The Potentiation Role of Hepatopoietin on Activator Protein-1 Is Dependent on Its Sulfhydryl Oxidase Activity*

Received for publication, April 17, 2003, and in revised form, September 17, 2003
Published, JBC Papers in Press, September 18, 2003, DOI 10.1074/jbc.M304057200

Xiaoxiao Chen‡§, Yong Li‡, Kaibua Wei‡, Li Li‡, Wanli Liu‡, Yunping Zhu‡, Zongyin Qiu§, and Fuchu He‡∥**

From the *Department of Systems Biology, Beijing Institute of Radiation Medicine, Chinese National Human Genome Center at Beijing, Beijing 100850, China, ‡Department of Clinical Biochemistry, Chongqing University of Medical Sciences, Chongqing 400016, China, §National Center of Biomedical Analysis of China, Beijing 100850, China, and ¶College of Life Science and Technology, Tsinghua University, Beijing 100051, China

Hepatopoietin (HPO) is a novel hepatotropic growth factor that stimulates hepatocyte proliferation by two pathways. In the first, intracellular HPO specifically modulates the activator protein-1 (AP-1) pathway through JAB1 (Jun activation domain-binding protein 1), whereas in the second, extracellular HPO triggers the mitogen-activated protein kinase pathway by binding its specific receptor on the cell surface. In this report we demonstrate that HPO is a flavin-linked sulfhydryl oxidase, and the invariant CXXC (Cys-Xaa-Xaa-Cys) motif in HPO is essential for the enzyme activity of HPO but not for its dimerization nor for its binding ability with JAB1. Two intramolecular disulfides were identified in HPO by mass spectrometry, one of which is formed by the redox CXXC cysteine residues. HPO site-directed mutants (Cys/Ser) at active sites, which lost sulfhydryl oxidase activity, could not increase c-Jun phosphorylation and failed to potentiate JAB1-mediated AP-1 activation. However, the mutants still have mitogenic stimulation and mitogen-activated protein kinase activation effects on HepG2 cells. Thus, it can be concluded that the potentiation role of HPO on AP-1 is dependent on its sulfhydryl oxidase activity.

Hepatopoietin (HPO) is a novel hepatotropic growth factor that stimulates hepatocyte proliferation by two pathways. In the first, intracellular HPO specifically modulates the activator protein-1 (AP-1) pathway through JAB1 (Jun activation domain-binding protein 1), whereas in the second, extracellular HPO triggers the mitogen-activated protein kinase pathway by binding its specific receptor on the cell surface. In this report we demonstrate that HPO is a flavin-linked sulfhydryl oxidase, and the invariant CXXC (Cys-Xaa-Xaa-Cys) motif in HPO is essential for the enzyme activity of HPO but not for its dimerization nor for its binding ability with JAB1. Two intramolecular disulfides were identified in HPO by mass spectrometry, one of which is formed by the redox CXXC cysteine residues. HPO site-directed mutants (Cys/Ser) at active sites, which lost sulfhydryl oxidase activity, could not increase c-Jun phosphorylation and failed to potentiate JAB1-mediated AP-1 activation. However, the mutants still have mitogenic stimulation and mitogen-activated protein kinase activation effects on HepG2 cells. Thus, it can be concluded that the potentiation role of HPO on AP-1 is dependent on its sulfhydryl oxidase activity.

Hepatopoietin (HPO) is a novel hepatotropic growth factor that stimulates hepatocyte proliferation by two pathways. In the first, intracellular HPO specifically modulates the activator protein-1 (AP-1) pathway through JAB1 (Jun activation domain-binding protein 1), whereas in the second, extracellular HPO triggers the mitogen-activated protein kinase pathway by binding its specific receptor on the cell surface. In this report we demonstrate that HPO is a flavin-linked sulfhydryl oxidase, and the invariant CXXC (Cys-Xaa-Xaa-Cys) motif in HPO is essential for the enzyme activity of HPO but not for its dimerization nor for its binding ability with JAB1. Two intramolecular disulfides were identified in HPO by mass spectrometry, one of which is formed by the redox CXXC cysteine residues. HPO site-directed mutants (Cys/Ser) at active sites, which lost sulfhydryl oxidase activity, could not increase c-Jun phosphorylation and failed to potentiate JAB1-mediated AP-1 activation. However, the mutants still have mitogenic stimulation and mitogen-activated protein kinase activation effects on HepG2 cells. Thus, it can be concluded that the potentiation role of HPO on AP-1 is dependent on its sulfhydryl oxidase activity.

* This project was supported in part by the Chinese State Key Program of Basic Research, Natural Science Foundation of China, Chinese National Distinguished Young Scholar Award 39620514, State High Technology Major Project of China, and Beijing City Municipal Key Project. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed. Tel./Fax: 8610-68171208; E-mail: hef@nic.bmi.ac.cn.

† The abbreviations used are: HPO, hepatopoietin; rhHPO, recombinant human HPO; ALR, augmenter of liver regeneration; DTT, dithiothreitol; ERV1, essential for respiration and vegetative growth; SOX, sulfhydryl oxidase; JAB1, Jun activation domain-binding protein 1; AP-1, activator protein-1; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; PMF, peptide mass fingerprinting; DMEM, Dulbecco's modified Eagle's medium; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

†† This paper is available online at http://www.jbc.org

49022 This paper is available online at http://www.jbc.org
molecular mechanism of their actions has remained unclear, especially the precise relationship between the cytokine and enzyme activities. Whether the biological functions of HPO in mammals are created by its enzyme activity or its cytokine effect separately or by their interaction is a mystery.

In this report we intend to discuss the relationship between the enzymatic activity and the intra/extracellular cytokine effect of HPO in cellular signal transduction. Considering the importance of the conserved CXXC motif of HPO in its SOX activity, the dimerization of HPO and HPO-JAB1 interaction in its cytokine effects, we investigated the alteration of these factors by single or double cysteine to serine site mutagenesis of CXXC. Then the mitogenic stimulation and MAPK activation effect by the extracellular HPO mutants were detected. We further investigated whether the intracrine HPO mutants could increase phospho-c-Jun levels and AP-1 activation. Here we demonstrated that the enzymatic activity of HPO might be a key regulator in intracellular mediation of the AP-1 pathway through JAB1.

MATERIALS AND METHODS

Disulfide Bond Analysis of HPO by Mass Spectrometry

In-gel Digestion—Anaerobic purification is performed to avoid disulfide bond formation, which may occur in aerobic purification. The isoelectric focus-separated HPO band with a pI 6.44 was excised, washed in 100 mM NH₄HCO₃, destained in 100 mM NH₄HCO₃, 50% ACN three times, and dried by vacuum (SpeedVac). The gel was rehydrated in 100 µl of bovine trypsin or Glu-C (Sigma) buffer (0.01 µg/µl) and incubated for 16 h. For Glu-C (Sigma) digestion, phosphate buffer of pH 4.0 was used. Peptide extraction was carried out first in 100 µl of 5% trifluoroacetic acid (Merck) at 40 °C and then in 2.5% trifluoroacetic acid, 50% ACN for 1 h. The resulting digest was dried in vacuum and dissolved in 10 µl of 0.1% trifluoroacetic acid, 60% ACN for mass spectrometry analysis.

Digest Reduction—The digest was dissolved in 100 µl of 50 mM NH₄HCO₃, 1 µl of DTT (10 µg/µl) was added at 50 °C for 1 h, and 10 µl of 5% trifluoroacetic acid was added for acidification and dilution.

Mass Spectrometry—Peptide mass fingerprinting (PMF) of HPO was performed on Kratos Kompact SEQ matrix-assisted laser desorption ionization time-of-flight mass spectrometry with an acceleration voltage of 20 kV in positive mode. All spectra were acquired in linear mode. The matrix of a-cyano-4-hydroxycinnamic acid was dissolved in 0.1% trifluoroacetic acid, 60% ACN, and calibration was carried by external bovine insulin (5 pmol, Sigma).

Preparation of Recombinant Proteins

HPO open reading frame (390 base pairs, GenBank™ AF124603) (21) was amplified by PCR. The in vitro synthesis of HPO (15 kDa) is performed by PCR, and the cDNA of JAB1 in pRK2 (DNA-BD vector) was inserted into the pCMV-Myc expression vector (Invitrogen). The HPO (wild type and mutants) cDNAs were cotransfected into COS-7 cells using LipofectAMINE 2000. After 24 h, the cells were rinsed three times with ice-cold phosphate-buffered saline and lysed in 1 ml of lysis buffer (0.1M Tris-HCl (pH 7.5), 0.3 M NaCl, 2 µM Protein A/G agarose (Santa Cruz Bio-technology) was added to 900 µl of the lysate. The proteins were blotted onto Hybond-P membranes (Amersham Biosciences). The membranes were first blocked by incubation in NaCl/Tris/Tween containing 5% fat-free milk overnight at 4 °C, sequentially incubated with mouse anti-His (C-term) monoclonal antibody (Invitrogen) or mouse anti-Myc monoclonal antibody (Cell Signaling Technology) and peroxidase-conjugated secondary antibody, and then detected by Western blotting luminal reagent (Santa Cruz Biotechnology). Finally, the various Western blotting bands were scanned by GS-710 calibrated imaging densitometer (Bio-Rad).

His Pull-down Assay

For pull-down experiments, 100 µg of purified recombinant His-JAB1 fusion protein (see above for details) was immobilized on 100 µl (resuspended 50% bed resin) of nickel nitritolriatic acid-agarose beads (Qiagen), incubated for 15 min at room temperature, and then washed with 50 volumes of 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 8.0. The plasmids of pCMV-Myc-HPO (wild type and mutants) were transfected into COS-7 cells using LipofectAMINE 2000 (Invitrogen). After being transfected for 24 h, the cells were rinsed three times with ice-cold phosphate-buffered saline and lysed in 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 8.0 (Qiagen), with a mixture of protease inhibitor (Roche Applied Science) by sonication on ice. Six 15-s bursts at 75 W were used, with a 10-s cooling period between each burst. The lysate was centrifuged at 10,000 rpm for 30 min, and the crude protein extracts (1 mg) were incubated with the His-JAB1 fusion protein-immobilized nickel nitritolriatic acid-agarose for 1 h at 4 °C, then washed twice with 50 volumes of 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8.0 (Qiagen), and eluted with 50 volumes of 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8.0 (Qiagen). The solution was detected by anti-Myc monoclonal antibody. The blots were revealed by Western blotting luminal reagent.

Co-immunoprecipitation

The full-length cDNA of JAB1 was subcloned into pcDNA3 expression vector (Invitrogen). The HPO (wild type and mutants) cDNAs were inserted into the pCMV-Myc expression vector with a Myc epitope. COS-7 cells were cotransfected with both pcDNA3-JAB1 coding for JAB1 and either pCMV-Myc-HPO coding for HPO fusion or pCMV-Myc coding for only Myc using LipofectAMINE 2000. After 24 h of transfection, the cells were harvested, and 30 µg of protein was used for immunoprecipitation (20 µl of protein A/G agarose (Santa Cruz Biotechnology) was added to 900 µl of the cell lysate and incubated 4 h at 4 °C for preimmunoprecipitation. The mixture was centrifuged at 10,000 g for 3 min at 4 °C, and the supernatant was incubated with a rabbit anti-JAB1 polyclonal antibody (for JAB1) (Santa Cruz Biotech-
RESULTS

Identification of Two Intramolecular Disulfide Bonds of HPO by Mass Spectrometry—The SOX protein has a redox-active disulfide bridge formed by the enzyme active site CXXC cysteine residues, whereas the thiol-disulfide state of HPO is not clear. Thus, we performed the following experiments by mass spectrometry with 15-kDa rhHPO, resulting in the identification of two intramolecular disulfide bonds.

Peptide mass fingerprinting before and after DTT reduction of HPO trypic digests showed that there were four peptides involving disulfide bonds (Fig. 1, A and B). The base peak of m/z 1970.4 shifted to m/z 1971.9 after reduction, so there was an intramolecular disulfide bond in this peptide. The sequence of this peptide (searched by disulfide bond analysis software S Mass analyzer) is ACFTQWLCHLHNEVNR, with the disulfide bond formed between Cys-90 and Cys-96. The Glu-C PMF before/after DTT reduction showed a similar result since there was a similar m/z shift, from m/z 2115.2 to m/z 2117.3, corresponding to the sequence TRTRACFTQWLCHLHNE (Fig. 1, C and D). The other intramolecular disulfide bond, formed between Cys-67 and Cys-70, was also identified by m/z shift in PMFs before/after DTT reduction; the shift was from m/z 1473.7 to m/z 1475.5 in trypic PMF and from m/z 2294.2 to m/z 2294.8 in Glu-C PMF, corresponding to the sequences FYP-CEECAEDLR and MAQFIHLFSKFPYCEECAE, respectively.

Of the 8 cysteines in the 15-kDa HPO molecule, 4 take part in the formation of two internal disulfide bonds (Fig. 1E). The one between Cys-67 and Cys-70 agrees with the SOX redox-active disulfide, which is adjacent to FAD moieties. The other disulfide bond between Cys-90 and Cys-96 has been identified for the first time. The CXXC motif and the redox-active disulfide are conserved in Erv1p, Erv2p, 15-kDa rat ALR, and 15-kDa HPO. However, their disulfide bridges are different because of amino acid sequence disagreements (28, 29) (Fig. 1E).

HPO (15 kDa) Is a FAD-linked Sulfhydril Oxidase and the CXXC Motif Is Essential for Its Enzymatic Activity—HPO exists as 2 isoforms at 15 and 23 kDa in liver cells (21). The different spliced forms of HPO not only reside in various cellular locations but also might have different functions. To examine the FAD-linked SOX activity of 15-kDa HPO, 3 cysteine to serine mutants of CXXC active disulfide bond were designed: HPOC67S, HPOC70S, and HPOC90S. Cys-90 in another internal disulfide was also changed into serine (HPOC90S), as a control. We expressed the C terminus hexahistidyl-tagged recombinant proteins of HPO wild type and mutants in E. coli and purified them to homogeneity. Then the dialyzed proteins were directly used for spectroscopy to identify the bound FAD. The purified proteins are bright yellow after 24 h of dialysis at 4 °C, and Fig. 2 shows that the HPO protein spectra are all characteristic for a FAD moiety but exhibit distinct differences from free FAD. The absorbance maximum of free FAD at 450 nm is shifted by about 5 nm in the wild type HPO protein. The HPO Cys → Ser mutant (at positions Cys-67, Cys-70, Cys-67/Cys-70 and Cys-90) proteins can still bind FAD, indicating that they fold normally when overexpressed in E. coli. The FAD content of the protein was calculated from the absorption at 460 nm by using an extinction coefficient of 10 mM/cm, a value that is typical for flavin-containing proteins (27, 30, 31). The ratio of the wild type HPO protein concentration and the flavin concentration was about 0.78, indicating that one HPO monomer binds one FAD molecule normally. The FAD molecule can be released from the protein by boiling or by precipitation of the protein in 10% trichloroacetic acid, demonstrating that it is firmly but not covalently bound to HPO. The fluorescence of the released flavin increases on transfer from neutral to acidic pH; this is a characteristic of FAD, not FMN (32, 16).
Reduced lysozyme was then used as the substrate in a standard enzyme assay for SOX. The reactions were started by the addition of the purified HPO proteins to the substrate mixture. At different time points, aliquots were withdrawn and analyzed for their thiol content (12). The results demonstrated that the active site C\textsuperscript{XX}C mutants (HPO\textsubscript{C67S}, HPO\textsubscript{C70S}, and HPO\textsubscript{C67S/C70S}) lost the enzyme activity completely because of the spoiling of the redox-active disulfide and that HPO wt and HPO\textsubscript{C90S} were able to oxidize lysozyme in a time-dependent manner, whereas HPO\textsuperscript{C90S} exhibited slightly decreased activity compared with its wild type (Fig. 3). Like Erv1p/Erv2p, the redox-active residues Cys-67 and Cys-70 in HPO might be proximal to FAD (Fig. 1E), and the redox-active disulfide transfers 2 electrons from the substrate protein to FAD, then to oxygen. Considering the role of the disulfide bond between Cys-90 and Cys-96 in the HPO-FAD interaction, the single 

![Image](image_url)
cysteine mutation changed the optimal status of HPO-FAD binding and diminished the enzyme activity to some degree but not very seriously. Taken together, 15-kDa HPO exhibits FAD-linked sulfhydryl oxidase activity, and the CXXC motif is essential for its enzymatic activity.

**HPO Cys → Ser Mutation in the CXXC Motif Could Disturb Neither the HPO Dimerization nor HPO-JAB1 Interaction**—We previously reported that HPO exists naturally in a homodimeric form. Here we tested the dimerization of the four Cys to Ser mutant HPOs (HPOC67S, HPOC70S, HPOC67S/C70S, and HPOC90S) that had been demonstrated to disturb the enzymatic activity of HPO. The HPO mutants were expressed in prokaryotic cells (E. coli) or in mammalian cells (COS-7), and then their homodimers were detected in nonreducing but denaturing conditions. Western-blotting analysis with anti-His or anti-Myc antibody revealed a series of 30-kDa proteins in the absence of reducing agent, suggesting that mutant HPOs can form homodimers as wild type (Fig. 4, A (E. coli) and B (COS-7)). The addition of reducing agent to the sample resulted in the detection of a single band of 15 kDa, corresponding to the monomeric form of HPOwt. The results shown in Fig. 4, A and B, revealed that the substitution of these three cysteines with serine did not interfere in the dimerization of HPO, i.e. mutant HPO could also form homodimer in *in vitro* and *in vivo*.

Considering that the interaction of HPO with JAB1 is essential for the c-Jun phosphorylation and AP-1 activating effects, we investigated the interaction between JAB1 and Cys → Ser HPO mutants through *in vitro* and *in vivo* assays. First, the Matchmaker™ two-hybrid system 2 (Clontech) was used. The pACT2-HPOwt or a set of pACT2-HPO Cys → Ser mutants separately were cotransfected with pAS2-JAB1 into yeast Y190. Then the cells were cultured in SD/leu/−/trp−/his− medium or SD/leu/−/trp−/his− medium, whereas the controls (pAS2+ACT2, pAS2-JAB1+pACT2, pAS2+pACT2-HPOwt) could not (Fig. 4, C, D, and E). The binding ability between HPOs and JAB1 was quantified by the liquid galactosidase assay, and the galactosidase activity was expressed in Miller units. The similar data in Miller units show that the HPO cysteine mutants bind JAB1 as efficiently as wild type HPO (Fig. 4F). These results indicate that in the yeast two-hybrid system cysteine mutant HPO fusion protein can fold correctly and interact specifically with JAB1 as does wild type HPO.

We further verified the specific association between HPO mutants and JAB1 by His pull-down assay and co-immunoprecipitation of HPO-JAB1 complexes. The pull-down assay was performed as described under "Materials and Methods." The ectopically expressed HPO (Myc-HPO) in COS-7 cells bound to the His-tagged JAB1 fusion protein specifically (Fig. 4G).

For co-immunoprecipitation assay, the plasmids pcDNA3-JAB1 and pCMV-Myc-HPO (HPOwt, HPOC67S, HPOC70S, HPOC67S/C70S, and HPOC90S, respectively) were cotransfected into COS-7 cells. Both proteins co-expressed in COS-7 cells, and the HPO-JAB1-specific complexes in *in vitro* were precipitated by anti-JAB1 antibody. HPO was detected by anti-Myc Western blot when the anti-JAB1 antibody but not the control antibody was used for co-precipitation (Fig. 4H). Together, all these protein-protein interaction assays in *in vitro* or *in vivo* with those of the yeast two-hybrid interaction assay indicate that the cysteine mutant HPO specifically binds to JAB1 both in *in vitro* and *in vivo*, i.e. HPO Cys → Ser mutation could not disturb the HPO dimerization or HPO-JAB1 interaction.

**HPO Cys → Ser Mutation in the CXXC Motif Could Not Disturb Its Stimulation of the MAPK Pathway in HepG2 Cells**—15-kDa rhHPO can stimulate proliferation of hepatocytes or hepatoma cells *in vitro*, and autocrine HPO can trigger the MAPK pathway by binding its specific receptor on the cell membrane. To determine whether the enzymatic activity is related to the mitogen-activating effect, wild type and the Cys → Ser mutant rhHPOs were added to the culture of 48-h serum-starved HepG2 cells. Both the wild type and the mutant re-combinant proteins (50 ng/ml) could stimulate the DNA synthesis and cell proliferation (Fig. 5). Furthermore, detection of the endogenous activated MEK and activated MAPK levels by Western blot with phospho-specific antibodies indicates that the enzyme active site mutants of HPO (HPOC67S, HPOC70S, HPOC67S/C70S, and HPOC90S)
and HPO$^{C67S/C70S}$ are still able to induce phosphorylation of MEK and MAPK in HepG2 cells (Fig. 6). The mutation of cysteine seemed not to tamper with the normal folding nor dimerization of HPO protein nor with the activation of the MAPK signaling pathway in HepG2 cells by ligating the receptors. These results suggest that the sulfhydryl oxidase activity
HPO Activates AP-1 in Redox Manner

of HPO does not participate in its extracellular cytokine effect through the MAPK pathway.

Potentiation Activity of HPO on AP-1 through JAB1 Depends on Its Sulfhydryl Oxidase Activity, and the Enzyme Active Site CXXC Is Also Essential for HPO to Increase c-Jun Phosphorylation—Because the enzyme activity is not essential to the extracellular activity of HPO and the intracellular HPO-JAB1 interaction could activate the AP-1-dependent promoter, we detected the relationship between the enzymatic and intracellular activity when HPO triggers the AP-1 pathway through interaction with JAB1. To study the enzymatic effect on the AP-1 pathway, we performed the luciferase assays with Cys → Ser mutant HPOs. COS-7 cells in the 24-well plate were cotransfected with 100 ng of plasmid of AP-1-driven luciferase reporter gene, 10 ng of pRL-TK, 50 ng of pcDNA3-c-Jun, 200 ng of pcDNA3-JAB1, and 200 ng of pcDNA3-HPOC70S or the mutant HPOs (and the pcDNA3-HPOC70S/C70S was set as a negative control) for 48 h. The reporter gene activity was then measured. The results indicated that expression of HPO (wild type and HPOC90S) in COS-7 cells enhanced potentiation of AP-1 reporter gene activity induced by cotransfected JAB1, whereas the enzymatic active site CXXC mutation HPOs (HPOC67S, HPOC70S, and HPOC67S/C70S) and the deleted HPOC1–60 did not (Fig. 7A), indicating that potentiation activity of HPO on AP-1 through JAB1 depends on its sulfhydryl oxidase activity.

Because c-Jun in HPO intracellular signaling may be phosphorylated via a c-Jun N-terminal kinase-independent pathway, we also detected the levels of c-Jun and phosphorylated c-Jun in COS-7 cells cotransfected as in Fig. 7A except omitting both reporter genes. The data indicated that only HPOC90S increased the level of phosphorylated c-Jun, and neither the CXXC mutation HPOs nor HPOC1–60 increased the phosphorylation of c-Jun (Fig. 7B), in concordance with HPO-induced enhancement of potentiation of AP-1 activity through JAB1 (Fig. 7A). However, neither HPOC90S nor its mutant had any effect on the expression of transfected c-Jun (Fig. 7B). These results conform to the data of AP-1 reporter gene activation assay (Fig. 7A). Taken together it was concluded that
HPO interacts with JAB1 to activate AP-1 transcription activity by potentiating phosphorylation of c-Jun in a MAPK-independent fashion. It was also concluded that this effect depends on the integrity of the CXXC enzyme active site, which could provide a novel intracellular signaling pathway shortcut by redox regulation. This implies that the intracellular function of HPO could be mediated by the redox-dependent pathway through JAB1 and c-Jun.

**DISCUSSION**

In this paper we offer a previously unknown molecular link between the enzymatic redox function of HPO and its cytokine role. We demonstrated the necessity of cysteine residues in the CXXC catalytic center for the intracellular potentiation of AP-1 activity; we also proved that the extracellular cytokine effect of HPO, i.e., stimulation of MAPK pathway via its receptor, did not associate with its sulfhydryl oxidase activity.

Over the past years the ERV/ALR family proteins were found to have the ability to catalyze disulfide bond formation (12–17) in the same way as quiescin-sulfhydryl oxidase family proteins, which contain a N-terminal thioredoxin domain and an Erv1p homologous domain closer to the C terminus (30, 33). All these proteins contain a conserved CXXC motif essential for its catalytic action. Members of the ERV/ALR family function in a range of diverse cellular localization, including nuclear, cytosol, ER (endoplasmic reticulum), mitochondria, and extracellular space. According to the latest data, there are three major physiological roles of the ERV/ALR family. First, they participate in one of the pathways of disulfide bond generation (Erv2p (14, 16) and Er10R (15, 17)). Second, Erv1p and HPO23 (23-kDa HPO) are functional orthologs, which are largely located in the mitochondria intermembrane space (34). In this compartment Erv1p/HPO23 supports the export of Fe/S cluster from mitochondria, contributing to the biogenesis of cytosolic Fe/S proteins and to cellular iron homeostasis (34). Finally, ALR/HPO23 was found to be a hepatotropic growth factor (8–10) that is dissimilar to Erv1p and Erv2p.

HPO could be naturally dimerized at the protein level, and its gene could be alternatively spliced at the transcription level. We previously identified that HPO has 2 forms (15 kDa and 23 kDa) in human liver tissues by Western blotting analysis using a rabbit anti-HPO polyclonal antibody (21). Intriguingly, 15-kDa HPO (15 kDa) in human liver tissues by Western blotting analysis using a rabbit anti-HPO polyclonal antibody (21). Intriguingly, 15-kDa HPO has a fascinating task in the nucleus, whereas in the cytosol and extracellular space, HPO23 plays the same role as Erv1p, which contains an N-terminal thioredoxin domain and an Erv1p homologous domain closer to the C terminus (30, 33).

Here we focus on the relationship between the cytokine effect and enzyme activity of HPO. Our results show that the redox CXXC motif is not essential for the extracellular cytokine activity. HPO is an autocrine growth factor unlike other typical growth factors (35), and the effects of HPO are liver-specific. The MAPK pathway is well demonstrated to be an important growth-related pathway in liver regeneration, which was activated by HPO through its specific receptors leading to DNA synthesis. The proper structure of HPO homodimer matching the receptor is important for activating the MAPK pathway. The CXXC mutant of HPO dimerized normally, indicating that its three-dimensional structure did not change too much by Cys → Ser site mutation. That might be why the absence of enzymatic activity does not weaken the mitogen effect of extracellular HPO by stimulating MAPKs.

On the contrary intracellular HPO modulates the AP-1 signaling pathways *in vivo* by interaction with JAB1 independent of the potential HPO receptor. The AP-1 component c-Jun is a critical regulator of hepatocyte proliferation and survival during liver development and regeneration (36). The rapid upregulation of HPO expression and AP-1 activity (37–39) in immediate early phase of liver regeneration suggests that the remarkably rapid activation of transcription factors could be initiated by the intracellular signal(s) of hepatocytes and that HPO might be one of these signal molecules.

HPO activated AP-1 by increasing c-Jun phosphorylation independent of both c-Jun N-terminal kinase and extracellular signal-regulated kinase 1/2, and the co-localization site of HPO with JAB1 is the nucleus (10). Considering that the CXXC Cys → Ser mutants devoid of enzyme activity can also bind to JAB1 and that they can neither increase phospho-c-Jun levels nor activate the AP-1-dependent promoter, our data show that the CXXC motif, the N-terminal amino acid sequences of HPO, and SOX activity are indispensable to its intracellular cytokine effect via JAB1/HPO interaction. The HPO-JAB1 may provide a molecular basis for several prominent HPO activities.

Redox processes have been implicated in various biologic processes, including signal transduction, gene expression, and cell proliferation. Several molecules have been identified as redox regulators in cell activation, in which redox regulation of transcription factors, including AP-1, NF-κB, Myb, and Ets, are important (40–42). The activity of several transcription factors is post-translationally altered by redox modification(s) of specific cysteine residue(s). Endogenous JAB1/CSN5 (COP9 signalosome 5) is a subunit of COP9 complex and is found to be incorporated into the COP9 signalosome, a multi-protein complex involved in modulating signal transduction, gene transcription, and protein stability (43–47). COP9 is reported to have kinase activity that phosphorylates IkBo and c-Jun (48, 49). As well, JAB1 exists in two different complexes in mammalian cells; one is 450 kDa in size, nuclear, and identical to the conventional COP9 signalosome complex, and the other is much smaller and located in the cytoplasm (50). Recently, we found that endogenous HPO can be co-localized with endogenous JAB1/CSN5, CSN1, and CSN8 in the nucleus of HepG2 cells (data not shown), which means that HPO can interact not only with JAB1 but also with the COP9 signalosome complex in *nucleus in vivo*. We speculate that HPO possibly interacts with JAB1-containing COP9 signalosome using its enzyme activity to modify the redox status of JAB1 and/or COP9, leading to the phosphorylation of c-Jun, and resulting in the stabilization of the transcription factor *in vivo* accompanied by elevated AP-1 activity. Other thiol oxidoreductase and thiol antioxidants, such as protein disulfide isomerase, thioredoxin, and the thioredoxin system, might participate in this molecular cascade of redox regulation (16, 51).

Although many missing pieces of this seductive puzzle have not yet been found, a novel mechanism for the molecular cascade of intracellular redox regulation of AP-1 mediated by HPO and JAB1/COP9 has begun to emerge. According to our knowledge, HPO is the first intracrine growth factor that can modify the redox status of JAB1 and/or COP9, leading to the phosphorylation of c-Jun, and resulting in the stabilization of the transcription factor *in vivo* accompanied by elevated AP-1 activity. Other thiol oxidoreductase and thiol antioxidants, such as protein disulfide isomerase, thioredoxin, and the thioredoxin system, might participate in this molecular cascade of redox regulation (16, 51).

**Acknowledgment**—We thank Dr. Brian Lim for linguistic suggestions.
The Potentiation Role of Hepatopoietin on Activator Protein-1 Is Dependent on Its Sulphydryl Oxidase Activity
Xiaoxiao Chen, Yong Li, Kailhua Wei, Li Li, Wanli Liu, Yunping Zhu, Zongyin Qiu and Fuchu He

J. Biol. Chem. 2003, 278:49022-49030.
doi: 10.1074/jbc.M304057200 originally published online September 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304057200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 20 of which can be accessed free at http://www.jbc.org/content/278/49/49022.full.html#ref-list-1