Intracrine hepatopoietin potentiates AP-1 activity through JAB1 independent of MAPK pathway


*Department of Genomics and Proteomics, Beijing Institute of Radiation Medicine, Chinese National Human Genome Center at Beijing, 27 Taiping Road, Beijing 100850, P. R. China; †National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing 100101, P. R. China; ‡Peking University School of Life Sciences, Beijing 100871, P. R. China

Corresponding author: Fuchu He, Department of Genomics and Proteomics, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, P. R. China. E-mail: hefc@nic.bmi.ac.cn

ABSTRACT

Many growth factors and cytokines are involved in liver regeneration. Of them, only hepatopoietin (HPO)/ALR (augmenter of liver regeneration) is a specifically hepatotrophic factor originally identified from the cytosol of regenerating or hyperplastic hepatic cells. Previous reports indicate that extracellular HPO triggers the MAPK pathway by binding its specific receptor on the cell surface. However, its function in the cytosol of hepatocytes is unclear. Here we identified that JAB1 (Jun activation domain-binding protein 1), a co-activator of AP-1, which is essential for liver regeneration, specifically interacts with intracellular HPO. JAB1 colocalizes with HPO in nuclei of hepatic cells or COS-7 cells. As an intracrine factor, the intracellular function of HPO is to increase c-Jun phosphorylation independent of c-Jun amino-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) -1 and -2, and leads to potentiation of JAB1-mediated AP-1 activation. Amino acids 1-63 of HPO molecule are sufficient to bind to JAB1, but the full-length HPO is necessary for its intracellular signaling. Taken together, these results elucidate a novel mechanism of intracrine cytokine signaling by specifically modulating the AP-1 pathway through JAB1, in a MAPK-independent fashion.

Key words: intracellular signaling • augmenter of liver regeneration

Hepatopoietin (HPO)/augmenter of liver regeneration (ALR), a hepatotrophic growth factor, was first discovered in the cytosol of liver parenchymal cells and was produced in an autocrined way during liver regeneration or organogenesis (1–3). The action of HPO is liver-specific, different from the effects of other well-known hepatic stimulators such as insulin, epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth
factor (IGF-1), and transforming growth factor (TGF-α), which can stimulate proliferation of a wide variety of cell types (4–6). Recently, we characterized the mitogenic effect of HPO on hepatoma cell lines and demonstrated the specific existence of HPO receptor on the membranes of these cells (4, 5). HPO stimulates proliferation of hepatocytes and enhance 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 (4, 5).

In addition, the cytoplasmic functions of two members of HPO/ALR family, ERV1 (yeast homologue of HPO) required for mitochondrial biogenesis and E10R (a viral member of HPO/ALR family) participating in a cytoplasmic pathway of disulfide bond formation (7–9), suggest that HPO/ALR, might also has some important unknown intracellular action. The existence of HPO in the nucleus and cytosol of liver tissues further implicates that HPO could have intracellular function in hepatocytes. So, to identify the putative intracellular protein(s) that can regulate the intracellular functions of HPO through physical interaction, we used the full-length HPO fused to the GAL4 DNA-binding domain (DBD) as a bait in the yeast two-hybrid system to screen a human fetal liver cDNA library. We identified several types of clones that interacted specifically when tested for nutritional selection and β-galactosidase activity. One of these clones contained a cDNA insert with almost the entire coding sequence (amino acids 39–335) of human JAB1 (c-Jun activating domain-binding protein1) (10).

JAB1, a co-activator of c-Jun/AP-1 transcription factor, enhances binding of c-Jun-containing AP-1 complex to their DNA consensus site and increases transactivation of an AP-1-dependent promoter (10). Some molecules can potentiate or inhibit AP-1 transactivation activity by interacting with JAB1, indicating that JAB1 could be involved in some intracellular signaling pathways (11, 12). In this report, we further demonstrated the association of HPO and JAB1 in vitro or in vivo and investigated the biological effect of interaction of HPO with JAB1 on the modulation of AP-1-mediated transcription.

MATERIALS AND METHODS

Yeast two-hybrid screen and interaction assay

The Matchmaker™ two-hybrid system 2 (Clontech, Palo Alto, CA) was used according to the protocols provided by the manufacturer. We subcloned the full-length of HPO into pAS2-1 vector to generate a fusion protein with the GAL4 DNA binding domain. The resulting bait plasmid pAS2-HPO was used to screen a pACT2-human fetal liver cDNA library (Clontech) by the yeast two-hybrid method by using the manufacturer protocols. Briefly, yeast transfecants were cultured on Trp⁻ Leu⁺ His⁻ selection medium, and the resultant colonies were tested for β-galactosidase activity. After segregation, plasmids containing cDNA insert were recovered from yeast cells and tested to see whether they could confer His-requirement in Y190 cells when transformed with the plasmid pAS2-HPO. The entire coding sequence of the human JAB1 cDNA
was isolated from the human fetal liver cDNA library (Clontech) by PCR method.

For interaction assay, different combinations of the cDNAs encoding the putative and rational designed domain sequences derived from HPO in pACT-2 and the cDNA of JAB1 in pAS-2 were cotransfected into Y190 cells and plated on the triple-selection medium. The binding between constructs derived from HPO and JAB1 was quantified by the liquid β-galactosidase assay according to the manufacturer protocol, and the β-galactosidase activity was expressed in Miller units.

GST pull down experiments

JAB1 and HPO cDNAs were amplified by PCR and sequenced to confirm sequence integrity and inserted into pGEX-4T-2 (Amersham, Buckinghamshire, U.K.), respectively. GST fusion proteins were expressed in E. coli and purified as described (13). Human rHPO was purified from E. coli as described (5). For overexpression in mammalian cells, the full-length cDNA of HPO was subcloned into the pFlag-CMV-2 expression vector (provided by Yiping Wu, Beijing Institute of Biotechnology, China) in-frame with a Flag epitope. Crude cell extracts containing endogenous JAB1 and Flag-tagged exogenous HPO expressed in COS-7 cells transfected by pFlag-CMV-2-HPO were prepared as described (13). For binding assay in vitro (13), GST-JAB1 fusion protein immobilized on Sepharose beads (Amersham) were incubated with soluble rHPO or Flag-tagged HPO expressed in COS-7 cells 1 h at 4°C, washed with 50 volumes of binding buffer, eluted with 25 mM glutathione, resolved on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected by anti-HPO or anti-Flag antibodies (Stratagene, La Jolla, CA). Just as the same, we incubated the immobilized GST-HPO fusion protein with crude cells extracts containing endogenous JAB1 and detected the eluted complex by anti-JAB1 antibody (kindly provided by Elisabetta Bianchi, Scientific Institute San Raffaele-DIBIT, I-20132 Milano, Italy). The blots were revealed by enhanced chemiluminescence (ECL) (Amersham).

Co-immunoprecipitation

The full-length cDNA of HPO was subcloned into pEGFP expression vector (Clontech) in-frame with a GFP fusion protein. The JAB1 cDNA was inserted into the pHA expression vector (provided by Yiping Wu) in-frame with a HA epitope. COS-7 cells were cotransfected with both pHAB1 coding for HA-JAB1 fusion and either pEGFP-HPO coding for GFP-HPO fusion or pEGFP (Clontech) coding for only GFP using Lipofectamine (GIBCO, Rockville, MD). After 48 h, the cells were harvested and lysed in 200 µl of lysis buffer (phosphate buffered saline containing 10 mM CHAPS, 5 µg/ml chymostatin, 5 µg/ml leupeptin, 5 µg/ml pepstatin A, 1 mM Na$_3$VO$_4$, and 1 mM NaF) for 1 h on ice and centrifuged at 12,000 $\times$ g for 10 min at 4°C. 20 µl of the cell lysate was used for Western blot analysis to compare protein expression levels. For co-immunoprecipitation, 100 µl of the cell lysate was incubated with a mouse anti-HA
monoclonal antibody (for HA-JAB1) (Roche, Mannheim, Germany) at 4°C for 2 h, and 15 µl of protein A/G–Sepharose beads (Amersham) was added to the reaction for another 2 h of incubation. The Sepharose beads was then washed three times with lysis buffer and resuspended in SDS-PAGE loading buffer for immunoblot analysis.

For Western blot analysis, the polyacrylamide gel was transferred to a PVDF membrane (Millipore, Bedford, MA). Duplicate blots were made from the same set of immuno-precipitation experiments. One blot was probed with rabbit anti-GFP (Clontech) to detect GFP-HPO in the HA-JAB1-GFP-HPO immunocomplex, and the other was probed with a mouse anti-HA monoclonal antibody to monitor the amount of HA-JAB1 protein that had been immuno-precipitated by the anti-HA antibody in each reaction. The blots were revealed by ECL (Amersham). We analyzed interaction of endogenous JAB1 with endogenous HPO in lysates of human liver tissues (BioChain, Hayward, CA). Endogenous JAB1-HPO complexes were precipitated with anti-JAB1 antibody in comparison with control antibody and immuno-blots analyzed with anti-HPO antibody.

**Immunoflorescence assay and living colors study**

For colocalization study of endogenous JAB1 and HPO in vivo, HepG2 cells were fixed in 30% paraformaldehyde, permeabilized in 0.5% Triton X-100, stained with anti-HPO rabbit polyclonal and anti-JAB1 mouse monoclonal antibodies, and incubated with fluorescein (FITC)-linked anti-mouse and Texas red (TR)-linked anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). For living colors study, COS-7 cells were cotransfected with pRFP-JAB1 (pRFP/pDsRed1-N1, Clontech) coding for RFP-JAB1 fusion and pEGFP-HPO coding for GFP-HPO fusion using Lipofectamine (GIBCO). After 48 h, the cells were fixed in 30% paraformaldehyde, and the nuclei were stained by Hoechst 33342. All cell samples were viewed by a confocal laser-scanning microscope (Leica, Bensheim, Germany).

**Transactivation assays (or luciferase assays)**

COS-7 cells were plated on 3.5-cm wells at a density of 2 × 10⁵/well and were cotransfected with AP-1 driven luciferase reporter gene (Stratagene), together with the indicated cDNAs, using the FuGene 6 reagent (Roche). 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, respectively, pcDNA3-c-Jun coding for c-Jun, pcDNA3-JAB1 coding for JAB1, pcDNA3-HPO coding for HPO, pcDNA3-ΔHPO¹⁻⁶³ coding for deleted HPO (amino acids 1–63), pcDNA3-ΔHPO⁶³⁻¹²⁵ coding for deleted HPO (amino acids 63–125), pEF-BOS-antisense-JAB1 coding JAB1 antisense, and pEF-BOS-antisense-IL12Rβ2 coding IL12Rβ2 antisense as a control (provided kindly by Elisabetta Bianchi). Total DNA was kept constant by the addition of the appropriate amount of pcDNA3 for all transfections. At 48 h after transfection, the cells were lysed and assayed for luciferase activity by using reporter gene assay
kits (Roche). We normalized luciferase activity for transfection efficiency by using a cotransfected β-galactosidase expression vector.

**Antibodies and protein analysis**

Cell lysis, immuno-precipitation, and immuno-blotting have been described (13). Polyclonal anti-c-Jun, anti-JNK antibodies, and monoclonal anti-p-c-Jun, anti-p-JNK antibodies were from Santa Cruz Biotechnology. Polyclonal antibodies against active-MAPK and ERK1/ERK2 were from Promega. We detected HA-tagged proteins by using mouse monoclonal antibody against a HA-peptide (anti-HA antibody, Santa Cruz). Flag-tagged proteins were detected using mouse monoclonal antibody against a flag-peptide (anti-flag antibody, Stratagene). GFP-tagged proteins were detected by using rabbit polyclonal antibody against GFP (anti-GFP antibody, Clontech). Polyclonal rabbit antibody against human HPO was generated against bacterially produced recombinant protein.

**RESULTS**

**The interaction of HPO with JAB1 in the yeast two-hybrid system**

During the course of screening for HPO-interacting proteins using the yeast two-hybrid system, we identified several types of clones that interacted specifically when tested for nutritional selection and β-galactosidase activity. Several clones were identified as different sizes of HPO, indicating the oligomerization of HPO. One clone contained a cDNA insert with almost the entire coding sequence (amino acids 39–335) of human JAB1 (10). We respectively subcloned JAB1 full-length cDNA or mutant JAB1 (amino acids 39-335) in pAS2, forming pAS2-JAB1 or pAS2-△JAB1, subcloned HPO cDNA in pACT2, forming pACT2-HPO, and further determined the interaction of HPO with JAB1 or △JAB1, which can be assayed by using the yeast two-hybrid system. Data in Figure 1a demonstrate that cells expressing both HPO and JAB1 or △JAB1 grow well on His⁻ SC medium, suggesting that an interaction occurs between HPO and JAB1 or △JAB1 molecules. β-galactosidase activity assays produce consistent results with His selection experiments (Fig. 1b).

**The N-terminal half sequence of HPO involved in association with JAB1**

To determine which part of HPO molecule is essential for association with JAB1, we performed the yeast two-hybrid study by using different fragments of HPO and the cDNA of JAB1 (Fig. 2). For interaction, the deletion fragments of HPO were subcloned in pACT2 vector in-frame with the transcription activation domain of Gal4 and JAB1 was fused with the DNA-binding domain (pAS2-JAB1). The binding between various fragments of HPO and JAB1 was quantified by the liquid β-galactosidase assay. The results show that N-terminal region (1–63) of HPO is sufficient for binding to JAB1 while the C-terminal half sequence of HPO was not required (Fig. 2).
The interaction of HPO with JAB1 in vitro or in vivo

We further verified a specific association between JAB1 and HPO by GST pull-down and co-immuno-precipitation experiments of JAB1-HPO complexes. Purified soluble recombinant HPO (rHPO) and ectopically expressed HPO (Flag-HPO) in COS-7 cells bound to a GST-JAB1 fusion protein, not to GST alone (Fig. 3a, left and middle). GST-HPO protein, but not GST alone, specifically coprecipitated the endogenous JAB1 protein from cell extracts (Fig. 3a, right). We also observed specific complex formation in vivo between JAB1 and HPO when both proteins were co-expressed in COS-7 cells. Complexes of GFP-tagged HPO and HA-tagged JAB1 were specifically precipitated (Fig. 3b). Furthermore, endogenous JAB1-HPO complexes were precipitated from nuclear extracts of human fetal liver (Fig. 3c, the upper) and adult liver tissues (Fig. 3c, the lower). Specific co-precipitation of HPO was detected by anti-HPO Western blot when anti-JAB1 antibody but not control antibody was used for coprecipitation. Together, these protein–protein interaction assays in vitro or in vivo combined with those of the yeast two-hybrid screen indicate that HPO specifically binds to JAB1 both in vitro and in vivo, and implicate that the intracellular function could be mediated by JAB1-dependent pathway.

Similar distribution or colocalization of HPO with JAB1 within cells

The association between HPO and JAB1 was confirmed further in colocalization of the two proteins in vivo (Fig. 4). The endogenous HPO and JAB1 in human hepatoma line HepG2 cells were visualized by double immuno-fluorescence staining. HPO was localized in both cytoplasm and nucleus with a relative stronger staining of the nucleus (Fig. 4a). And HPO/ALR and its yeast homologue ERV were reported previously to be located in cytosol of yeast cell (7). Similarly, the endogenous JAB1 was localized primarily in nucleus except in nucleolus (Fig. 4a) (14). Figure 4a obviously demonstrated that endogenous HPO and endogenous JAB1 have a similar pattern of distribution or colocalization in HepG2 cell. In addition, transiently expressed GFP-HPO gather in nuclear periphery of COS-7 cells (Fig. 4b), inconsistent with the localization of endogenous HPO in HepG2 cells. The localization of transiently expressed RFP-JAB1 in COS-7 cells is also different from the endogenous JAB1 in HepG2 cells. Single RFP-JAB1 localizes in the cytoplasm of COS-7 cells rather in the nucleus like in HepG2 cells (Fig. 4b). When HPO and JAB1 were cotransfected into COS-7 cells, the distribution of the two proteins changed (Fig. 4c). A part of GFP-HPO proteins was presented diffusely in the cytoplasm and colocalized with RFP-JAB1 between the two nuclei in a binucleated cell. The fact that HPO colocalized with JAB1 implicated their physiological interaction.

Regulation of AP-1 activity by the physical interaction of HPO with JAB1

JAB1 could activate the AP-1-dependent promoter (10–12). To study the function of the HPO-JAB1 interaction, we determined whether HPO modulates JAB1-induced AP-1 activity.
We reasoned that HPO, by binding to JAB1, could modulate this AP-1 activity. So, COS-7 cells were transfected with an AP-1-driven luciferase reporter gene in the presence of c-Jun, JAB1, and HPO, and the reporter gene activity was measured. The results indicated that overexpression of JAB1 caused an increase in the relative activation levels of the AP-1 reporter and that expression of HPO further enhanced potentiation of AP-1 reporter gene activity that was induced by cotransfected JAB1 (Fig. 5a). HPO appeared to increase JAB1-induced AP-1 activity in a concentration-dependent manner (Fig. 5a). The effect of HPO in activating JAB1-induced AP-1 activity peaked when 0.2 µg HPO cDNA was cotransfected. Furthermore, ectopical expression of HPO alone could potentiate AP-1 activity presumably through endogenous JAB1 (Fig. 5b). These results indicated a synergy in enhancing transactivation of AP-1 activity between HPO and JAB1. To confirm further the role of HPO in potentiation of JAB1 on AP-1 activity, we studied the effect of suppressed expression of JAB1. Overexpression of JAB1 antisense constructs resulted in an obvious reduction of endogenous JAB1 level (Fig. 5c, the lower panel) and significantly inhibited the activation of the AP-1 reporter gene induced by intracellularly expressed HPO (Fig. 5c, the upper panel). The deletion mutants of HPO; i.e., either HPOΔ1–63 or HPOΔ63–125, were unable to potentiate AP-1 activity in response to JAB1, although HPOΔ1–63 was capable of binding to JAB1. These results together indicate that JAB1 is involved in the intracellular signaling transduction from HPO to AP-1.

**JAB1 increases phospho-c-Jun levels and intracrine HPO enhances this effect of JAB1**

JAB1 can activate JNK activity and increase phosphorylation of c-Jun, leading to the activation of AP-1 promoter activity (12). To determine whether HPO regulates this function of JAB1 by stimulating JNK activity, we then detected the levels of c-Jun and phosphorylated c-Jun in COS-7 cells cotransfected as in Figure 5a except by omitting both reporter genes. The results indicated that JAB1 actually increased the level of phosphorylated c-Jun (Fig. 6a), in agreement with potentiation of AP-1 activity induced by JAB1 (Fig. 5a) and that HPO further enhanced this function of JAB1 (Fig. 6a), in concordance with HPO-induced enhancement of potentiation of AP-1 activity induced by JAB1 (Fig. 5a). These results conform to the data of AP-1 reporter gene activation assay (Fig. 5a). However, neither JAB1 nor HPO had any effect on expression of transfected c-Jun, endogenous JNK, or its phosphorylation (Fig. 6a). These data showed that c-Jun in HPO intracellular signaling may be phosphorylated via JNK-independent pathway as previous reported (15).

Our previous data indicate that extracellular HPO triggers MAPK/ERK pathway in hepatoma cells by its specific receptor (4, 5). To determine whether HPO activates AP-1 activity via MAPK/ERK pathway in an extracellular triggering way such as in HepG2 cells, besides physical interaction with JAB1, we investigated whether HPO activates ERK in COS-7 cells, which were used as host cells to demonstrate the physical interaction of HPO/JAB1 in vivo and its biological consequence; that is, the potentiation of AP-1 activation. COS-7 cells were transfected with HPO
cDNA. The results demonstrated that ectopically expressed HPO was not secreted into extracellular media at all like observation from hepatocytes (16) but in an intracrine form (Fig. 6b) as similar as FGF (17, 18). Unlike the positive control EGF (19, 20), neither recombinant HPO added to the culture media nor ectopically expressed HPO within cells could activate MAPK/ERK activity in COS-7 cells (Fig. 6c). Taken together, these data indicate that HPO interacts with JAB1 to activate AP-1 transcription activity by potentiating phosphorylation of c-Jun in a MAPK-independent fashion and that intracrine HPO with JAB1 represents a new intracellular short-cut signaling pathway, which could provide a novel mechanism in triggering of immediately early response transcription factors during initiation of liver regeneration.

DISCUSSION

Cytokines and growth factors with mitogenic effects induce potentiation of AP-1 transactivation function by triggering several signal transduction pathways (21). Our data show that intracrine HPO interacts with JAB1 in vivo to regulate AP-1 transcriptional activity, indicating a new mechanism for AP-1 activation by cytokines and growth factors. According to our knowledge, HPO is the first intracrine growth factor identified to trigger AP-1 pathway through intracellular interaction with a transcriptional co-activator. In addition, ERV/HPO family has thiol oxido-reductase to participate in a cytoplasmic pathway of disulfide bond formation (9). Also, another cytokine MIF (macrophage migration inhibitory factor) as a redox enzyme and regulator of cell differentiation can bind to JAB1 and acts broadly to negatively regulate JAB1-controlled pathways (12, 22, 23). Presently, increasing evidence has indicated that cellular redox status modulates various aspects of cellular events, such as proliferation and apoptosis, wherein redox regulation of transcription factors, including AP-1, NF-κB, Myb, and Ets, is an important issue (24). Considering the similar characteristics between MIF and HPO that both intracellularly interact with JAB1 and act as redox enzyme and regulator of cell growth, the regulation of JAB1 function or AP-1 transcriptional activity by both HPO or MIF may be associated with their redox enzyme activity.

JAB1 is a subunit of COP9 complex containing at least eight subunits (15, 25). The plant COP9 is involved in light-mediated signal transduction (26). COP9 is reported to have kinase activity that phosphorylate IκBα and c-Jun (15, 25). Our data implicate that HPO possibly increase the phosphorylation of c-Jun under mediation of JAB1-containing COP9 signalosome, leading to the activation of AP-1 activity. JAB1 is seemingly regulated by different proteins to redistribute in cells and to undergo different functions. For example, after interaction with integrin LFA-1 that transduces signals, JAB1 moves from an extranuclear to a nucleus compartment to induce AP-1 activation (11). JAB1 also can interact with p27kip1 in nucleus and direct the movement of it from the nucleus to the cytoplasm for proteins breakdown (14). Apparently JAB1 is a protein with multifunctional characteristics. In our work, we found the colocalization of HPO with JAB1 in nucleus. So it can be speculated that the COP9 complex could represent an intracellular signal crossroad, where signals from the extracellular or intracellular environment are coordinated with
transcriptional activation and with the regulation of the related functions of cells.

Because the specific receptor of HPO exists only on the surface of liver cells (4), the action of HPO to trigger MAP kinase pathway might be liver-specific (5). However, the intracellular function of HPO to stimulate AP-1 activity through JAB1 indicates a new pathway of HPO signaling to regulate gene expression, which may contribute to the physiological regulation of HPO-originated tissues, such as testis, kidney, brain, and liver (8). Whether the dual signaling pathways of HPO cooperate with each other or act separately remains to be elucidated. It is possible that extracellular HPO secreted as an autocrine growth factor maintains the autonomous growth of liver cells and intracellular HPO as an intracrine factor triggers AP-1 pathway by regulation of JAB1 for immediately early response when its intracellular level is increased after partial liver hepatectomy or liver injury. The rapid upregulation of HPO expression and AP-1 activity during liver regeneration might support this hypothesis (27–29), which would be valid for further investigation.

ACKNOWLEDGMENT

We thank Elisabetta Bianchi for kindly providing anti-JAB1 mAb, JAB1, and IL-12R β2 subunit antisenses; Xianwu Li for help in reporter gene assays; and Lin Mei, and Yi Rao for both scientific and linguistic suggestions. This project was supported partially by Chinese State Key Program of Basic Research, Natural Science Foundation of China, National Distinguished Young Scholar Award of China, State High-tech Program of China, and Beijing City Municipal Key Project.

REFERENCES


signalosome-directed c-Jun activation/stabilization is independent of JNK. *J. Biol. Chem.* **274**, 35297–35300


*Received July 5, 2001; revised September 13, 2001.*
Figure 1. Interaction between HPO and JAB1 in yeast. A) Yeast cells harboring the combination of pACT2-HPO and pAS2-JAB1 or pAS2-ΔJAB1 plasmids were streaked on synthetic media without histidine. Left, pACT2-HPO + pAS2-JAB1; right, pACT2-HPO + pAS2-ΔJAB1; top, pACT2-HPO + pAS2 empty vector only; bottom, pAS2-JAB1 + pACT2 empty vector only. B) Yeast cells extracts were assayed for β-galactosidase activity, and average values from four independent assays are presented. 1) control; 2) pACT2-HPO + pAS2-JAB1; 3) pACT2-HPO + pAS2-ΔJAB1. The error bar indicates SD. Control indicates a study with the plasmid alone. β-galactosidase activity is indicated in Miller units.
Figure 2. Binding region of HPO protein with JAB1 identified in yeast. Binding between different fragments of HPO and full-length JAB1 was quantified by liquid β-galactosidase assay. Results are an average of four independent studies and error bar indicates SD. Control indicates a study with the plasmid alone. β-galactosidase activity is indicated in Miller units (upper panel). The constructs derived from HPO used in this study are shown (lower panel). Number indicates amino acid position.
Figure 3. HPO interacts with JAB1 in vitro and in vivo. A) rHPO and flag-HPO expressed in COS 7 cells were incubated with immobilized GST, GST-JAB1 fusion proteins. Bound proteins were eluted with excess glutathione and analyzed by immunoblotting with anti-HPO rabbit polyclonal and anti-flag mouse monoclonal antibodies (left and middle). Extracts of no-transfected cells were incubated with immobilized GST, GST-HPO fusion proteins. Bound proteins were eluted with excess glutathione and analyzed by immunoblotting with anti-JAB1 monoclonal antibodies (right). B) COS 7 cells were cotransfected with HA-tagged JAB1 and either GFP-tagged HPO or GFP control vector. The cells lysates were incubated with a mouse anti-HA antibody in the protein A/G–Sepharose beads. The immuno-complex was resolved on SDS-PAGE and was analyzed by immunoblotting with anti-GFP antibody. Of the total cell lysate used in immuno-precipitation, one-tenth was loaded onto lanes 1 and 2 as an indication of the relative expression level for GFP and GFP-HPO. Lanes 4 and 5 are from cells expressing HA-JAB1/GFP and HA-JAB1/GFP-HPO, respectively. Lane 3 is a control for lane 5 with mouse mock antibody (normal IgG). A duplicate blot was also probed with anti-HA antibody to monitor the amounts of JAB1 protein precipitated in each reaction (bottom). C) Endogenous HPO-JAB1 complex were immunoprecipitated from nuclear extracts of human fetal liver (upper) and human adult liver (lower) with anti-JAB1 antibody and HPO was detected by immuno-blotting with anti-HPO antibody. Precipitation with mouse mock antibody (normal IgG) served as controls. Two blots were also probed with anti-JAB1 antibody to monitor the amounts of JAB1 precipitate in each reaction (bottoms of the upper and lower panels).
Figure 4. JAB1 colocalizes with HPO. A) HepG2 cells were stained for HPO (red) and JAB1 (green) by indirect immunofluorescence. Arrowheads indicate nucleolus. B) Localization of HPO and JAB1. COS 7 cells were transfected with GFP-HPO or RFP-JAB1 constructs, respectively. The nuclei were stained by Hoechst 33342 (blue). C) Colocalization of JAB1 with HPO. COS 7 cells were cotransfected with RFP-JAB1 and GFP-HPO constructs. Arrowheads indicate the field of colocalization. All cell samples were visualized by confocal microscopy (Leica), and all white bars indicate 20 µm.
Figure 5. Regulation of AP-1 activity by HPO interacting with JAB1. A) HPO enhances JAB1-mediated activation of AP-1 reporter gene activity in COS 7 cells. COS 7 cells were cotransfected with AP-1-driven-luciferase reporter, c-Jun, JAB1, and HPO or its mutants. B) HPO stimulates AP-1 transcriptional activity in COS 7 Cells. COS 7 cells were cotransfected with AP-1-driven-luciferase reporter, c-Jun, and HPO or its mutant. C) Endogenous JAB1 contributes to the activation of AP-1 transcriptional activity induced by HPO in COS 7 cells. COS7 cells were cotransfected with AP-1-driven-luciferase reporter, c-Jun, HPO, and one of the indicated antisense constructs. The lower panel indicates the reduction of endogenous JAB1 by JAB1 antisense or IL-12R β2 subunit antisense (control antisense). As a loading control, β-actin content in the cell extracts was analyzed by immunoblotting. Each luciferase activity was normalized to a cotransfected β-galactosidase expression vector. Shown are representative examples at least three independent experiments performed in duplicate. The error bar indicates SD.
**Figure 6.** JAB1 increases phospho-c-Jun levels and intracrine HPO enhances this effect of JAB1. A) COS 7 cells were cotransfected with the indicated cDNAs (designed as in Fig. 5a). The cell lysates were resolved on SDS-PAGE and were analyzed by immunoblotting with anti-phospho-c-Jun (specific for c-Jun p39 phosphorylated on serine-63), anti-c-Jun (specific for c-Jun; non-cross-reactive with Jun b or Jun D), anti-phospho-JNK (reactive with phosphorylated JNK1, JNK2, and JNK3, non-cross-reactive with ERK1, ERK2, or p38), anti-JNK1 antibodies (reactive with JNK1 p46, JNK2 p54, and JNK3), respectively. β-actin immuno-blots served as a control. B) COS 7 cells were transfected with pFLAG-CMV-2-HPO and pcDNA3-HPO vectors, respectively. The lysates (L) and culture supernatants (S) were resolved on SDS-PAGE and detected by anti-flag and polyclonal anti-HPO antibodies, respectively. C) Serum-starved COS 7 cells were stimulated for 5 min with EGF (10 ng/ml) or HPO (50 ng/ml) and lysed (5). These cell lysates and other cell lysates through transfection with pcDNA3-HPO vector were detected by antibodies against phospho-MAPK or MAPK.