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Direct association of hepatopoietin with thioredoxin constitutes a redox signal transduction in activation of AP-1/NF-κB

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Abstract

It has been demonstrated that growth factors quiescin Q6 family was created by the fusion of the sulfhydryl oxidase fragment of the yeast essential for respiration and vegetative growth (ERV)1 prototype [an orthologue of hepatopoietin (HPO)] and thioredoxin (TRX)/disulfide isomerase domain during evolution. In this paper, our results demonstrated that two components of this composite protein, i.e., HPO and TRX, were involved in the same signal transduction and interacted physically in eukaryocyte. When HPO and TRX were cotransfected into COS7 cells, the activity of activator protein-1 (AP-1) and NF-κB was evidently enhanced compared with the transfection with HPO or TRX alone, at the same time, the phosphorylation of c-Jun was increased. They were colocalized in the cells. By Co-IP and GST pull-down experiments, we found that HPO could physically interact with TRX, which was also confirmed by yeast two-hybrid assay. By further investigation, we found both HPO and TRX were sensitive to cellular oxidative state. HPO dimer is in its natural state and could be reduced by dithiothreitol (DTT) in vitro and in vivo. Under the treatment of oxidants such as H_2O_2 and diamide, the amount of HPO monomer was decreased significantly and assembled into dimer, and the free thiol in TRX was oxidized. HPO could transfer oxidizing equivalents to TRX via direct thiol-disulfide exchange in vitro, the redox state of TRX was also affected by HPO in vivo. Taken together, it was implicated that the oxidizing equivalents might flow from HPO to TRX and then to substrate protein by the dimerization of HPO, and its interaction with TRX finally activates the redox-sensitive transcription factor, suggesting a new redox signal pathway conducted by thiol-disulfide transformation in eukaryocytic cytoplasm.

Keywords: Hepatopoietin; Thioredoxin; Reduction; Oxidization; Disulfide; Signal pathway

1. Introduction

Hepatopoietin (HPO) is a novel hepatotrophic growth factor, which is involved in the process of liver regeneration in rat, mouse and human [1]. It belongs to the family of essential for respiration and vegetative growth (ERV) 1/augmenter of liver regeneration (ALR). Homologs of this family have been found in a large number of lower and higher eukaryocytes and some viruses [2–4]. Recently, ALR was identified as a sulfhydryl oxidase by its ability to oxidize thiol groups of protein substrates and the presence of an FAD moiety in the carboxy-terminal domain and the formation of dimer in vivo [5]. It has also been shown that the effect of HPO on activator protein-1 (AP-1) is dependent on its sulfhydryl oxidase activity [6]. ERV2, a member of ERV1/ALR in yeast, is an essential element of the pathway for the formation of disulfide bonds within the endoplasmic reticulum [7]. E10R, a viral member of the ERV1/ALR protein family, participates in a cytoplasmic pathway of

Abbreviations: ALR, Augmenter of liver regeneration; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; AP-1, Activator protein-1; ASK, Apoptosis signal-regulating kinase; DBD, DNA-binding domain; DTT, Dithiothreitol; EMSA, Electrophoretic mobility shift assay; ERV, Essential for respiration and vegetative growth; GFP, Green flourescence protein; HPO, Hepatopoietin; JNK, c-Jun NH_2-terminal kinase; rhHPO, Recombinant human hepatopoietin; Ref-1, Redox factor 1; ROS, Reactive oxygen species; TCA, Trichloroacetic acid; TRX, Thioredoxin.

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disulfide bond formation [8] and is responsible for the oxidation of the viral G4L gene product, which is homologous to glutaredoxin. A common character of the proteins in this family is that they are involved in the redox reaction by the regulation of disulfide bond formation.

It has been known that the family of FAD-dependent sulfhydryl oxidase/quiescin-Q6-related genes contains thioredoxin (TRX) and yeast ERV1 domains [4]. If a composite protein is uniquely similar to two component proteins no matter whether they are in the same species or not, the component proteins are most likely to interact or be involved in the same signal transduction [9,10]. We inferred that HPO could function in conjunction with TRX by which it plays an important role in sensing the extracellular redox signals.

TRX is a key molecule in the maintenance of cellular redox balance. In addition to the cytoprotective action against oxidative stresses, it is involved in various cellular processes, including gene expression, signal transduction, proliferation and apoptosis [11,12]. Both HPO and TRX have conserved CXXC motifs as their enzymatic active site. These cysteines in the redox regulatory domain are reactive and can be covalently linked to other proteins by forming disulfide bridges. Recently, a class of signaling factors was identified, which use conserved cysteine motif such as CXXC or CXXS as redox-sensitive sulfhydryl switches to modulate specific signal transduction cascades that have similar redox-sensitive sites [13]. The identity of the amino acids separating the two cysteines in the CXXC motif and protein location influences redox properties of CXXC-containing proteins, these proteins may serve as reductants or oxidants [14].

Two major mechanisms involving reversible modification of amino acid side chains to modulate protein activity are phosphorylation/dephosphorylation by kinase and phosphatase systems and reduction/oxidation by thiol-dependent enzyme [15]. Whereas many signaling processes involving phosphorylation are well understood in terms of mechanisms and identities of participating enzymes, redox regulation of cellular processes remains a poorly characterized area. To explore the molecular mechanism of thiol-disulfide transferring-dependent redox regulation in eukaryotic cytoplasm, we investigated the role of HPO within the complex cellular redox network during oxidative stress, the interaction between HPO and TRX and its effect on the activation of transcription factor. These results suggest that HPO might play an important role in response to oxidative stress and that ERV1/ALR/HPO family proteins function in conjunction with thioredoxin by which the disulfide bonds are transferred to a substrate protein.

2. Materials and methods

2.1. Cell culture, transient transfection and luciferase assay

Cells used in this experiment were maintained as monolayer cultures and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a humidified 5% CO_2 incubator at 37 °C. For transient transfection the day before transfection, the cells were treated as follows: (1) Remove growth medium; (2) Wash cells using 1×PBS for three times and discard the wash solution; (3) Add trypsin–EDTA (0.5% trypsin, 5.3 mM EDTA·4Na) to the flask and swirl the flask briefly. Discard all but enough trypsin–EDTA to leave a thin film of liquid over the cells. Incubate the cells at 37 °C for 5 to 10 min. Monitor the cells during incubation under a microscope; (4) Add growth medium to the cell suspension; (5) Transfer the cell suspension to a 15-ml centrifuge tube. Centrifuge at 100 ×*g* for 4 min. Remove the supernatant by aspiration; (6) Resuspend the cells in 5 ml of growth medium. Centrifuge at 100 ×*g* for 4 min. Remove the supernatant by aspiration; (7) Resuspend the cells in 5 ml of growth medium. Count the number of viable cells; (8) Plate them in 24-well tissue culture plates at a density of 2×10^5 cells per well so that they would be 90–95% confluent on the day of transfection. After a 24-h attachment period, transfection was performed with LipofectAMINE™ 2000 Reagent (Invitrogen) following the manufacturer's instructions. After 24-h incubation, cells were harvested in passive lysis buffer (Promega). Luciferase assays were conducted with the Dual Luciferase Assay System (Promega) according to the manufacturer's recommendations, with a modification for 10 μl of cell lysate in 50 μl of firefly/renilla luciferase assay reagent. To normalize the transfection efficiency, the firefly luciferase activity was divided by the renilla luciferase activity from the internal control pRL-TK. All transfection and reporter assays were performed independently at least three times, each in triplicate, as described in the figure legends. The results are presented as means ±S.D.

2.2. The construction of the vectors and fusion proteins

Human TRX and HPO cDNAs were amplified by PCR and sequenced to confirm sequence integrity, then they were subcloned to the following vectors at the indicated sites to form fusion proteins (Table 1).

2.3. Expression and purification of His-tagged TRX

Overnight cultures of *Escherichia coli* strain BL21(DE3) transformed with pET24a(+)-TRX were diluted 1:100 in 1 l

Table 1
The construction of the vectors and fusion proteins

Vectors	Inserted sites	Fusion protein
pDsRed1-N1-Trx	*Eco*RI/*Bam*HI	RFP-TRX
pcDNA3.1 (+)A–Trx	*Bam*HI/*Eco*RI	Myc-His-TRX
pACT2-Trx	*Nco*I/*Xho*I	AD-TRX
pET24a(+)-Trx	*Nde*I/*Xho*I	His-TRX
pEGFP-HPO	*Bam*HI/*Hin*dIII	GFP-HPO
pGEX-4T-2-HPO	*Eco*RI/*Xho*I	GST-HPO
pFlag-CMV-2-HPO	*Bam*HI/*Eco*RI	Flag-HPO
pAS2-HPO	*Eco*R1/*Bam*HI	BD-HPO