Hepatopoietin interacts directly with COP9 signalosome and regulates AP-1 activity

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Abstract Hepatopoietin (HPO)/augmenter of liver regeneration (ALR) is a specific hepatotrophic growth factor, which plays a key role in liver regeneration. Our previous study indicated that HPO executes its function by an inter-reactive network of the autocrine, paracrine and endocrine pathways. Recently, we have demonstrated that intracellular HPO interacts with Jun activation domain-binding protein 1 (JAB1) and leads to potentiation of activating protein-1 (AP-1) activity in a MAPK independent fashion. JAB1 is the fifth subunit of the COP9 signalosome (CSN), which is first identified as a suppressor of plant morphogenesis. A protein complex kinase activity associated with the CSN has been reported but not identified yet. In this report, we investigated further the association of HPO with the whole CSN. HPO exists in a complex with the eight-component CSN, both when purified from glycerol gradient centrifugation and when reciprocal immunoprecipitated from the lysates of transfected COS-7 cells. Intracellular HPO colocalizes with endogenous CSN in nucleus of hepatic cells. In addition, intracellular function of HPO that increases the phosphorylation of c-Jun leading to potentiate the AP-1 activity is inhibited by curcumin, a potent inhibitor of CSN-associated kinase. Taken together, these results elucidate a novel relationship of intracellular growth factor, HPO with large protein complex, CSN, which suggests a possible linkage between CSN and liver regeneration.

Keywords: Liver regeneration; Signal transduction; Growth factor

1. Introduction

Hepatopoietin (HPO)/augmenter of liver regeneration (ALR) is a novel polypeptide mitogen that originated from liver and specifically augments hepatocytes proliferation [1]. Yang et al. [2] firstly cloned the cDNA of HPO (rhHPO) from human fetal liver lysates encoding 125 amino acids and 15 kDa in molecular weight. Recombinant human HPO could stimulate proliferation of cultured hepatocytes as well as hepatoma cells in vitro, promote regeneration and recovery of damaged hepatocytes, and rescue acute hepatic failure in vivo [3,4]. HPO exerts its hepatrophic activities through paracrine and autocrine pathways. Wang et al. characterized the mitogenic effect of paracritic HPO on hepatoma cell lines and demonstrated the existence of HPO specific receptor on the surface of these cells, and elucidated that HPO stimulates proliferation of hepatocytes and enhances liver regeneration by activating the mitogen-activated protein kinase cascade (MAPK signaling pathway) under the mediation of HPO receptor, leading to DNA synthesis of hepatic cells [5,6]. The existence of HPO in the nucleus and cytosol of liver tissues further implicates that HPO could have intracellular function in hepatocytes. We used the full-length HPO as bait in the yeast two-hybrid system to screen a human fetal liver cDNA library. Among the specifically interacting clones was Jun activation domain-binding protein 1 (JAB1), co-activator of c-Jun/activating protein-1 (AP-1) transcription factor, which enhances binding of c-Jun-containing AP-1 complex to their DNA consensus site and increases transactivation of an AP-1-dependent promoter [7]. Lu et al. [8] further demonstrated the association of HPO and JAB1 in vitro or in vivo and suggested the biological effect of interaction of HPO with JAB1 on the specific modulation of AP-1-mediated transcription in a MAPK-independent fashion.

JAB1 is also known as a subunit (CSN5, S5) of mammalian COP9 signalosome (CSN) that is highly conserved from yeast to human [9]. The CSN was first identified in *Arabidopsis thaliana* as an eight-subunit complex involved in the suppression of light-dependent development [10], and it has now been found to participate in diverse cellular and developmental processes in various eukaryotic organisms. Two known activities associated with the CSN are deneddylation and phosphorylation [11,12]. The associated deneddylase regulates the activity of specific Skpl-Cullin-F-box protein complex E3 ubiquitin ligases [13]. The CSN-associated kinase phosphorylates transcription regulators such as p105, IκBα, c-Jun [9], and p53 [14], which determine their stability towards the ubiquitin-26S proteasome system. In contrast to c-Jun N-terminal kinase (JNK), CSN-specific phosphorylation only modifies full-length of c-Jun and leads to its stabilization in HeLa cells, resulting in

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Abbreviations: PBS, phosphate-buffered saline; HPO, hepatopoietin; JAB1, Jun activation domain-binding protein 1; MAPK, mitogen-activated protein kinase; AP-1, activating protein-1; JNK, c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis; COP9 signalosome (CSN), constitutive photomorphogenesis 9

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increased AP-1 transcriptional activity [15]. Although none of the CSN subunits contain a recognizable kinase domain and the CSN kinase has not yet been identified, some protein kinases have been found to associate with CSN kinase activity. Recently, Sun et al. [16] proposed that inositol-1,3,4-trisphosphate-5/6-kinase (5/6-kinase) is the previously described CSN-associated kinase in that they could be co-purified from bovine brain, and both phosphorylated the same substrates. Uhle et al. also proved that kinase activity of CSN are most likely due to protein kinase CK2 and protein kinase D, which are recruited by CSN in order to regulate Ub-proteasome-dependent degradation [17]. Herein, we demonstrate that intracrine HPO associated with CSN and regulated AP-1 activity, which suggests a novel mechanism of intracrine cytokine that exerts its signaling modulation function through protein complex.

2. Materials and methods

2.1. Chemicals, antibody and plasmid constructions

Chemicals were purchased from Sigma–Aldrich unless stated otherwise. Reagents for SDS–polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-Rad. Tissue culture reagents were purchased from Invitrogen Life Technologies Inc. HepG2 is the human hepatoma cell line from ATCC.

Myc-tagged proteins were detected using mouse monoclonal antibody against a Myc-peptide (Cell signal technology). Anti-CSN5 (rabbit polyclonal antibody), anti-c-Jun (rabbit polyclonal antibody), anti-p-c-Jun (mouse monoclonal antibody), anti-actin (goat polyclonal antibody), peroxidase-conjugated goat-anti-mouse IgG, rabbit-anti-goat IgG, goat-anti-rabbit IgG and ECL luminescence kit were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HPO mouse monoclonal antibody was a gift from Dr. Qing-ming Wang. Anti-CSN1 (S1), -CSN2 (S2) and -CSN8 (S8) rabbit polyclonal antibodies were kindly provided by Professor Ning Wei and Xing-wang Deng (Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, USA).

For the immunoprecipitation and glycerol gradient centrifugation, the full-length cDNA of HPO was subcloned into pCMV-Myc (Clontech) expression vector in-frame with a Myc fusion protein. For the Luciferase transactivation assay, full-length cDNA of HPO and JAB1 were all subcloned into pcDNA3 (Invitrogen Life Technologies).

2.2. Protein assay and Western blotting

Cells were rinsed three times with ice-cold phosphate-buffered saline (PBS) and lysed in the lysis buffers stated in each experimental step. Cell debris was removed by centrifugation at $1000 \times g$ for 10 min at 4 °C and the supernatants were collected for further treatments. Protein concentration was determined as described by Bradford [18] with bovine albumin serum (Roch) to make a standard curve. The protein samples were fractionated via 10–15% SDS–polyacrylamide gel electrophoresis and transformed to the PVDF membranes (Amersham Pharmacia Biotech). The membranes were first blocked by incubation in TBS-T containing 5% non-fatty milk overnight at 4 °C, then incubated sequentially with primary antibody and HRP-conjugated secondary antibody, and detected using an ECL luminescence kit. Finally, the acquired bands in the membranes were scanned by GS-710 Calibrated Imaging Densitometer (Bio-Rad) and precisely quantified using PDQUEST 6.0 software package (Bio-Rad).

2.3. Cell culture, treatment and transient transfection

COS-7 cells and HepG2 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 g/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified 5% CO_2 atmosphere. For transient transfection, cells were seeded in tissue culture plates for 18–24 h prior to infection. When the cell confluence reached 90–95%, transfections were performed using Lipofectamine 2000 reagent (Invitrogen Life Technologies) as recommended by the manufacturer. In some experiments, cells were treated with curcumin which was obtained from Sigma–Aldrich Chemical Co. Stock solutions of curcumin (20 mM) were prepared in 100% DMSO and cells were treated with 50 μM curcumin in combination for 3 h.

2.4. Immunoprecipitation

COS-7 cells were transfected with pCMV-Myc-HPO coding for Myc-HPO fusion or pCMV-Myc vector using lipofectamine reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. After 48 h incubation, the cells were rinsed three times with ice-cold PBS and lyzed in the lysis buffer (PBS containing 10 mM CHAPS, 5 μg/ml chymostatin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 mM Na_3VO_4 and 1 mM NaF). For immunoprecipitation, the cell lysates were incubated with anti-Myc and anti-CSN1, CSN2, CSN5 (JAB1) and CSN8 antibodies, respectively, rotated at 4 °C for 3 h. Protein A/G–Sepharose beads (Santa Cruz Biotech) were added to the reaction for another 3 h of incubation. The Sepharose beads were then washed three times with lysis buffer and resuspended in 1× SDS–PAGE loading buffer for immunoblotting analysis.

2.5. Immunofluorescent staining

For physiological colocalization study of endogenous CSN and HPO in vivo, HepG2 cells grown on 35 mm glass bottom microwell cell culture dishes (MatTek corporation) were rinsed three times in PBS and fixed in 4% paraformaldehyde for 15 min, followed by permeabilization in 0.2% Triton X-100 for 15 min. Washed three times with PBS, the samples were incubated with the blocking solution (3% BSA and 5% newborn calf serum in PBS) for 1 h. The primary antibody diluted in blocking solution was incubated with the samples for 2 h. Unbound antibodies were washed with PBS containing 0.1% Tween 20 (PBS-T) for 3×5 min, and then were stained with fluorescein isothiocyanate (FITC)-linked anti-mouse and Rhodamine Red-linked anti-rabbit IgG (Santa Cruz Biotechnology). After being washed with PBS-T for 3×5 min, the samples were double stained with 1 μg/ml 4′,6-diamidino-α-phenylinodole (DAPI). All cell samples were viewed through a confocal laser-scanning microscope (Bio-Rad Radiance 2100), using the program of Laser Sharp 2000.

2.6. Luciferase assays

COS-7 cells were plated on 24 wells plates at a density of 1×10^5/well and were co-transfected with AP-1 driven luciferase reporter gene (Stratagene), together with the indicated cDNAs, using the Lipofectamine 2000 reagent. The AP-1 driven luciferase reporter gene is a reporter plasmid encoding for the firefly luciferase gene driven by several copies of an AP-1 enhancer. The indicated cDNAs were pcDNA3-c-Jun coding for c-Jun, pcDNA3-JAB1 coding for JAB1 and pcDNA3-HPO coding for HPO, respectively. Total DNA was kept constant by the addition of the appropriate amount of pcDNA3 for all transfections. At 16 h after transfection, cells were either treated for 3 h with the indicated concentrations of 50 μM curcumin or left untreated (DMSO as the control). Luciferase assays were performed at 3 h after treatment with the Dual Luciferase Assay System (Promega) as recommended by the manufacturer. We normalized luciferase activity for transfection efficiency by using a co-transfected internal control, pRL-TK vector. The results were recorded on an Ultra-weak Luminescence Analyzer. Representative results from more than three independent experiments performed in triplicate are shown as fold induction or compared with the control. Activities varied <10% between transfection experiments. The results were presented as means ± S.D.

2.7. Glycerol gradient centrifugation

For separation experiment, 1.5×10^7 COS-7 cells transfected with pCMV-Myc-HPO or pCMV-Myc vector or HepG2 cells were lysed in lysis buffer containing 20 mM HEPES, pH 7.6, 140 mM NaCl, 1 mM DTT, 10% glycerol, 0.1% Nonidet P-40 and Complete™ protease inhibitor tablets (Roche Molecular Biochemicals) and subjected to density gradient centrifugation as described [9,15]. Briefly, total cell lysates went through three freeze–thaw cycles in liquid N_2 and were centrifuged at 4 °C, $1000 \times g$ for 10 min to remove debrits. The supernatants were filtered through a 0.45 μm filter (Millipore) before being separated on 10–40% density glycerol gradient centrifugation. For glycerol gradient centrifugation analysis, 1 ml of cell lysate was layered onto 10 ml of a 10–40% linear glycerol gradient containing 10 mM Tris–HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol. After centrifugation for 24 h at 27 000 rpm in a

Beckman Ti40 rotor at 4 °C, 0.6-ml fractions were collected and proteins from fractions 1 to 16 were precipitated by trichloroacetic acid, separated by SDS–PAGE, and analyzed by standard Western blot using antibodies against Myc and CSN subunits.

3. Results

3.1. HPO interacts with endogenous COP9 signalosome

We have demonstrated that HPO interacted with JAB1, the fifth subunit of CSN, both in vivo and in vitro [8]. To further verify the specific association between HPO and the whole CSN, we performed immunoprecipitation experiments to obtain the specific complex formation in vivo between HPO and CSN protein complex. Myc-tagged HPO was expressed in COS-7 cells and anti-Myc or anti-CSN subunits antibodies specifically precipitated complexes of Myc-HPO and endogenous CSN. Specific precipitation of HPO was detected by anti-Myc Western blot when anti-CSN subunits antibodies (CSN1, CSN2, CSN5 and CSN8) but not control antibody was used for immunoprecipitation, and precipitated CSN was shown by anti-CSN1, -CSN2, -CSN5 and -CSN8 antibodies (Fig. 1A). Moreover, anti-Myc monoclonal antibody could also pull down CSN complex as well as HPO (Fig. 1B), since HPO can form homodimer in vivo [19]. The physiological interaction of HPO with CSN by their reciprocal coimmunoprecipitation implicated that the intracellular function of HPO could be associated with CSN.

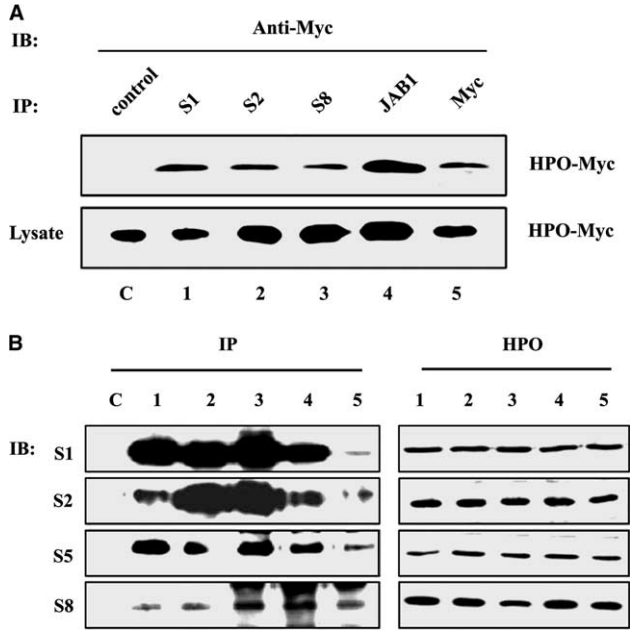

Fig. 1. The reciprocal immunoprecipitation suggested the assembly of HPO–CSN complex. (A) COS-7 cells were transfected with Myc-tagged HPO. At 48 h after transfection, the cells were lysed and cell lysates were incubated with anti-Myc antibody or anti-S1, -S2, -S8, as well as anti-JAB1 antibodies. The immuno-complex was conjugated by protein A/G–Agarose beads and then the immunoprecipitates were resolved on SDS–PAGE. The aliquots were analyzed by immunoblotting with anti-Myc antibody. Lane 1 is a control for the other lanes with rabbit mock antibody (normal IgG). Lane 5 is a positive control, since HPO can form homodimer. A duplicate blot was also probed with anti-Myc antibody to monitor the HPO protein inputted in each precipitation reaction (bottom). (B) CSN was immunoprecipitated either by anti-HPO mouse monoclonal antibodies (Lane 5) or anti-S1, -S2, -S5 and -S8 antibodies (Lanes 1, 2, 3 and 4). And the precipitated CSN was detected by immuno-blotting with the related antibodies of the subunits. Precipitation with mouse mock antibody (normal IgG) served as negative controls (Lane C). Four lines of blots were also probed with anti-Myc antibody to monitor HPO precipitated in each reaction (right panel).

3.2. Physiological colocalization of HPO with COP9 signalosome within cells

The association between HPO and CSN was confirmed further in colocalization experiments (Fig. 2). The endogenous HPO and CSN in human hepatoma line HepG2 cells were visualized by double immuno-fluorescence staining. HPO was stained with green color and was observed mainly in nucleus and very small portion in cytoplasm. Similarly, the endogenous CSN was detected by its three subunits (CSN1, CSN2 and CSN8), respectively, that stained with red color. The identical localization of these subunits was obtained that the CSN existed primarily in nucleus (Fig. 2A–C) [14]. The merged figure of each individual detection obviously demonstrated that endogenous HPO and endogenous CSN had a similar pattern of distribution or colocalization in HepG2 cell. The fact that HPO colocalized with CSN implicated their physiological interaction and the nucleus is the exact subcellular compartment for the function performing of HPO through CSN.

3.3. Co-purification of HPO and CSN in vivo further confirmed the natural binding

Because endogenous CSN can co-immunoprecipitate ectopical HPO and vice versa, we attempted to confirm the natural binding of HPO and CSN by transfection of COS-7 cells with plasmid containing Myc-tagged HPO, and a glycerol gradient was performed on COS-7 cell lysates. The subunits of CSN were detected in the complex using the antibodies against CSN1, CSN2, CSN5 and CSN8; and expressed HPO was also identified in the complex by the anti-Myc as well as anti-HPO antibody, since the molecular weight of HPO is only 15 kDa, which is much smaller than that of CSN (Fig. 3A). Further significant co-purification of HPO and CSN was obtained by the same method in HepG2 cells, which contain endogenous HPO expression (Fig. 3B). The data presented demonstrate that HPO is in fact associated with CSN, as indicated by immunoprecipitation. Therefore, the native interaction actually provided the structural bases for the functional association between HPO and CSN.

3.4. Curcumin inhibited AP-1 activity increment caused by transient expression of HPO

CSN has been identified that phosphorylates cellular regulators, such as p105, IκBα, c-Jun [9], and p53 [13], designating their stabilization towards the ubiquitin proteolysis system. CSN-specific phosphorylation of c-Jun leads to stabilization of the transcription factor in HeLa cells, resulting in increased AP-1 transcriptional activity [15]. Curcumin (Diferuloylmethane) has been reported to be the most effective inhibitor of the CSN kinase activity [14,20]. As the component of CSN, JAB1 could activate the AP-1-dependent promoter [18,21,22]. In our previous results, HPO could modulate this AP-1 activity by binding to JAB1. To test whether this process, further mediated by CSN, we used curcumin to perform inhibitory relative luciferase activity assays, in order to detect the effect

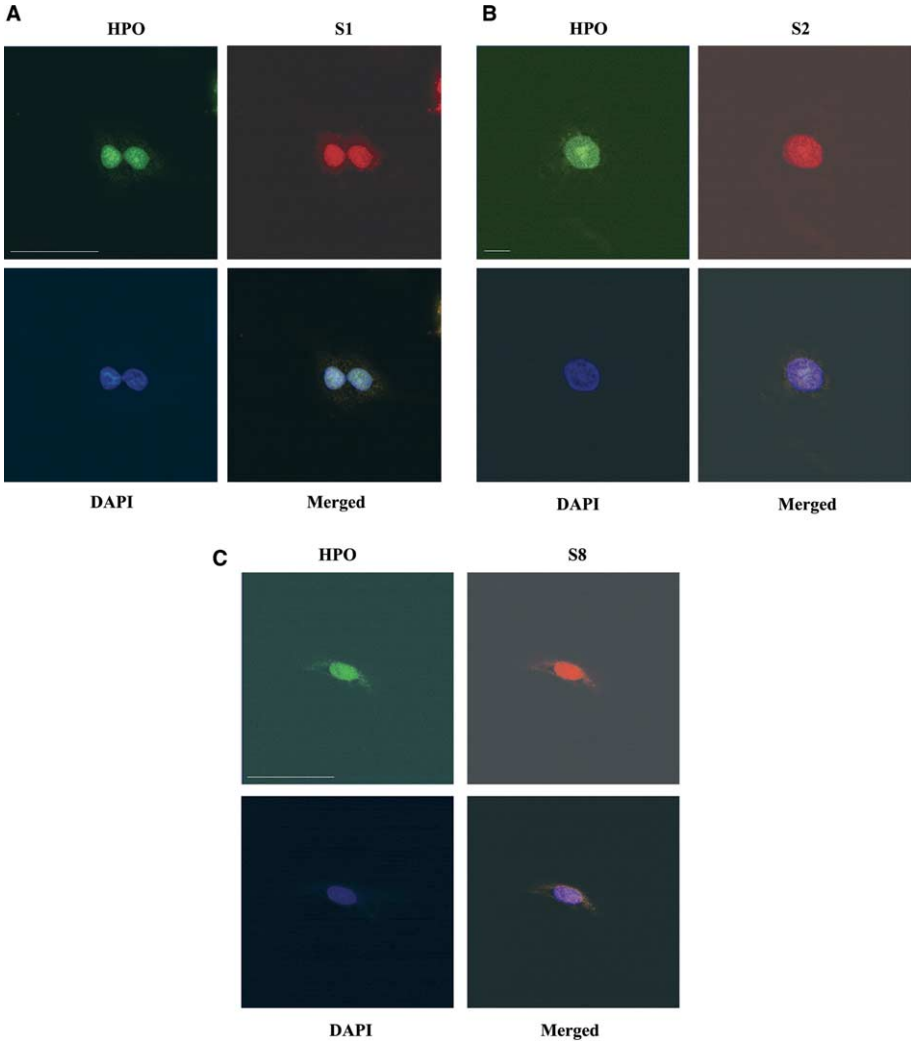

Fig. 2. HPO colocalizes physically with CSN in HepG2. HepG2 cells were stained for endogenous HPO (green) and the three subunits of CSN (red), S1, S2 and S8 in each experiment of A, B and C, respectively, by indirect immunofluorescence. Cells were double stained with DAPI (blue) to identify the nuclei in the corresponding fields. Merged pictures indicated the nucleic colocalization of HPO and the COP9 complex. All cell samples were visualized by confocal microscopy (Bio-Rad), and the white bar indicated in A and C was 50 μm, and 10 μm in B.

on the activation of AP-1 activity modulated by HPO. So, COS-7 cells were co-transfected with an AP-1-driven luciferase reporter gene in the presence of c-Jun, JAB1, and HPO, and at 16 h after transfection, cells were either treated for 3 h with 50 μM of curcumin or left untreated (DMSO as the control). The dual reporter gene activity was measured at 3 h after treatment. The results indicated that curcumin completely inhibited the HPO potentiation of AP-1 reporter gene activity (Fig. 4A). Furthermore, the activation of AP-1 activity by ectopical expression of HPO was also blocked by the indicated concentration of curcumin (Fig. 4B). These results together demonstrated the involvement of CSN in the intracellular signaling transduction pathway of HPO.

3.5. Curcumin inhibited the potentiation role of HPO on c-Jun

We have demonstrated that HPO-activated AP-1 promoter activity and increased phosphorylation of c-Jun in a JNK-independent way [8]. To further determine whether CSN mediated this reaction, we then detected the levels of c-Jun as well as phosphorylated c-Jun in COS-7 cells transfected with HPO, JAB1 or both, and treated or left untreated with curcumin. The results indicated that ectopically expressed HPO increased the phosphorylated c-Jun concentration, which conformed to our former results and curcumin actually decreased the level of phosphorylated c-Jun (Fig. 5) that elevated by HPO, in agreement with the data suggested in AP-1 reportor gene transactivation assay (Fig. 4A). Meanwhile, we also observed that c-Jun level was also affected by curcumin treatment, which indicated a subsequent instability of this protein (Fig. 5) towards the Ubiquitin-26S proteasome system.

4. Discussion

CSN complex consists of eight subunits that are highly conserved from plants to mammals [23]. This complex has a role in cell-signaling processes, which is manifested by its associated novel kinase activity and by the involvement of its subunits in regulating multiple signaling pathways, cell-cycle progression and developmental control. JAB1, the fifth

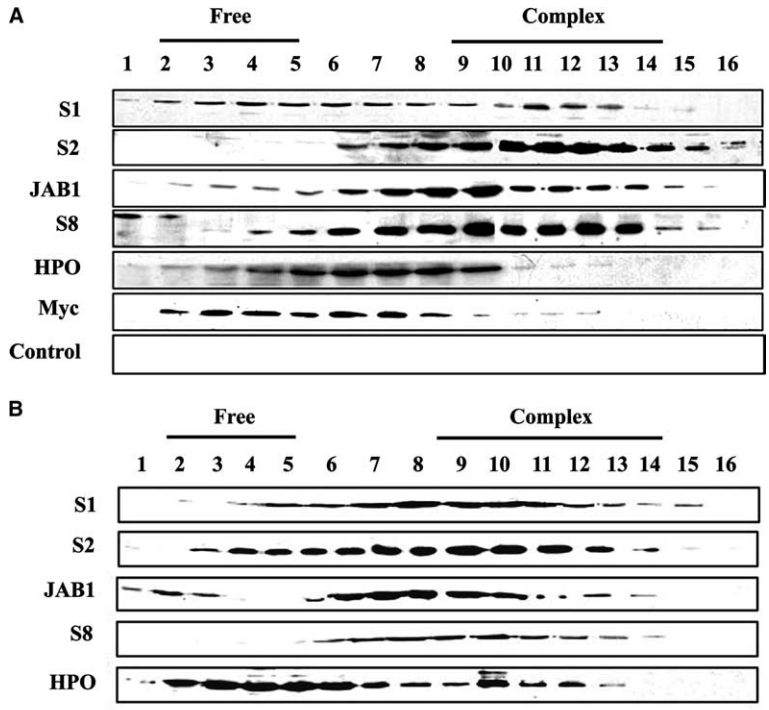

Fig. 3. Co-purification of HPO and CSN by glycerol gradient centrifugation. (A) COS-7 cells were transfected with Myc tagged HPO or pCMV-Myc vector (control) and lysed in lysis buffer at 48 h after transfection. The cell lysates were fractionated through 10–40% glycerol gradient by centrifugation for 24 h at 27 000 rpm and 4 °C. Fractions of 1–16 were analyzed by immunoblotting using the antibodies shown to the left of the panels. (B) HepG2 cells were fractionated and detected using the same methods as in (A).

subunit of CSN, is a co-activator of AP-1, which has been shown to interact with a variety of cytokines, growth factors or nuclear receptors. We have identified that JAB1 specifically interacted with intracellular HPO both in vivo and in vitro, which induced the potentiation of AP-1 transactivation function [8]. In this report, our data further demonstrated that HPO associated with the whole CSN using several criteria.

The fact that the majority of endogenous JAB1 is in the CSN complex prompted us to investigate whether other components of CSN participate in the regulation of HPO. Intracrine HPO could co-purify with CSN through glycerol density gradient centrifugation. Meanwhile, the reciprocal coimmunoprecipitation could identify the physiological interaction of HPO with CSN. In addition, initial characterization of the CSN complex indicated that it is a large molecular weight and multi-functional protein complex predominantly in the nucleus [24–26]. In our work, we found the colocalization of HPO with CSN mainly in the nucleus, which suggested the existence in the same subcellular distribution and provided the exact compartment for the direct interaction taken place.

Four individual subunits of CSN had been implicated, respectively, in the AP-1-mediated transcription activation, and purified CSN was reported to exhibit serine/threonine protein kinase activity towards c-Jun, IκBα, p105, and p53. None of the CSN subunits contain a recognizable kinase domain, however. c-Jun, the specific form of transcription factor AP-1, has been implicated in several cellular processes including normal cell growth, differentiation, proliferation and cell transformation [27]. We reported previously that HPO increased the phosphorylation of c-Jun via JAB1 in a JNK independent manner. If the phosphorylation step is mediated by CSN kinase, the interference of its kinase activity will affect the intracellular effect of HPO. The data presented here clearly demonstrated that the CSN is a key player in the functional regulation of HPO. The AP-1 activation potentiated by HPO is sensitive to curcumin. Curcumin is a dietary pigment, which has been described as a suppressor of tumor initiation and promotion [28]. Curcumin is also known as a strong inhibitor of AP-1 activation [29] and in consequence, a potent inhibitor of the CSN-associated kinase activity [20]. Although curcumin is also reported to inhibit the JNK pathway, as shown before under our conditions, the JNK pathway is inactive [30], and the inhibitory effects of curcumin on phosphorylated c-Jun levels and AP-1 activity, which increased by HPO are most likely due to the inhibition of the CSN kinase. In addition, the transcription factor stability of c-Jun towards Ubiquitin-26S proteasome system is also mediated by CSN-specific phosphorylation [9,15]. Through the inhibition of kinase activity, curcumin affected the phosphorylation of c-Jun executed by HPO and the consequence was the ubiquitination and subsequent degradation of c-Jun by the 26S proteasome.

Recently, CSN has become a focus of interest for many researchers for its function at the interface between signal transduction and ubiquitin-dependent proteolysis [31,32], which is required for the proper progression of the cell cycle in yeast and is essential for development in plants and *Drosophila* [33,34]. The new relationship of intracellular growth factor, HPO with CSN that we described here suggested a possible linkage between CSN and liver regeneration. Liver growth is regulated by an inter-reactive network of the various autocrine, paracrine and endocrine factors. The role of HPO as a stimulator of hepatocyte proliferation in liver regeneration has been systematically investigated for years. HPO is constitutively expressed in hepatocytes in an inactive form and released

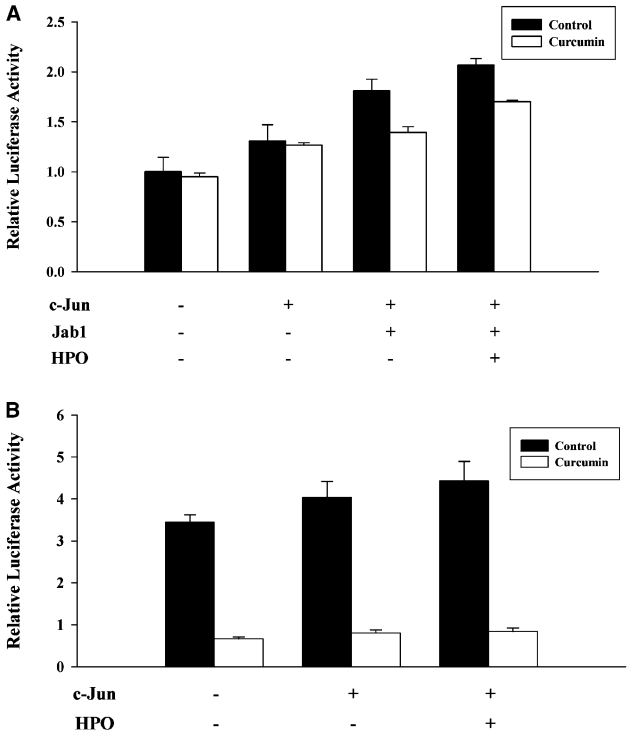

Fig. 4. Curcumin inhibited the potentiation of AP-1 activity by HPO via CSN. (A) HPO enhanced JAB1-mediated activation of AP-1 reporter gene activity in COS-7 cells. This process could be abrogated by curcumin, the potent inhibitor of CSN associated kinase activity, as analyzed by the AP-1 luciferase reporter assay. COS-7 cells were co-transfected with AP-1-driven-luciferase reporter, c-Jun, JAB1 and HPO. (B) Similarly, the AP-1 activity induced by HPO through endogenous CSN was completely inhibited by curcumin. COS-7 cells were cotransfected with AP-1-driven-luciferase reporter, c-Jun, and HPO without ectopical JAB1. At 16–19 h after transfections, the cells were incubated with 50 μM curcumin for 3 h. Each luciferase activity was normalized to a cotransfected pRL-TK vector. Shown are representative examples of at least three independent experiments performed in triplicate. Activities varied <10% between transfection experiments. The *error bar* indicates S.D.

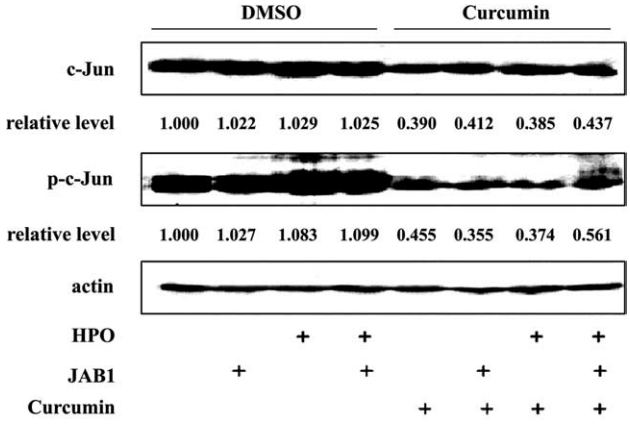

Fig. 5. Curcumin inhibited the potentiation role of HPO on c-Jun. The levels of c-Jun as well as phosphorylated c-Jun in COS-7 cells transfected with HPO, JAB1 or both as indicated, and treated or left untreated with curcumin, were detected by immunoblotting analysis using the antibodies shown to the left of the panels. The line of actin indicated the equal loading as an internal control. The relative levels of c-Jun and p-c-Jun, as measured by densitometry, are indicated below the corresponding panels.

from cells in an active form during liver regeneration [1]. As to our knowledge, HPO is the first intracrine growth factor identified to trigger AP-1 pathway through intracellular interaction with a multicomponent protein regulator complex. Taken together, with the increasing evidence concerning the role of CSN in signal transduction and developmental control, this versatile multicomponent complex serves as an important integrator of cellular events. It can be speculated that HPO as an intracrine factor triggers AP-1 pathway by regulation of CSN for immediate early response when its intracellular level is increased after partial liver hepatectomy or liver injury and the CSN complex could represent an intracellular signal platform, where signals from the extracellular or intracellular environment are coordinated with transcriptional activation and with the regulation of the related functions of cells [8]. It would be of interest to further investigate the concrete interaction pattern of HPO and CSN complex. This may shed light on the mechanism of intracrine HPO signaling and how the regulation of the CSN acts as the capacity for liver regeneration.

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