THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 49, Issue of December 5, pp. 49022–49030, 2003
Printed in U.S.A.

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‡, Kaihua 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 100084, China

Hepatopoietin (HPO) is a novel hepatotrophic 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 C*XX*C (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 C*XX*C 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)[1]/augmenter of liver regeneration (ALR) is a novel human hepatotrophic growth factor. Since LaBrecque *et al.* (1) first reported hepatic stimulator substance in 1975, HPO has recently been the subject of intense investigation (2–10). Recombinant HPO can stimulate proliferation of hepatocytes as well as hepatoma cells *in vitro*, promote liver regeneration and recovery of damaged hepatocytes, and rescue acute hepatic failure *in vivo* (6, 7). In 1999 we identified the existence of HPO-specific receptor on the surface of these cells (8). Furthermore, we proposed that extracellular HPO stimulates proliferation of hepatocytes and enhances liver regeneration by activating the MAPK signaling pathway under the mediation of HPO receptor (9). Intriguingly, we further found that intracellular HPO can specifically modulate the AP-1 pathway through JAB1 via a MAPK-independent pathway and that HPO enhances the increased phosphorylation level of c-Jun through JAB1 but has no effect on the expression of transfected c-Jun or endogenous c-Jun N-terminal kinase nor on phosphorylation of c-Jun N-terminal kinase (10).

Cytokines and growth factors stimulate AP-1 activity through several pathways (11), whereas the intracrine HPO regulates AP-1 transcriptional activity by an additional mechanism different from other cytokines and growth factors with mitogenic effects. It seems strange that intracellular and extracellular cytokine HPO have dissimilar actions in signal transduction. Recently, it was reported that the ERV1/HPO family belongs to sulfhydryl oxidase (SOX) participating in disulfide bond formation (12–17). The SOX proteins contain a conserved C*XX*C motif and a non-covalent FAD adjacent to C*XX*C, which are vital to their catalytic activity (18, 19). Sulfhydryl oxidases generally form dimers *in vivo* and catalyze the oxidation of sulfhydryl groups to disulfides according to the general reaction $2R\text{-}SH + O_2 \rightarrow RSSR + H_2O_2$ (18), which is different from the flavoprotein families (20). HPO also has the C*XX*C motif and displays the homodimer form *in vitro* and *in vivo* (21). It is reasonable to hypothesize that the enzymatic function of HPO might provide an approach to uncover why HPO displays a dual nature.

Although unusual for a cytokine, protein mediators have been shown previously to function both as a cytokine and as an enzyme. These peculiar proteins that display both cytokine and enzyme activities are called "cytozymes." For example, human thioredoxin/adult T cell-derived factor is a disulfide reductase, but it is also an autocrine growth factor that has an extracellular growth-promoting effect. Intracellularly, thioredoxin/adult T cell-derived factor is involved in the regulation of protein-protein or protein-nucleic acid interactions through the reduction/oxidation of protein cysteine residues (22, 23). Macrophage migration inhibitory factor is another cytokine with a critical role in the immune and inflammatory response, but it is also a thiol oxidoreductase (24, 25). Macrophage migration inhibitory factor can specifically interact with JAB1 and then act broadly to negatively regulate JAB1-controlled pathways (25). The evidence implies that the mechanism for the macrophage migration inhibitory factor-JAB1 antagonism might be enzyme-based but is not understood yet.

Despite the important progress that has been made with respect to the elucidation of the biological role of cytozymes, the

* 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 Tech Program 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: hefc@nic.bmi.ac.cn.

[1] 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.

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 effects of HPO in cellular signal transduction. Considering the importance of the conserved C*XX*C 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 C*XX*C. 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 bridges formation, which may occur in aerobic purification. The isoelectric focus-separated HPO band with a pI 6.44 was excised, washed in 100 mM NH_4HCO_3, destained in 100 mM NH_4HCO_3, 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 dried digest was dissolved in 10 μl of 50 mM NH_4HCO_3, 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 spectroscopy with an acceleration voltage of 20 kV in positive mode. All spectra were acquired in linear mode. The matrix of α-cyano-4-hydroxylcinnic 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* cysteine to serine mutants of HPO (15 kDa) at positions 67, 70, 67/70, and 90 were constructed using the overlap extension PCR amplification method (26). JAB1 open reading frames were amplified by PCR using the following primers: primer 1, 5′-TG GCG GCG TCC GGG AGC GGT-3′; primer 2, 5′-CCG CTC GAG AGA GAT GTT AAT TT. All the sequences were verified by sequencing. The restriction sites NdeI and XhoI were used for cloning the PCR fragments into the hexahistidyl tag vector pET-24a (+) (Novagen).

Recombinant proteins were expressed in *Escherichia coli* strain BL21 (DE3) (Clontech), and the C-terminal His_6-tagged proteins were purified using a His_6 spin purification kit (Pierce) according to the manufacturer's protocols. Purification to homogeneity was verified by SDS-polyacrylamide gels and by antibody tests. In a final step the proteins were dialyzed against 50 mM Tris-HCl, pH 8.0.

Spectroscopy of the Recombinant HPO Proteins and Enzyme Assay for Sulfhydryl Oxidase

The dialyzed HPO (wild type and mutants) proteins were directly used for spectroscopy. The absorption range is from 350 to 550 nm. Under identical conditions a reference of 7 μM pure FAD (Sigma) was measured.

Preparation of reduced lysozyme (Sigma) as a substrate for HPO proteins was done as described elsewhere (27). Enzyme assays were done according to the protocol described by Jeung-Eun Lee *et al.* (12).

Identification of HPOs Homodimers

The recombinant His-tagged HPOs were expressed in *E. coli* (see above for details), and cells were prepared under nonreducing conditions. Aliquots were separated by a 15% SDS-polyacrylamide gel with or without 10 mM DTT in the sample buffer.

The HPO^{wt} and mutants (HPO^{C67S}, HPO^{C70S}, $HPO^{C67S/C70S}$, HPO^{C90S}) cDNAs were inserted into the pCMV-Myc expression vector (Clontech) in-frame with an Myc epitope. The pCMV-Myc-HPOs were transiently expressed in COS-7 cells grown in 35-mm dishes (Corning Inc., Corning, NY) and maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (HyClone). Cell extracts were fractionated on 15% SDS/PAGE in the presence or absence of 10 mM DTT in the sample buffer.

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 luminol reagent (Santa Cruz Biotechnology). Finally, the various Western-blotting bands were scanned by GS-710 calibrated imaging densitometer (Bio-Rad).

Yeast Two-hybrid Interaction Assay

The Matchmaker™ two-hybrid system 2 (Clontech) was used according to the manufacturer's protocols for interaction assay. Different combinations of the cDNAs encoding HPO^{wt} (15 kDa) or its mutants in pACT2 (AD vector) and the cDNA of JAB1 in pAS2 (DNA-BD vector) were cotransfected into Y190 cells and plated on the SD/$Trp^-Leu^-His^-$ triple-selection medium (Clontech). The binding ability between different HPO and JAB1 was quantified by the liquid galactosidase assay according to the manufacturer's protocol (Clontech), and the β-galactosidase activity was expressed in Miller units.

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 nitrilotriacetic acid-agarose Beads (Qiagen), incubated for 15 min at room temperature, and then washed with 50 volumes of 50 mM NaH_2PO_4, 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 3 times with ice-cold phosphate-buffered saline and lysed in 50 mM NaH_2PO_4, 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 12,000 rpm for 30 min, and the crude protein extracts (1 mg) were incubated with the His-JAB1 fusion protein-immobilized nickel nitrilotriacetic acid-agarose for 1 h at 4 °C, then washed twice with 50 volumes of 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8.0, and eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8.0 (Qiagen). The elution was detected by anti-Myc monoclonal antibody. The blots were revealed by Western blotting luminol 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 in-frame with a Myc epitope. COS-7 cells were cotransfected with both pcDNA3-JAB1 coding for JAB1 and either pCMV-Myc-HPO coding for Myc-HPO fusion or pCMV-Myc coding for only Myc using LipofectAMINE 2000. After 24 h of transfection, the cells in a 24-cm^2 flask (Corning) were rinsed 3 times with ice-cold phosphate-buffered saline and lysed in 1 ml of lysis buffer (0.1 M Tris-HCl (pH 7.5), 0.3 M NaCl, 2 μM NaF, 2% Tween 20, 0.4% Nonidet P-40, 20% glycerol, and mixture protease inhibitor) for 30 min on ice and sonicated by 3 10-s bursts at 75 W with a 10-s cooling period between each burst. The lysate was centrifuged at 12,000 rpm for 20 min at 4 °C. 100 μl of the supernatant was separated and used for Western blot analysis to compare protein expression levels. For co-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 1000 × *g* for 3 min at 4 °C, and the supernatant was incubated with a rabbit anti-JAB1 polyclonal antibody (for JAB1) (Santa Cruz Biotech-

nology) at 4 °C for 4 h. The purified rabbit IgG was incubated as a control antibody with another set of supernatant in comparison with the rabbit anti-JAB1 antibody. 50 μl of protein A/G-agarose beads was then added to the reaction for another 16 h of incubation at 4 °C. Finally, the agarose beads were 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 Hybond-P membrane. Duplicate blots were made from the same set of immunoprecipitation experiments. One blot was probed with mouse anti-Myc monoclonal antibody to detect Myc-HPO in the JAB1-Myc-HPO immunocomplex, and the other was probed with a rabbit anti-JAB1 antibody to monitor the amount of JAB1 protein that had been immunoprecipitated by the anti-JAB1 antibody in each reaction. The blots were revealed by Western blotting luminol reagent.

The Mitogenic Effect of HPO and Its Mutants

HepG2 cells were seeded into 96-well plates (1×10^4 cells/well) in DMEM supplemented with 10% fetal calf serum. Cells were changed to serum-free DMEM 48 h before stimulation to eliminate hormones and growth factors and then stimulated for 12 h with recombinant human hepatopoietin (rhHPO) wild type or mutants (50 ng/ml) with equal volumes of DMEM or with 10% serum DMEM. The cells were pulse-labeled with [^{3}H]thymidine (5 μCi/well) for 8 h and harvested onto glass fiber filters. Incorporation of [^{3}H]thymidine was measured in a scintillation counter.

Identification of MEK and MAPK activation by HPOs (Wild Type and Mutants)—HepG2 cells were cultured without serum for 48 h before stimulation with 50 ng/ml rhHPO^{wt} or its mutants for 5 min. Cell lysates (50 mg) were fractionated by 12% SDS-polyacrylamide gel electrophoresis, transferred to Hybond-P membranes, and probed with antibodies against phospho-MEK1/2 (Cell Signaling Technology), MEK1/2 (Cell Signaling Technology), or phospho-MAPK or MAPK (extracellular signal-regulated kinase 1/2) (Promega).

Transactivation Assays (or Luciferase Assays)

COS-7 cells were plated on 24-well tissue culture plates (Corning) at a density of 5×10^4/well and cotransfected with AP-1-driven luciferase reporter gene (Stratagene) together with the indicated cDNAs using the FuGENE 6 reagent (Roche Applied Science). 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; pcDNA3-HPO coding for HPO; pcDNA3-HPO$^{\Delta 1-60}$, coding for deleted HPO (amino acids 1–60); pcDNA3-HPO^{C67S}, HPO^{C70S}, HPO$^{C67S/C70S}$, and HPO^{C90S}, coding for HPO mutants. 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 the dual luciferase assay system (Promega) according to the manufacturer's protocols. To normalize the transfection efficiency, the firefly luciferase activity was divided by the Renilla luciferase activity from the internal control vector of pRL-TK (Promega).

Detection of the Phospho-c-Jun Levels in COS-7 Cells

COS-7 cells were plated on 35-mm tissue culture plates (Corning) at a density of 1×10^5/well and cotransfected with pcDNA3-c-Jun, pcDNA3-JAB1, and pcDNA3-HPO^{wt}, HPO$^{\Delta 1-60}$, HPO^{C67S}, HPO^{C70S}, HPO$^{C67S/C70S}$, and HPO^{C90S} using the FuGENE 6 reagent. 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 detected by immunoblotting.

Antibodies and Other Reagents

Lysozyme, 5,5′-dithiobis(2-nitrobenzoic acid), FAD, and DTT were from Sigma. Polyclonal antibodies against active MAPK and extracellular signal-regulated kinase 1/2 were from Promega. Monoclonal mouse antibody against 15-kDa human HPO was generated against bacterially produced recombinant protein by our laboratory. Polyclonal antibodies against phospho-MEK1/2 and MEK1/2 were from Cell Signaling Technology. Polyclonal anti-c-Jun, anti-JAB1, anti-actin antibodies, monoclonal anti-p-c-Jun antibodies, protein A/G PLUS-agarose, and Western-blotting luminol reagent were from Santa Cruz Biotechnology. Peroxidase-conjugated anti-rabbit, anti-goat, and anti-mouse IgG were from Jackson Immuno Research Laboratories, Inc. Hybond-P membrane were from Amersham Biosciences.

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 C*XX*C 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 tryptic 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 Ssanalyzer) is ACFTQWLCHLHNEVNR, with the disulfide bond formed between Cys-90 and Cys-96. The Glu-C PMF before/after DTT reduction agreed with this 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 tryptic PMF and from *m/z* 2292.4 to *m/z* 2294.8 in Glu-C PMF, corresponding to the sequences FYPCEECAEDLR and MAQFIHLFSKFYPCEECAE, respectively.

Of the 8 cysteines in the 15-kDa HPO molecule, 4 take part in the formation of two internal disulfide bounds (Fig. 1*E*). The one between Cys-67 and Cys-70 agrees with the SOX redox-active disulfide, which is adjacent to FAD moiety. The other disulfide bound between Cys-90 and Cys-96 has been identified for the first time. The C*XX*C 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. 1*E*).

HPO (15 kDa) Is a FAD-linked Sulfhydryl 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 C*XX*C active disulfide bound were designed: HPO^{C67S}, HPO^{C70S}, and HPO$^{C67S/C70S}$. Cys-90 in another internal disulfide was also changed into serine (HPO^{C90S}) 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 rhHPO 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).

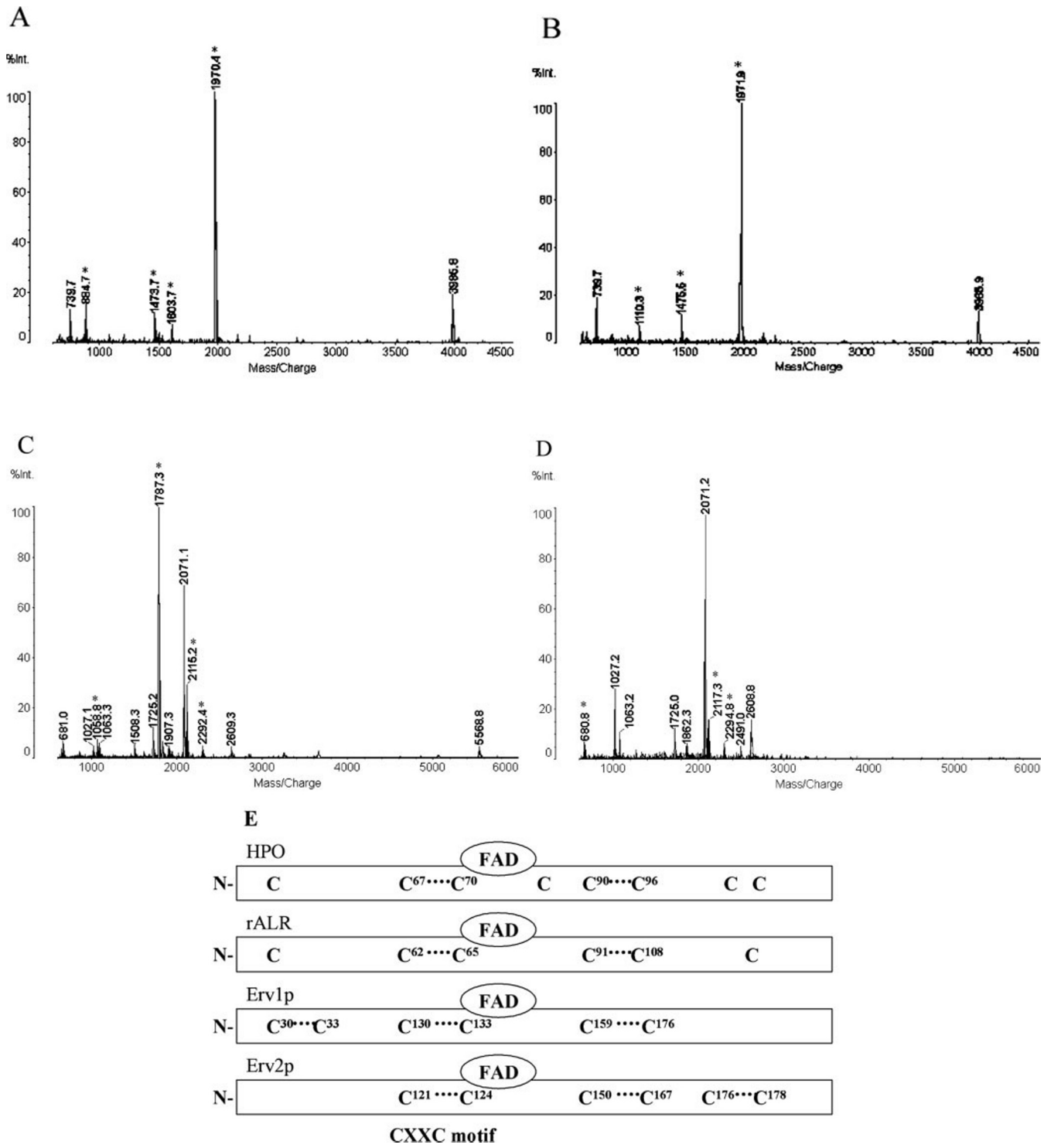

FIG. 1. **15-kDa HPO has two internal disulfides.** In-gel digestion condition and mass spectrometry method was described under "Materials and Methods." Peptide mass fingerprinting of 15-kDa HPO is shown in panels *A–D*. *A*, trypsin, non-reduction. *B*, trypsin, reduced by DTT. *C*, Glu-C, non-reduction. *D*, Glu-C, reduced by DTT. *, cysteine-containing peptides. *E*, comparison of the cysteine residues in Erv1p, Erv2p, 15-kDa rat ALR, and 15-kDa HPO. The C*XX*C motif/redox-active disulfide that is adjacent to FAD moiety is conserved in these four proteins. The *superscript numbers* give the positions of the amino acid residues in the proteins. Disulfide bonds are shown by *dotted lines* between Cys residues.

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*XX*C mutants (HPO^{C67S}, HPO^{C70S}, and $HPO^{C67S/C70S}$) lost the enzyme activity completely because of the spoiling of the redox-active disulfide and that HPO^{wt} and HPO^{C90S} were able to oxidize lysozyme in a time-dependent manner, whereas HPO^{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. 1*E*), 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

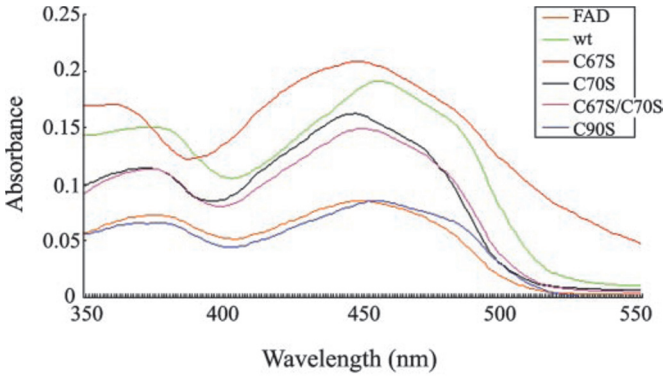

FIG. 2. **Spectroscopy of purified 15-kDa HPOs.** The absorbance spectrum was recorded in the range of 350–550 nm using the purified 15 kDa HPOs (wild type (*wt*) or mutants) and pure FAD (as control). The concentrations of the various samples have been adjusted to different levels to get distinguishing absorption levels and curves. All HPO proteins exhibit a spectral character for FAD but distinct differences from free FAD. *wt*, *C67S*, *C70S*, *C67S/C70S*, *C90S* represent wild type HPO, HPO^{C67S}, HPO^{C70S}, $HPO^{C67S/C70S}$, HPO^{C90S}, respectively, which are also designations used in all the following figures.

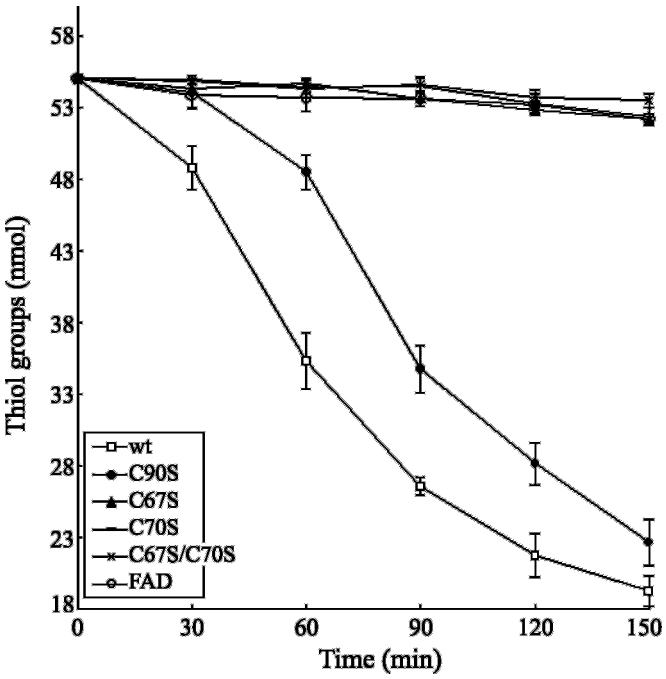

FIG. 3. **The 15-kDa HPO exhibits sulfhydryl oxidase activity dependent on the active motif C*XXC*.** Reduced lysozyme corresponding to 55-nmol thiol groups was incubated with HPO^{wt}, HPO^{C67S}, HPO^{C70S}, $HPO^{C67S/C70S}$, HPO^{C90S}, or pure FAD without added protein, respectively. The oxidation of thiol groups was detected spectrophotometrically at 412 nm after the addition of 5,5′-dithiobis(2-nitrobenzoic acid). The time-dependent decrease of extinction indicates the oxidation of the thiol groups. The *error bar* indicates ± S.D. Statistical analysis was carried out using Microcal Origin (Microcal Software). *wt*, wild type.

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 C*XXC* 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 (HPO^{C67S}, HPO^{C70S}, $HPO^{C67S/C70S}$, and HPO^{C90S}) 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 HPO^{wt}. 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 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-HPO^{wt} 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^- medium or SD/leu^-/trp^-/his^- medium (Fig. 4, *C*, *D*, and *E*). Cells cotransfected with pAS2-JAB1 and pACT2-HPOs could grow in SD/leu^-/trp^-/his^- medium, whereas the controls (pAS2+ACT2, pAS2-JAB1+pACT2, pAS2+pACT2-HPO^{wt}) 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. 4*F*). 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. 4*G*).

For co-immunoprecipitation assay, the plasmids pcDNA3-JAB1 and pCMV-Myc-HPO (HPO^{wt}, HPO^{C67S}, HPO^{C70S}, $HPO^{C67S/C70S}$, and HPO^{C90S}, respectively) were cotransfected into COS-7 cells. Both proteins co-expressed in COS-7 cells, and the HPO-JAB1-specific complexes *in vivo* 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. 4*H*). Together, all these protein-protein interaction assays *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 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 recombinant 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 (HPO^{C67S}, HPO^{C70S},

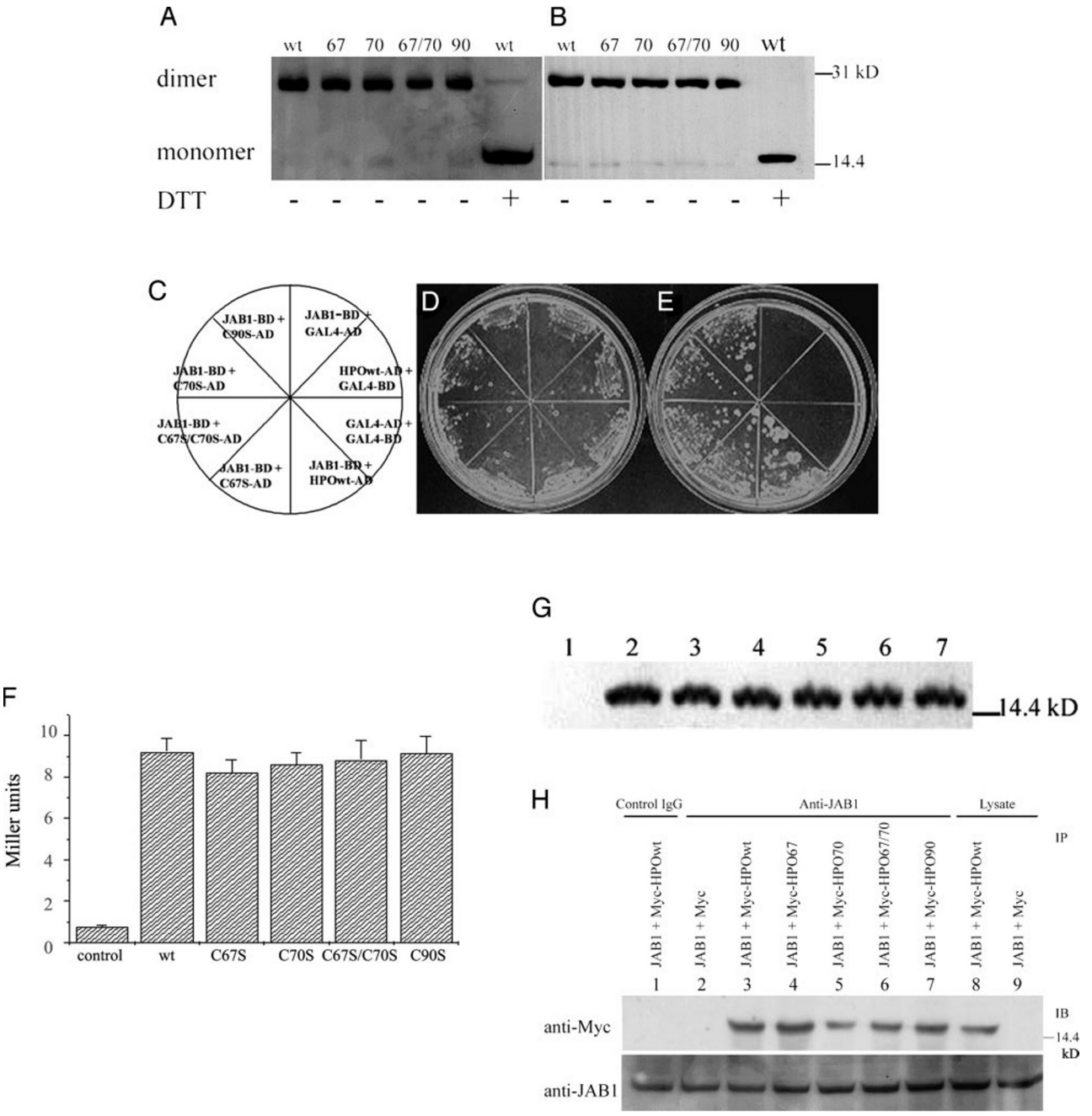

FIG. 4. **The Cys → Ser mutation of HPO destroying its enzyme activity disturbed neither HPO dimerization nor HPO-JAB1 interaction *in vivo*/*in vitro*.** *A*, recombinant His-tagged HPOs (wt or mutants) were expressed in BL21 (DE3), and cells were prepared under nonreducing conditions. Aliquots were separated by a 15% SDS-polyacrylamide gel with or without 10 mM DTT in the sample buffer. The proteins were detected by Western blotting with anti-His monoclonal antibody. *B*, Myc-HPOs (wild type (*wt*) or mutants) were expressed in COS-7 cells. Cell extracts were fractionated on 15% SDS/PAGE in the presence or absence of 10 mM DTT in the sample buffer. The Western blot was performed using anti-Myc monoclonal antibody. *C*, the indication of yeast cells containing vectors in each pie slice of the culture plate. *D*, growth of transformants coexpressing HPO and JAB1 on selective leu^-/trp^-medium. *E*, growth of transfected yeast cells on $leu^-/trp^-/his^-$ medium. *F*, β-galactosidase activity is indicated in Miller units. The yeast cell containing the vector pACT2 and pAS2 were used as negative control (*bars*, S.D. of triplicate samples). Statistical analysis was carried out using Microcal Origin (Microcal Software). *G*, COS-7 cells were transfected with pCMV-Myc or pCMV-Myc-HPO (wild type and mutants), respectively. After 24 h of transfection, protein extracts of COS-7 cells (*lane 1*, pCMV-Myc; *lane 2*, Myc-HPO^{wt}; *lane 3*, Myc-HPO^{C67S}; *lane 4*, Myc-HPO^{C70S}; *lane 5*, Myc-$HPO^{C67S/C70S}$; *lane 6*, Myc-HPO^{C90S}) were incubated with the His-JAB1 fusion protein-immobilized nickel nitrilotriacetic acid-agarose. Bound proteins were eluted with elution buffer and analyzed by immunoblotting with anti-Myc monoclonal antibody. *Lane 7*, expression control. Protein extracts of pCMV-Myc-HPO^{wt}-transfected COS-7 cells. *H*, COS 7 cells were cotransfected with JAB1 and either Myc-tagged HPOs (wild type or mutants) or Myc control vector. The cell lysates were incubated with a rabbit anti-JAB1 antibody then with protein A/G-agarose. The immuno-complex was resolved on 15% SDS-PAGE and analyzed by immunoblotting with anti-Myc antibody. As an indication of the relative expression level for Myc-HPO, some of the total cell lysate used in immuno-precipitation was loaded onto *lanes 8* (pCMV-Myc-HPO^{wt}) and *9* (pCMV-Myc). *Lanes 2–7* are from cells expressing JAB1/Myc and JAB1/Myc-HPO (HPO^{wt}, HPO^{C67S}, HPO^{C70S}, $HPO^{C67S/C70S}$, and HPO^{C90S}), respectively. *Lane 1* is a control for *lane 3* with rabbit mock antibody (normal IgG). A duplicate blot was also probed with anti-JAB1 antibody to monitor the amounts of JAB1 protein (*bottom*). *IP*, immunoprecipitation; *IB*, immuno-blotting.

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

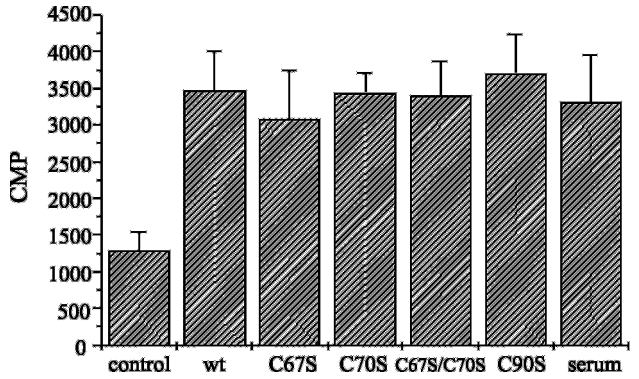

FIG. 5. **The Cys → Ser mutation of HPO destroying its enzyme activity could not disturb its mitogenic effect on HepG2 cells.** HepG2 cells in 96-well plates were serum-deprived for 48 h and incubated for 12 h with the indicated rhHPOs (wild type (*wt*) or mutants, 50 ng/ml) with DMEM as the negative control (*control*) or with 10% serum DMEM as the positive control (*serum*). Cells were pulse-labeled with [^{3}H]thymidine (5 μCi/well) for 8 h and harvested onto glass fiber filters. Then incorporation of [^{3}H]thymidine was measured in a scintillation counter, and results were expressed as median counts per minute from triplicate cultures. The *error bar* indicates S.D. Statistical analysis was carried out using Microcal Origin (Microcal Software). *CMP*, counts per minute.

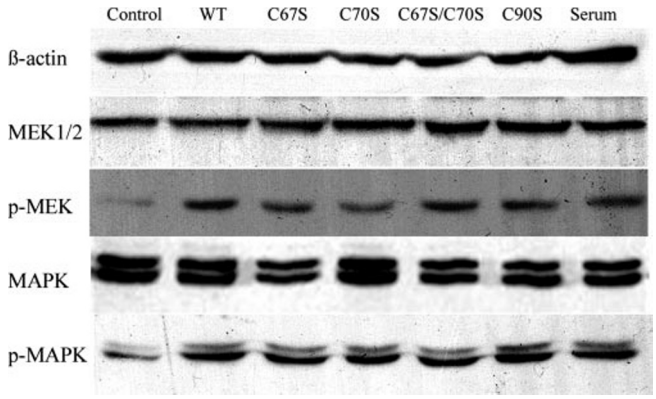

FIG. 6. **The Cys → Ser mutation of HPO destroying its enzyme activity could not disturb its activation of MEK and MAPK.** Serum-starved HepG2 cells were stimulated with 50 ng/ml rhHPOs (wild type (*WT*) or mutants) for 5 min. Cell incubation with DMEM was set as the negative control (*control*) or with 10% serum DMEM as the positive control (*serum*). The cell lysates were analyzed by antibodies against MEK, phospho-MEK, MAPK, or phospho-MAPK. As a loading control, β-actin content in the cell extracts was analyzed by immunoblotting.

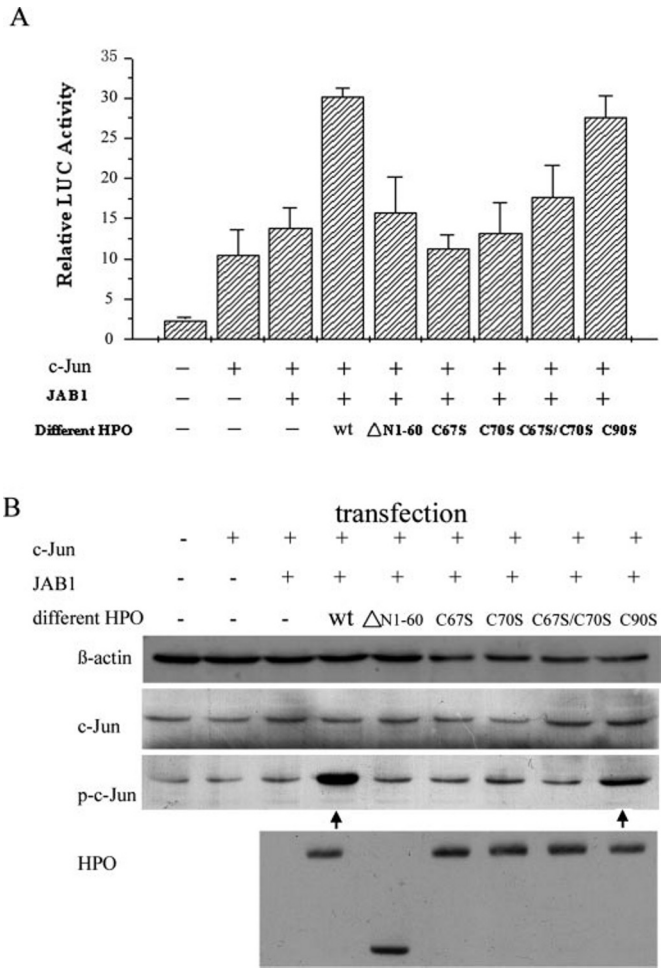

FIG. 7. **HPOs potentiation of AP-1 activity through JAB1 depends on its redox-active site C*XX*C, and the enzyme active site C*XX*C is essential for HPO to increase phosphorylation level of c-Jun.** *A*, COS-7 cells were cotransfected with AP-1-driven luciferase (*LUC*) reporter, pRL-TK, c-Jun, JAB1, and different HPO. Each luciferase activity was normalized to a cotransfected Renilla luciferase expression vector. Shown are representative examples at least three independent experiments performed in duplicate. The *error bar* indicates S.D. Statistical analysis was carried out using Microcal Origin (Microcal Software). *wt*, wild type. *B*, COS-7 cells were cotransfected with the indicated cDNAs. The cell lysates were resolved on 12% SDS-PAGE or on Tricine-SDS-PAGE (52) and then 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), and anti-HPO. β-Actin immunoblots served as a control. The *arrows* indicate the remarkable increased phospho-c-Jun level.

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-HPO^{wt} or the mutant HPOs (and the pcDNA3-HPO$^{\Delta 1-60}$ 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 HPO^{C90S}) in COS-7 cells enhanced potentiation of AP-1 reporter gene activity induced by cotransfected JAB1, whereas the enzymatic active site C*XX*C mutation HPOs (HPO^{C67S}, HPO^{C70S}, and HPO$^{C67S/C70S}$) and the deleted HPO$^{\Delta 1-60}$ did not (Fig. 7*A*), 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. 7*A* except omitting both reporter genes. The data indicated that only HPO^{wt} and HPO^{C90S} increased the level of phosphorylated c-Jun, and neither the C*XX*C mutation HPOs nor HPO$^{\Delta 1-60}$ increased the phosphorylation of c-Jun (Fig. 7*B*), in concordance with HPO-induced enhancement of potentiation of AP-1 activity through JAB1 (Fig. 7*A*). However, neither HPO^{wt} nor its mutant had any effect on the expression of transfected c-Jun (Fig. 7*B*). These results conform to the data of AP-1 reporter gene activation assay (Fig. 7*A*). 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 C*XX*C 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 C*XX*C 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 an N-terminal thioredoxin domain and an Erv1p homologous domain closer to the C terminus (30, 33). All these proteins contain a conserved C*XX*C 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 E10R (15, 17)). Second, Erv1p and HPO^{23} (23-kDa HPO) are functional orthologs, which are largely located in the mitochondria intermembrane space (34). In this compartment $Erv1p/HPO^{23}$ 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/HPO was found to be a hepatotrophic 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 rabbit anti-HPO polyclonal antibody (21). Intriguingly, 15-kDa HPO exists only in the nuclear fractions, and HPO^{23} exists mainly in the cytosol fractions (21). In the mitochondria intermembrane space, HPO^{23} plays the same role as Erv1p, whereas in the nucleus, 15-kDa HPO has a fascinating task based on redox regulation of AP-1 transcriptional activity through JAB1.

Here we focus on the relationship between the cytokine effect and enzyme activity of HPO. Our results show that the redox C*XX*C 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 C*XX*C 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 intracrine 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 C*XX*C 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 C*XX*C 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 IκBα 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 by immunofluorescence, and four subunits of COP9 including JAB1/CSN5 can be coprecipitated with HPO in COS-7 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 JAB1/COP9 signalosome complex by a redox manner. Elucidation of the molecular mechanisms of HPO action will be valuable for our understanding of liver organogenesis, regeneration, and oncogenesis. Considering that the precise substrate of ERV/ALR sulfhydryl oxidase family have not been identified so far, the significance of the redox-activating AP-1 pathway through JAB1/COP9 in HPO signaling may shed some light on the way to approach the natural substrates and provide mechanistic insight into cytozyme-mediated cellular redox regulation.

Acknowledgment—We thank Dr. Brian Lim for linguistic suggestions.

REFERENCES

1. LaBrecque, D. R., and Pesh, L. A. (1975) *J. Physiol.* (*Lond.*) **248,** 273–278
2. LaBrecque, D. R. (1991) *Dig. Dis. Sci.* **36,** 669–673
3. Hagiya, M., Francavilla, A., Polimeno, L., Ihara, I., Sakai, H., Seki, T., Shimonishi, M., Porter, K. A., and Starzl, T. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8142–8146
4. Giorda, R., Hagiya, M., and Seki, T. (1996) *Mol. Med.* **2,** 97–108
5. Yang, X. M., Xie, L., Xing, G. C., Wu, Z. Z., and He, F. C. (1998) *World J. Gastroenterol.* **4,** 100–102
6. Yang, X. M., Hu, Z. Y., Xie, L., Wu, Z. Z., Wu, C. T., and He, F. C. (1997) *Sheng Li Xue Bao* **49,** 557–561
7. Yang, X. M., Xie, L., He, H., Wei, H. D., Wu, Z. Z., and He, F. C. (1997) *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao* (*Shanghai*) **29,** 414–418
8. Wang, G., Yang, X. M., Zhang, Y., Wu, Z. Z., and He, F. C. (1999) *J. Biol. Chem.* **274,** 11469–11472
9. Li, Y., Li, M., Xing, G. C., Hu, Z. Y., Wang, Q. M., Dong, C. N., Chen, J. Z., Yang, X. M., Chen, H. P., Guan, K. L., Wu, C., Zhang, C. G., and He, F. C. (2000) *J. Biol. Chem.* **275,** 37443–37447
10. Lu, C. R., Li, Y., Zhao, Y. L., Xing, G. C., Tang, F., Wang, Q. M., Sun, Y. H., Wei, H. D., Yang, X. M., Wu, C., Chen, J. G., Guan, K. L., Zhang, C. G., Chen, H. P., and He, F. C. (2002) *FASEB J.* **16,** 90–92
11. Karin, M. (1995) *J. Biol. Chem.* **270,** 16483–16486
12. Lee, J.-E., Hofhaus, G., and Lisowsky, T. (2000) *FEBS Lett.* **477,** 62–66
13. Lisowsky, T., Lee, J. E., Polimeno, L., Francavilla, A., and Hofhaus, G. (2001) *Dig. Liver Dis.* **33,** 173–180
14. Gerber, J., Mühlenhoff, U., Hofhaus, G., Lill, R., and Lisowsky, T. (2001) *J. Biol. Chem.* **276,** 23486–23491
15. Senkevich, T. G., White, C. L., Koonin, E. V., and Moss, B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 12068–12073
16. Sevier, C. S., Cuozzo, J. W., Vala, A., Aslund, F., and Kaiser, C. A. (2001) *Nat. Cell Biol.* **3,** 874–882
17. Senkevich, T. G., White, C. L., Koonin, E. V., and Moss, B. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 6667–6672
18. Hoober, K. L., Joneja, B., White, H. B., III, and Thorpe, C. (1996) *J. Biol. Chem.* **271,** 30510–30516
19. Gross, E., Sevier, C. S., Vala, A., Kaiser, C. A., and Fass, D. (2002) *Nat. Struct. Biol.* **9,** 61–67
20. Massey, V. (1995) *FASEB J.* **9,** 473–475
21. Li, Y., Wei, K. H., Lu, C. R., Li, Y. X., Li, M., Xing, G. C., Wei, H. D., Wang, Qi. M., Chen, J. Z., Wu, C., Chen, H. P., Yang, S. C., and He, F. C. (2002) *Eur. J. Biochem.* **269,** 1–6
22. Nakamura, H., Nakamura, K., and Yodoi, J. (1997) *Annu. Rev. Immunol.* **15,** 351–369
23. Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, T., Matsui, H., Hamuro, J., Brown, R., Arai, K., Yokota, T., Wakasugi, N., and Yodoi, J. (1989) *EMBO J.* **8,** 757–764
24. Kleemann, R. Kapurniotu, A., Frank, R. W., Gessner, A., Mischke, R., Flieger, O., Juttner, S., Brunner, H., and Bernhagen, J. (1996) *J. Mol. Biol.* **280,** 85–102
25. Kleemann, R., Hausser, A., Geiger, G., Mischke, R., Burger-Kentischer, A., Flieger, O., Johannes, F. J., Roger, T., Calandra, T., and Bernhagen, J. (2000) *Nature* **408,** 211–216
26. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* (*Amst.*) **77,** 51–59
27. Hoober, K. L., Sheasley, S. L., Gilbert, H. F., and Thorpe, C. (1999) *J. Biol. Chem.* **274,** 22147–22150
28. Hofhaus, G., Lee, J. E., Tews, I., Rosenberg. B., and Lisowsky, T. (2003) *Eur. J. Biochem.* **270,** 1528–1535
29. Wu, C. K., Dailey, T. A., Dailey, H. A., Wang, B. C., and Rose, J. P. (2003) *Protein Sci.* **12,** 1109–1118
30. Hoober, K. L., Glynn, N. M., Burnside, J., Coppock, D. L., and Thorpe, C. (1999) *J. Biol. Chem.* **274,** 31759–31762
31. Hoober, K. L., and Thorpe, C. (1999) *Biochemistry* **38,** 3211–3217
32. Faeder, E. J., and Siegel, L. M. (1973) *Anal. Biochem.* **53,** 332–336
33. Benayoun, B., Esnard-Feve, A., Castella, S., Courty, Y., and Esnard, F. (2001) *J. Biol. Chem.* **276,** 13830–13837
34. Lange, H., Lisowsky, T., Gerber, J., Muhlenho, U., Kispal, G., and Lill, R. (2001) *EMBO Rep.* **2,** 715–720
35. Li, Y., Xing, G. C., Wang, Q. M., Li, M., Wei, H. D., Fan, G. C., Chen, J. J., Yang, X. M., Wu, C. Z., Chen, H. P., and He, F. C. (2001) *DNA Cell Biol.* **20,** 791–795
36. Behrens, A., Sibilia, M., David, J. P., Steinlein, U. M., Tronche, F., Schütz, G., and Wagner, E. F. (2002) *EMBO J.* **21,** 1782–1790
37. Taub, R. (1996) *FASEB J.* **10,** 413–427
38. Yang, X. M., Xie, L., Wu, Z. Z., Wu, C. T., and He, F. C. (1997) *Sheng Li Xue Bao* **49,** 599–601
39. Westwick, J. K., Weitzel, C., Leffert, H. L., and Brenner D. A. (1995) *J. Clin. Invest.* **95,** 803–810
40. Abate, C., Patel, L., Rauscher, F. J., and Curran, T. (1990) *Science* **249,** 1157–1161
41. Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3633–3638
42. Matés, J. M. (2000) *Toxicology* **153,** 83–104
43. Tomoda, K., Kubota, Y., Arata, Y., Mori, S., Maeda, M., Tanaka, T., Yoshida, M., Yoneda-Kato, N., and Kato, J. (2002) *J. Biol. Chem.* **277,** 2302–2310
44. Schwechheimer, C., and Deng, X. W. (2001) *Trends Cell Biol.* **11,** 420–426
45. Chamovitz, D. A., and Segal, D. (2001) *EMBO Rep.* **2,** 96–101
46. Bech-Otschir, D., Seeger, M., and Dubiel, W. (2002) *J. Cell Sci.* **115,** 467–473
47. Chamovitz, D. A., and Glickman, M. (2002) *Curr. Biol.* **12,** R232
48. Naumann, M., Bech-Otshir, D., Huang, X., Ferrell, K., and Dubiel, W. (1999) *J. Biol. Chem.* **274,** 35297–35300
49. Seeger, M., Kraft, R., Ferrell, K., Bech-otschir, D., Dumdey, R., Schade, R., and Gordon, C. (1998) *FASEB J.* **12,** 469–478
50. Chopra, S., Mattos, S. F., Lam, E. W., and Mann, D. J. (2002) *J. Biol. Chem.* **277,** 32413–32416
51. Arner, E. S., and Holmgren, A. (2000) *Eur. J. Biochem.* **267,** 6102–6109
52. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379