGF-supplemented conditions. In other words, our study identified proline as a novel cyclin-E_1 inducer. This is, to our knowledge, the first report to show that a stand-alone amino acid elicits cyclins, required for G_1/S progression. Thus, we will focus on the significance of cyclin-E_1, induced by proline, as described below.

Emerging evidence indicates that amino acids (such as leucine and glycine) enhance mTOR activation in various cells [35]. Proline also activates the mTOR pathway. For example, mTOR activation by proline in embryonic stem cell leads to epithelial differentiation [18], but no information is available about proline-induced mitogenic events. Notably, the proline-mediated induction of

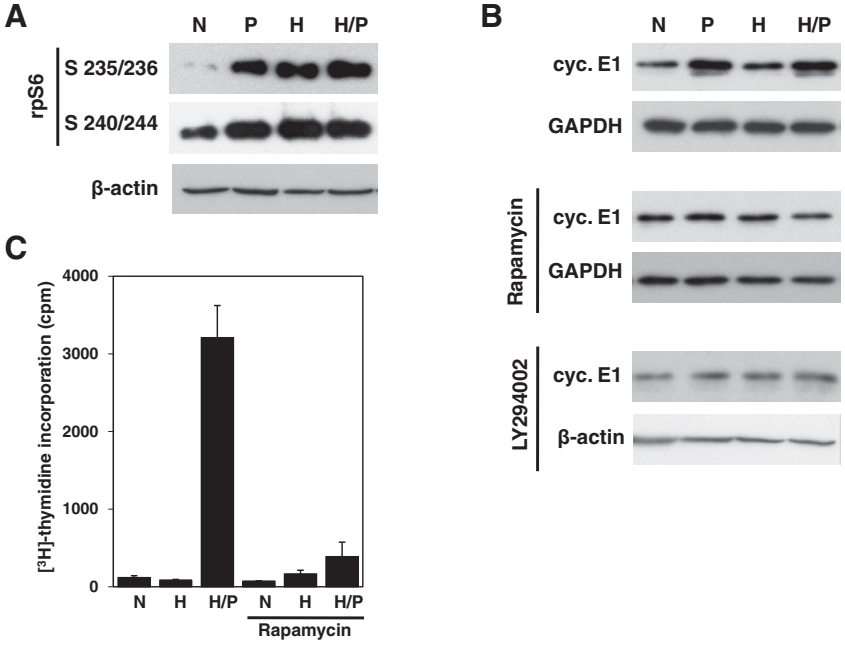

Fig. 3. mTOR pathway is required for cyclin-E_1 accumulation by proline. (A) Western blots for phospho-rpS6 (Ser 235/236 and Ser 240/244). Hepatocytes were cultured in DMEM with or without 30 μg/ml proline and 10 ng/ml HGF for 24 h. (B, C) Effects of rapamycin and LY294002 on cyclin-E_1 expression (B) or on DNA synthesis (C). Rapamycin (30 nM) or LY294002 (5 μM) were added to the culture medium with or without 30 μg/ml proline and 10 ng/ml HGF. N: non-treated control, P: proline, H: HGF.

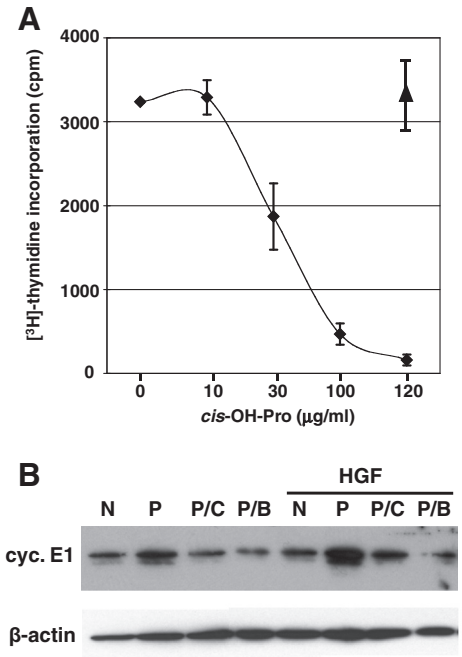

Fig. 4. Collagen synthesis inhibitors suppress cyclin-E_1 accumulation by proline. (A) Hepatocytes were cultured in WE for 24 h, and then 10 ng/ml HGF and various concentration of *cis*-OH-Pro were added. [^{3}H]-thymidine were added 8 h before assay of DNA synthesis. ▲: WE supplemented with 200 μg/ml proline. (B) *cis*-OH-Pro (120 μg/ml) or 2,2'-bipyridine (0.5 mM) were added to the culture medium with 30 μg/ml proline and/or 10 ng/ml HGF. Cell lysates were subjected to Western blot analysis for cyclin-E1. N: non-treated control, P: proline, C: *cis*-OH-Pro, B: 2,2'-bipyridine.

cyclin-E_1 was completely abolished by an mTOR-inhibitor, rapamycin, along with a loss in ribosome 40S rpS6 phosphorylation. With regard to these results, rpS6 activation was proved to be critical for cyclin-E_1 induction: hepatocyte G_1/S progression was inhibited in rpS6-deficient mice post-hepatectomy, and this was associated with a loss in cyclin-E_1 (but not cyclin-D_1) [36], implying that active rpS6 confers an up-stream cascade for cyclin-E_1 induction. Thus, we predict that the proline-specific mTOR → rpS6-activating cascade leads to the post-transcriptional up-regulation of cyclin-E_1, a key cyclin for liver regeneration [31–33].

Only proline can up-regulate cyclin-E_1 expression, suggesting a "specific" mechanism. Collagen metabolism is one of the proline-specific events, since intracellular conversion of proline to hydroxyproline by prolyl hydroxylase is critical for collagen helical triple assembly [37]. Notably, collagen synthesis inhibitors repressed proline-mediated cyclin-E_1 induction, leading to G_1/S progression arrest. With regard to this, collagen synthesis is known responsible for lipogenesis, glycogenesis and albumin production in hepatocytes [16,17], all of which are also linked to the mTOR pathway. Indeed, recent reports suggested that prolyl hydrolase, which can be activated by an excess of proline, enhances mTOR activity [38]. Although further studies are necessary to elucidate the collagen-related mechanism, we at least postulate that the up-regulation of cyclin-E_1 is one of the key mechanisms that defines proline as essential for growth factors, such as HGF, to produce the mitogenic outcomes.

In addition to cyclin-E_1, we also considered the contribution of cyclin-D_1, since this cyclin can dominate the mitogenic action in various cells, including hepatocytes. We found that proline markedly enhanced the HGF-mediated induction of cyclin-D_1 (Fig. 2A). In this process, proline did not modify HGF-mediated cyclin-D_1 mRNA up-regulation, but enhanced cyclin-D_1 protein *de novo* synthesis (not shown), suggesting a post-transcriptional mechanism. We next discussed the significance of up-regulated cyclin-D_1. Nelson et al. reported that G_1/S progression was arrested after the depletion of non-essential amino acids (including proline), while cyclin-D_1 cDNA transfection restored the loss of mitogenic actions [39], establishing a key role for cyclin-D_1 under the amino acid-starved conditions. Thus, the "synergic" effect of proline on HGF-induced cyclin-D_1 is also critical for inducing G_1/S progression.

Finally, we would like to discuss the possible events, related to HGF and proline, *in vivo*. Blood HGF levels markedly increase in rats after 70%-hepatectomy to trigger regenerative responses [40]. In contrast, serum proline levels decrease in hepatectomized rats [41], possibly due to local consumption by regenerating livers. Importantly, proline can enhance HGF production in stromal cells, such as stellate cells, in an mTOR-dependent manner [42]. Thus, proline may contribute to liver regeneration as a cyclin-D_1/E_1-provider in hepatocytes and as an HGF-inducer in stromal cells. Such a cross-talk event between the HGF-mediated repair system and the proline-mTOR-sensing pathway seems to be required for liver regeneration.

Acknowledgments

This work was supported by grants from the Ministry of Education, Science, Technology, Sports and Culture of Japan (No. 23590458 to S.M. and No. 21390079 and Global COE program to T.N.). We also thank Mrs. Yoshiko Yoneda for secretarial assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.052.

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