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Original Research Communication

Cell-Surface Events for Metallothionein-1 and Heme Oxygenase-1 Regulation by the Hemopexin–Heme Transport System

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ABSTRACT

A model has been developed for the hemopexin receptor-mediated heme transport system based on iron uptake in yeast. Two steps are required: reduction followed by oxidation by a multi-copper-oxidase. Furthermore, in the hemopexin system, the surface redox events have been linked with gene regulation. The impermeable Cu(I) chelator bathocuproinedisulfonate (BCDS) is shown here to abrogate heme oxygenase-1 (HO-1) mRNA induction by heme-hemopexin. A role for Cu(I) in the regulation of HO-1 and MT-1 (Sung *et al.*, 1999) by hemopexin supports the participation of electron transport processes at the cell surface as does competition by the reductase activator, ferric citrate, which inhibits the induction of MT-1 and HO-1 mRNA by heme–hemopexin. There is a key role for the hemopexin receptor because neither ferric citrate nor iron-transferrin alone regulates MT-1 or HO-1. Cell-surface copper is the first molecule to link the concomitant regulation of HO-1 and MT-1 by the hemopexin receptor. In addition, cytochrome b_5 and cytochrome b_5 reductase are implicated here in the response of cells to heme–hemopexin. Reduction of one or more electron donors of the reductase and oxidation of the electron acceptor, b_5 heme, leads to gene regulation, but only when heme–hemopexin is bound to its receptor. Protein kinase cascades, including JNK, are activated by the hemopexin receptor itself upon ligand binding but are modulated by a Cu(I)-dependent process likely to be heme uptake. Antiox. Redox Signal. 2, 753–765.

INTRODUCTION

HEMOPEXIN HAS TWO FUNCTIONS: to prevent heme-mediated oxidative damage to cells (Gutteridge and Smith, 1988; Vincent *et al.*, 1988) and to transport heme into cells that express hemopexin receptors (Davies *et al.*, 1979; Smith and Morgan, 1979; Taketani *et al.*, 1986). Binding of heme–hemopexin to hemopexin receptors results in profound changes in cellular physiology (Eskew *et al.*, 1999). Hemopexin receptor activation results in the transcription of genes, including heme oxygenase-1 (HO-1) and metallothionein-1 (MT-1), that play a protective role in the cellular response to oxidative stress (Dennery *et al.*, 1998; Poss and Tonegawa, 1997; van Lookeren Campagne *et al.*, 1999).

There are several similarities between the hemopexin and transferrin endocytic systems for the cellular uptake of heme and iron, respectively. A role for surface oxid-reductases for iron uptake has been shown in both yeast (for

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review, see Askwith and Kaplan, 1998) and mice (Yu and Wessling-Resnick, 1998). In yeast, ferric iron is reduced by a surface reductase (the products of the FRE1/FRE genes) and then oxidized by a surface multicopper oxidase, Fet3p. Oxidation is necessary for transport across the plasma membrane by a Fe(III)-specific transmembrane permease, Ftr1p. Both transferrin-dependent and transferrin-independent iron uptake in humans involve reduction of the iron, an iron [presumably Fe(II)] carrier for membrane transport, a stimulator of iron transport (STP) together with cellular copper sufficiency (Inman *et al.*, 1994; Yu and Wessling-Resnick, 1998). Based on the studies in yeast, we developed a model for heme uptake from heme–hemopexin (Fig. 1 and Smith, 1999). The heme-iron of ferric-heme–hemopexin complexes bound to the hemopexin receptor acts as an electron acceptor for an associated reductase analogous to Fre1p. Fre1p has a b-type heme protein, is a flavoprotein (Shatwell et al., 1996), and is generally considered to be NADPH-dependent. In the hemo-

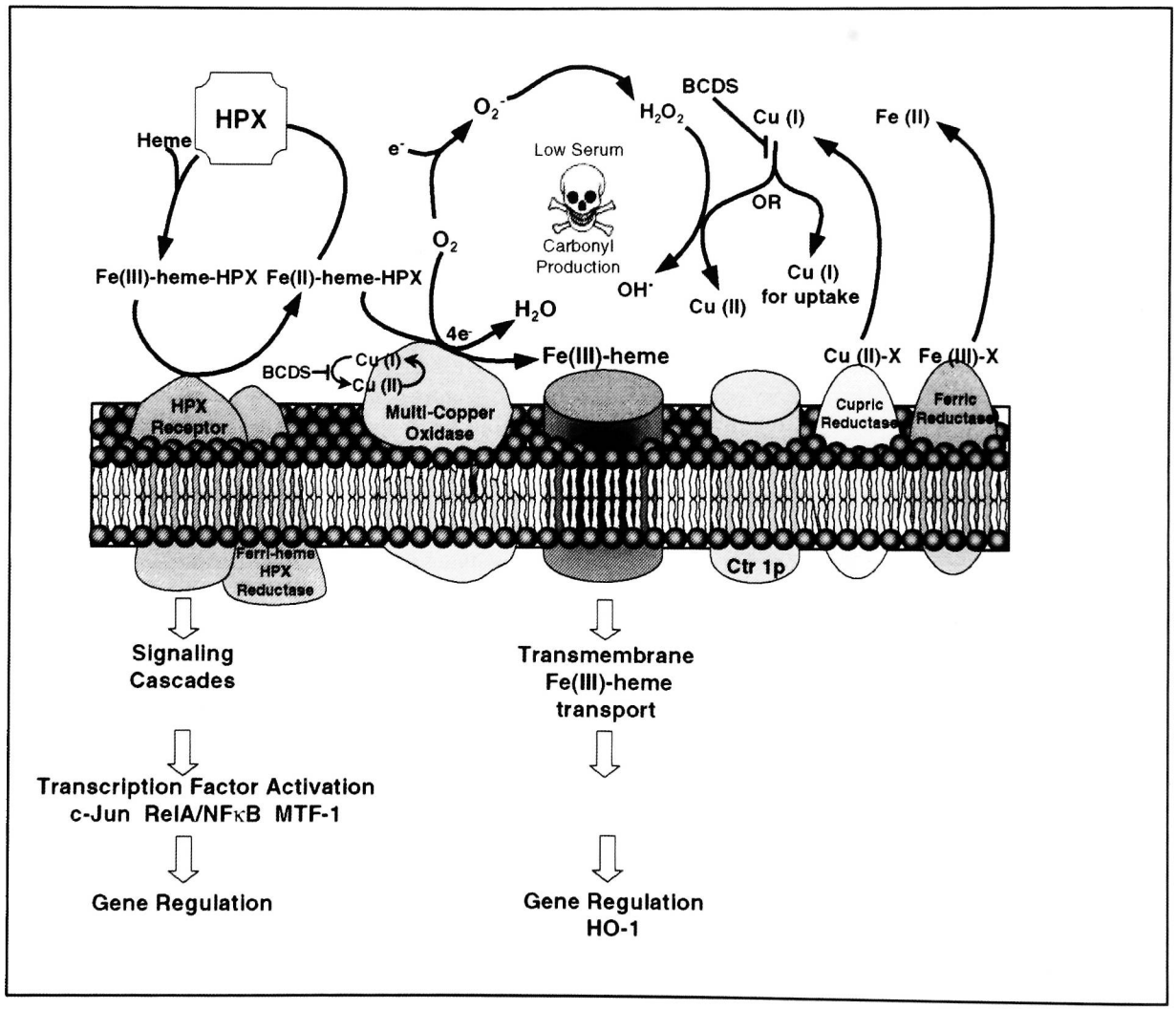

FIG. 1. Scheme of surface redox processes in the hemopexin system and their links with signaling cascades and gene regulation. Hemopexin (HPX)-bound ferric heme is a substrate for a reductase facilitating heme release from hemopexin. A copper-dependent oxido-reductase is also activated by heme–hemopexin. This step is proposed to facilitate heme uptake across the plasma membrane and to lead to increased transcription of HO-1. By analogy with yeast Fet3p, this is an integral membrane protein, although a copper ecto-enzyme has also been described (Dr. J. Morre, personal communication). Potential sites of action of the impermeable Cu(I) chelator BCDS are shown. When ligand binds to the hemopexin receptor, signaling cascades are activated leading to increased nuclear levels of transcription factors including phospho-c-Jun, and to activation of MT-1 transcription.

pexin system, heme transport requires ferriheme derived from a receptor-associated ferro-heme-(hemopexin)-O_2-oxidoreductase. This protein would be a copper-dependent oxidase analogous to Fet3p in the yeast plasma membrane. A heme transporter moves heme across the membrane. Electron transfer generates Cu(I) at the cell surface, where the cycling copper resides in the extracellular domain of the copper oxidase, thus accessible to the specific copper chelator bathocuproinedisulfonate (BCDS). This chelator inhibits Fet3p (de Silva *et al.*, 1997).

Furthermore, we proposed that these surface events are linked with gene regulation by the hemopexin receptor (Smith, 1999). In support of this, BCDS prevents the induction of MT-1 mRNA by hemopexin complexes (Sung, 1999). Here, we have carried out a series of investigations to substantiate and extend the model for the hemopexin receptor. First, we show using BCDS that surface events involving Cu(I) also contribute to the regulation of HO-1 by heme–hemopexin. Second, we show that ferric citrate, which is a known activator of surface reductases, inhibits the effects of hemopexin. Third, we address whether there is a role for cytochrome b_5/b_5 reductase in the surface processes using propylthiouracil (PTU), a specific inhibitor for the reductase (Lee, 1986). Finally, we show that surface copper is involved in the activation of the c-Jun N-terminal kinase (JNK) signaling cascade by hemopexin.

MATERIALS AND METHODS

Chemicals, chelators, and proteins

Heme and cobalt-protoporphyrin IX (CoPP) were obtained from Porphyrin Products (Logan, Utah); BCDS and cuprous chloride from Aldrich Chemical Co. (Milwaukee, WI), and propylthiouracil from Sigma (St. Louis, MO). BCDS is a sulfonated, and hence water-soluble, derivative of bathocuproine (2,9-dimethyl-4,7,-diphenyl-1,10-phenanthroline), which binds copper(I) specifically as a *bis*-chelate complex [$(BCDS)_2Cu(I)$], in which Cu(I) is stably coordinated between four nitrogen atoms. BCDS does not bind iron (Diehl, 1972; Wong *et al.*, 1984). The BCDS-copper (I) complex [$(BCDS)_2Cu(I)$] was made according to the procedure of Davis *et al.* (1995), stored under argon, and sterilized by filtration through a 0.2-μm nylon membrane before incubation with the cells. The final concentration of $(BCSD)_2Cu(I)$ was determined using a molar extinction coefficient at 483 nm of 12,250 ($A.M^{-1}\cdot cm^{-1}$). $(BCDS)_2Cu(I)$ complexes are stable and oxidized only slowly by air (Diehl, 1972). Hemopexin was isolated from rabbit serum (Pelfreeze, Rogers, AK) using published procedures (Morgan *et al.*, 1988a) and the heme and CoPP complexes formed (>90–95% saturation) by addition of 0.9–1.1 molar equivalents of heme or heme analog followed by dialysis to remove dimethylsulfoxide (DMSO). The complex concentrations and saturation were determined spectroscopically using published extinction coefficients (Morgan *et al.*, 1988a,b). Diferric-transferrin (Collaborative Biomed. Prod., Bedford, MA) was reconstituted in HEPES-buffered Dulbecco's modified Eagle medium (DMEM) medium containing glucose (4,500 mg/L) and bicarbonate.

Cell culture, RNA isolation, and Northern analysis

The growth, maintenance, and incubation conditions of mouse hepatoma (Hepa) cells and the effect of heme– and CoPP-hemopexin complexes on the expression of MT-1 and HO-1-mRNAs have been described previously (Smith *et al.*, 1993). Hepa cells were incubated in HEPES-buffered DMEM containing 0.5% fetal bovine serum (FBS) with either heme– or CoPP–hemopexin complexes in the presence or absence of BCDS at the concentrations indicated in Figure 2 legend and text. Experiments were carried out, first, for 1 hr to examine the more rapid effects involving surface events and signaling cascades or, second, for 3 hr to allow heme catabolism and induction of ferritin and HO-1 mRNA. Total RNA was subsequently isolated from rinsed cells as described below. Incubations of Hepa cells and measurement of steady-state mRNA levels using Northern blot analyses with riboprobes for MT-1, HO-1, and actin have been described previously (Ren and Smith, 1995). For the experiments with the specific cytochrome b_5 reductase inhibitor, cells

were incubated with propylthiouracil (PTU) for 10 min before addition of heme–hemopexin complexes. The mRNA was isolated using QI-Ashredder columns and RNeasy kits (both from Qiagen, Chatsworth, CA), fractionated on a 1% agarose gel, and, after transfer, covalently linked to a Nytran nylon membrane by exposure to UV light using an energy of 1.25 J/cm^2 in a Hoefer UVC 500 apparatus. Prehybridization was at 60°C for the HO-1 and MT-1 probes and at 68°C for the actin controls. The membranes were incubated for 14–16 hr with the riboprobes (~1×10^4 cpm/μg RNA loaded), and the hybridization incubation was terminated by dilution and three high-stringency washes at 65°C for the HO-1 and MT-1 probes. This was followed by a low-stringency wash at 60°C. Three high-stringency washes at 68°C were used to terminate the hybridization incubation for the control, actin or tubulin. The relative changes in mRNA levels from two to three individual experiments were assessed using Image software from the National Institutes of Health (NIH) (version 1.61) after calibration with a Kodak photographic step tablet #2 (lot#1523406). Images were scanned using a UMAX scanner (Model S-6E) with the Vista Scan version 2.3 scanning module.

Determination by immunoblotting of cytosolic ferritin levels and phosphorylation of c-Jun

Cellular protein was measured in aliquots of cell extracts by the bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL). Ferritin induction was determined by Western immunoblotting of mouse Hepa cell extracts after 3, 7, and 17 hr in serum-free medium containing as an inducer either 10 μM heme–hemopexin, 5 μM diferric transferrin, or 10–50 μM ferric citrate. Ferritin levels were determined in samples of cytosol (100,000 $\times$ g SN) heated at 70°C for 10 min followed by centrifugation at 12,000 $\times$ g, 5 min. after electrophoresis on a Tris-Tricine, 10–20% acrylamide gel to resolve the H- and L-ferritin subunits (Rogers *et al.*, 1990) using rabbit polyclonal anti-human spleen ferritin antibody (ICN Biomedicals Inc., Costa Mesa, CA).

Hepa cells (seeded at 1.3×10^6 cells per T-75 flask) were incubated in HEPES-buffered DMEM containing 0.5% FBS for 5–30 min with either 10 μM heme- or CoPP–hemopexin in the presence or absence of BCDS (50 μM) and cell extracts prepared as previously described (Eskew *et al.*, 1999). Cellular protein was measured in aliquots of cell extracts by the BCA method (Pierce Chemical Co., Rockford, IL). The transcription factor, c-Jun, is the principal substrate of JNK and the levels of phospho-c-Jun in cell extracts (40 μg protein) was estimated by immunoblotting using a polyclonal anti-phospho-c-Jun (Ser63) antibody followed by detection using ECL (Amersham, Arlington Heights, IL) and quantitation using the NIH Image program.

RESULTS

Effect of the specific copper chelator BCDS on HO-1 regulation by heme–hemopexin and comparison with MT-1

The data in hand support that the action of BCDS is rapid, occurs at the cell surface before catabolism of heme increases intracellular iron levels, and prevents the induction of MT-1 mRNA by heme–hemopexin. Here, we show that BCDS also prevents the extensive induction of HO-1 mRNA by heme–hemopexin (Fig. 2A). HO-1 is less sensitive to inhibition by BCDS than is MT-1. Inhibition by BCDS of MT-1 induction by 5 μM heme–hemopexin is apparent within 1 hr (Fig. 2B) and is inhibited more than 50% by equimolar concentrations of BCDS (6.25 μM; Sung *et al.*, 1999). Basal levels of HO-1 mRNA are not decreased when cells are incubated with 50 μM BCDS as are basal levels of MT-1 mRNA. To show that the effect of BCDS is due to its ability to chelate copper, cells were incubated with heme–hemopexin and a preformed copper–chelator complex [$(BCDS)_2Cu(I)$]. This complex does not prevent the induction by heme–hemopexin of MT-1 mRNA at 1 hr (Fig. 1 B, lane 4) or 3 hr (data not shown). Although $(BCDS)_2Cu(I)$ alone is without effect (Fig. 1B, lanes 5 and 6, respectively), it augmented induction of both MT-1 and HO-1 mRNA by heme–hemopexin. Possible mechanisms for this phenomenon, which the data show requires hemopexin receptor occupancy, are described below.

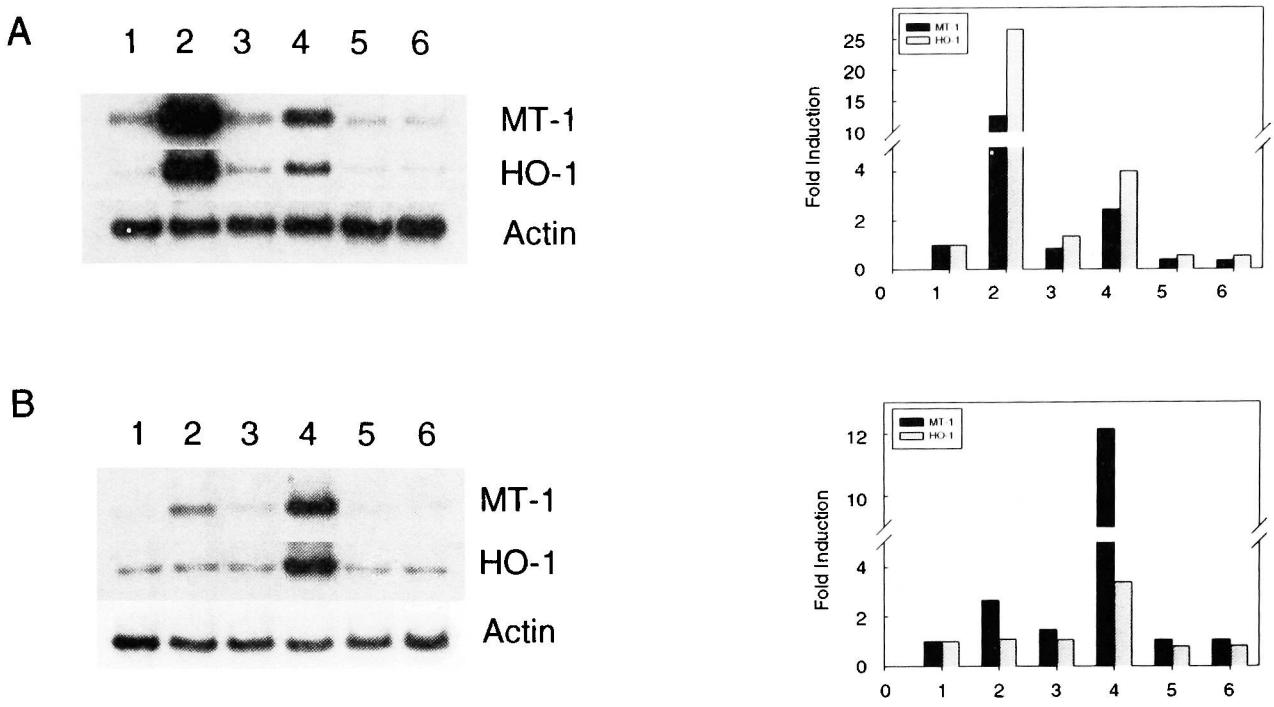

FIG. 2. Effect of BCDS on the induction of MT-1 and HO-1 mRNA by heme–hemopexin. The results of Northern analyses are shown. (**A**) Hepa cells were incubated for 3 hr with 10 μM heme–hemopexin in the presence or absence of BCDS. Lane 1, PBS control; lane 2, heme–hemopexin alone; lane 3, heme–hemopexin and 50 μM BCDS; lane 4, heme–hemopexin and 25 μM BCDS; lanes 5 and 6, 50 and 25 μM BCDS, respectively. (**B**) Northern blot analysis of the effect of BCDS and $(BCDS)_2Cu(I)$ on MT-1 and HO-1 induction by 5 μM heme–hemopexin after 1 hr. Lane 1, PBS control; lane 2, heme–hemopexin alone; lane 3, heme–hemopexin and 12.5 μM BCDS; lane 4, heme–hemopexin and 6.25 μM $(BCDS)_2Cu(I)$ BCS; lane 5, 12.5 μM BCDS; and lane 6, 6.25 μM $(BCDS)_2Cu(I)$. Actin controls are shown throughout in the lower panels. The histograms indicate the fold-induction relative to the PBS control or to heme–hemopexin-treated cells. The results of one representative experiment from a series of two to three independent experiments is shown.

Effect of ferric citrate and diferric transferrin on MT-1 and HO-1 mRNA expression induced by heme–hemopexin

Additional support for the model that participation of redox events from heme–hemopexin bound to the hemopexin receptor leads to gene regulation comes from competition studies with ferric citrate and iron–transferrin. These iron complexes are known to activate surface oxido-reductases for iron uptake by mammalian and yeast cells. Some reductases are known to interact with more than one partner, and those proposed to be activated by heme–hemopexin may be shared with those involved in iron uptake. Therefore, the model predicts that when heme–hemopexin is present at the cell surface together with these iron compounds, there would be competition for reductases and their associated coenzymes as well as for substrates such as oxygen for the copper-dependent oxidase needed for iron uptake. In either case, if these surface processes are linked with gene regulation, then effects on HO-1 and/or MT-1 induction by heme–hemopexin are predicted when these iron complexes are present.

Ferric citrate inhibits MT-1 mRNA induction by heme–hemopexin (Sung *et al.*, 1999, #5015). Only low concentrations of ferric citrate (2 and 4 μM) are required, essentially equimolar to the heme–hemopexin (Fig. 3). HO-1 mRNA induction by heme–hemopexin is also attenuated (Fig. 3B). Low concentrations of ferric citrate effectively inhibit MT-1 mRNA induction by 4 μM heme–hemopexin by 60% within 1 hr (see Sung *et al.*, 1999 and Fig. 3A) which is maintained for up to 3 hr (Fig. 2B).

The inhibitory effects of ferric citrate on the hemopexin system were more extensive than those of diferric-transferrin (5 and 10 μM). Diferric-transferrin also inhibited MT-1mRNA in-

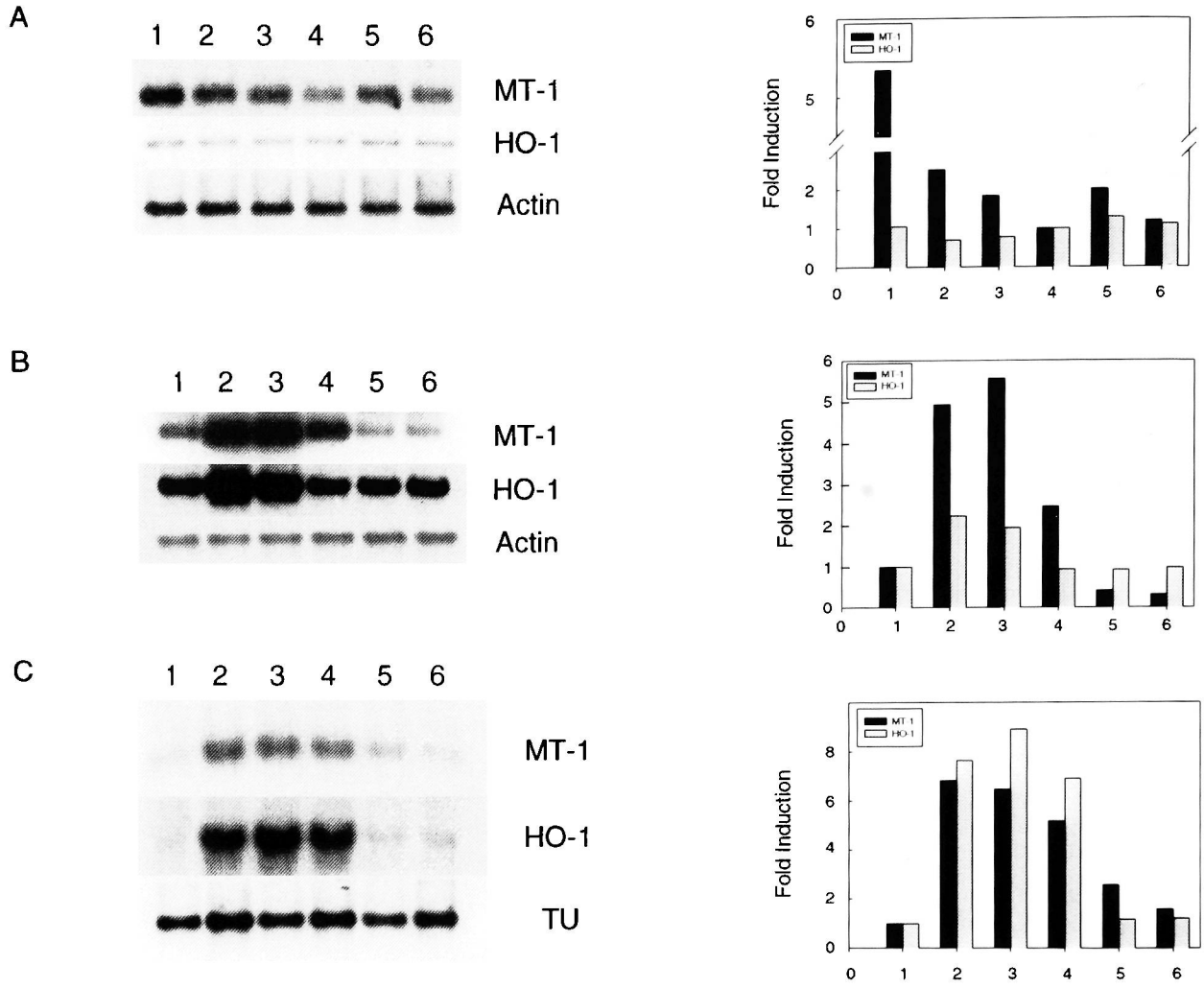

FIG. 3. Effect of ferric citrate or diferric transferrin and on the induction by heme–hemopexin of MT-1 and HO-1 mRNA. (**A**) Data from Hepa cells incubated for 1 hr either with 4 μM heme–hemopexin and 0, 2, and 4 μM ferric citrate (lanes 1–4, respectively) or with 2 or 4 μM ferric citrate alone (lanes 5 and 6, respectively). (**B**) Data from Hepa cells incubated for 3 hr either with PBS (lane 1), 10 μM heme–hemopexin alone (lane 2), or together with 2 or 4 μM ferric citrate (lanes 3 and 4, respectively); 2 or 4 μM ferric citrate (lanes 5 and 6, respectively). Actin controls are also presented as indicated. (**C**) Hepa cells incubated for 3 hr either with PBS (lane 1), 10 μM heme–hemopexin alone (lane 2), or with 5 or 10 μM diferric transferrin (lanes 3 and 4, respectively), 5 or 10 μM diferric transferrin alone (lanes 5 and 6, respectively). Also shown are histograms of the fold-increases in MT-1 and HO-1 mRNA levels relative to the PBS-treated cells calculated by densitometry of the autoradiograph depicted. The results of one representative experiment from a series of two to three independent experiments is shown.

duction by heme–hemopexin, but to a variable degree (ranging from 25% to 60% in three independent experiments). There were only minimal effects of diferric transferrin on HO-1 induction by heme–hemopexin. Significantly, neither ferric citrate (Fig. 3A) nor diferric-transferrin (Fig. 3B) alone induce MT-1 or HO-1 mRNA showing that activation of surface reductases or the copper-dependent oxidase alone does not induce the expression of MT-1 and HO-1. Thus, these data also show a key role for the hemopexin receptor in the regulation of both MT-1 and HO-1.

The inhibitory effect of ferric citrate on the hemopexin system (Sung *et al.*, 1999 and Fig. 3) occurs at concentrations significantly lower than those needed to induce ferritin protein (Fig. 3). Physiologically relevant concentrations of iron–transferrin or heme–hemopexin, based on their plasma concentrations of approxi-

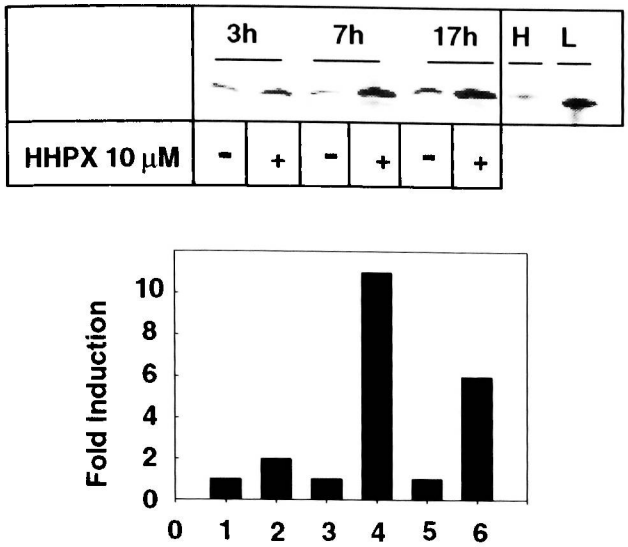

FIG. 4. Effect of heme–hemopexin on expression of cytosolic ferritin H- and L-subunits. Ferritin H- and L-chains in Hepa cell cytosol samples were resolved by SDS-PAGE using a 10–20% Tris-Tricine polyacrylamide gel and detected by Western immunoblotting with a rabbit polyclonal anti-ferritin antibody as described in Materials and Methods. The course of ferritin induction by 10 μM heme–hemopexin at 3, 7, and 17 hr compared with control cells incubated with PBS is shown. Purified recombinant human ferritin H (rH) and L (rL) chains (4 and 0.25 μg, respectively) were used as controls. In the accompanying histogram, the fold changes of one representative experiment from two independent experiments are given.

mately 15 μM, do induce ferritin, which is not rapidly turned over. Heme–hemopexin (10 μM) increases ferritin, predominantly the H-chain, approximately 10-fold within 7 hr, and levels remain elevated for at least 17 hr (Fig. 4). As expected, by increasingly intracellular iron pools, ferritin levels remain elevated, about five-fold higher than controls, 17 hr after 5 μM diferric transferrin (Sung *et al.*, 1999). These are not large changes in protein levels, but they are consistent with the capacity of each ferritin molecule to sequester 4,500 atoms of iron, equivalent to 4,500 heme–hemopexin complexes or 2,250 diferric–transferrin complexes. Ferritin induction by ferric citrate required 25 and 50 μM, which produced an approximately three-fold increase by 7 hr (data not shown). Extensive induction of ferritin routinely requires significantly higher concentrations of iron, *e.g.*, 100 μM heme or ferric–complexes (White and Munro, 1988).

Effect of propylthiouracil on MT-1 and HO-1 mRNA induction by heme–hemopexin

Cytochrome b_5 reductase acts as an electron donor at the inner surface of the plasma membrane (Villalba *et al.*, 1997) and is specifically inhibited by PTU (Lee and Kariya, 1986). Cytochrome b_5 reductase could be linked with the hemopexin receptor: directly by associated putative reductase or via transmembrane electron transport together with an electron carrier of

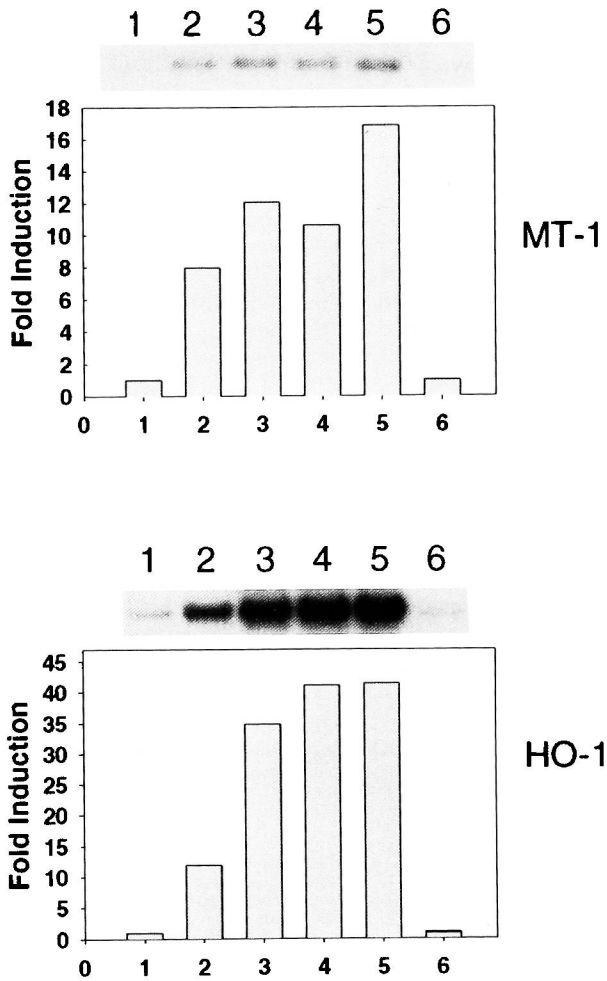

FIG. 5. Effect of PTU, a specific inhibitor of cytochrome b_5 reductase, on MT-1 and HO-1 mRNA induction by heme–hemopexin. The levels of MT-1 mRNA (**upper panel**) and HO-1 mRNA (**lower panel**) were detected by Northern analyses as described in Materials and Methods using published procedures. Shown are: Hepa cells incubated for 2 hr with either PBS (lane 1), 10 μM heme–hemopexin (lane 2), 10 μM heme–hemopexin after 10 min exposure to 0.5, 1.0, or 2.0 mM PTU (lanes 3–5, respectively), or with 0.5 mM PTU alone (lane 6). The fold changes are shown in the accompanying histograms using the data from one representative experiment out of a series of three independent experiments.

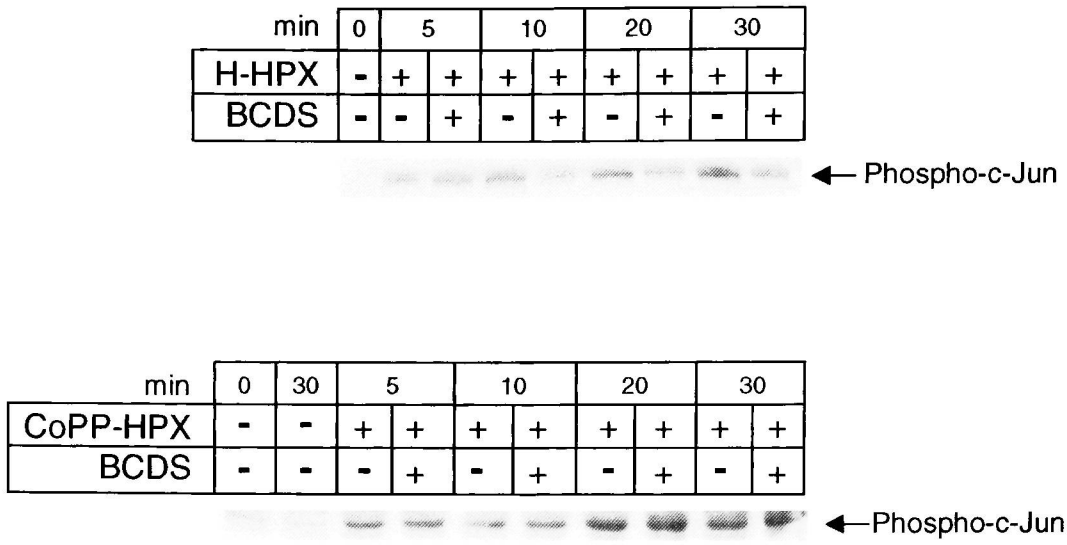

FIG. 6. Effect of the Cu(I) chelator BCDS on the phosphorylation of c-Jun by heme- or CoPP-hemopexin. Hepa cells were seeded at 2×10^6 cells per well in six-well plates and when approximately 80% confluent were incubated for up to 30 min in DMEM containing 0.5% FBS in the presence or absence of 10 μM heme- or CoPP-hemopexin. The upper and lower panels, respectively, show the effect of BCDS (50 μM) on the levels of phospho-c-Jun in response to heme-hemopexin or CoPP-hemopexin at the times indicated and detected by immunoblotting (New England BioLabs, Beverly MA) and quantitated as previously described (Eskew *et al.*, 1999). The results from one of two independent experiments are shown.

suitable redox potential such as a quinone or a heme prosthetic group. Increasing concentrations of PTU augment, in a dose-dependent manner, the induction of both MT-1 and HO-1 mRNA levels when cells are incubated with heme–hemopexin. This effect is maintained for up to 1–2 hr (Fig. 5). Significantly, PTU alone (0.5 mM, in Fig. 5, lane 6, to 5 mM, data not shown) does not alter MT-1 or HO-1 mRNA levels. Thus, occupancy of the hemopexin receptor is one requirement for the effects of PTU on HO-1 and MT-1.

Effect of BCDS on JNK activation by heme- and CoPP–hemopexin binding to the hemopexin receptor

When cells are incubated with heme–hemopexin, there is a transient increase in surface oxidation shown by carbonyl production. Oxidation of proteins including Ras has been shown to be part of the activation of signaling cascades like JNK, and heme–hemopexin also activates JNK (Eskew *et al.*, 1999). Therefore, we addressed whether BCDS inhibits JNK activation

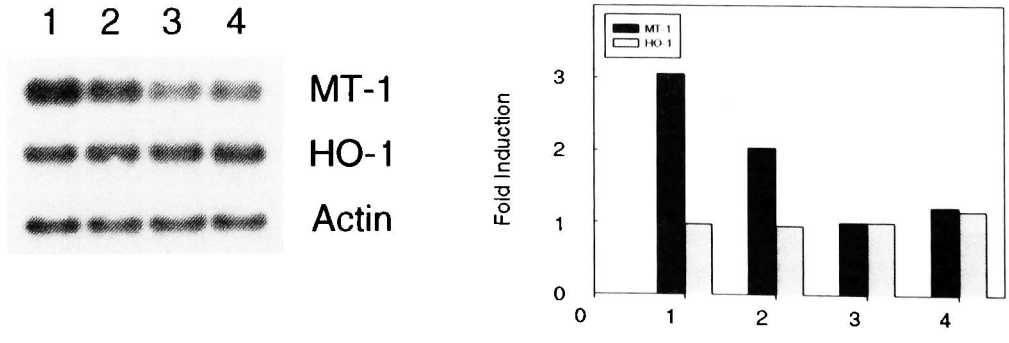

FIG. 7. Effect of BCDS on the induction of MT-1 mRNA by CoPP–hemopexin. Hepa cells were incubated for 1 hr with 10 μM CoPP-hemopexin in the presence or absence of BCDS and the results of Northern analyses are presented. Shown are: 10 μM CoPP–hemopexin alone (lane 1) with 25 μM BCDS (lane 2); PBS control (lane 3); and 25 μM BCDS (lane 4). The histogram indicates the fold-induction relative to the CoPP–hemopexin-treated cells of one representative experiment from a series of two to three independent experiments.

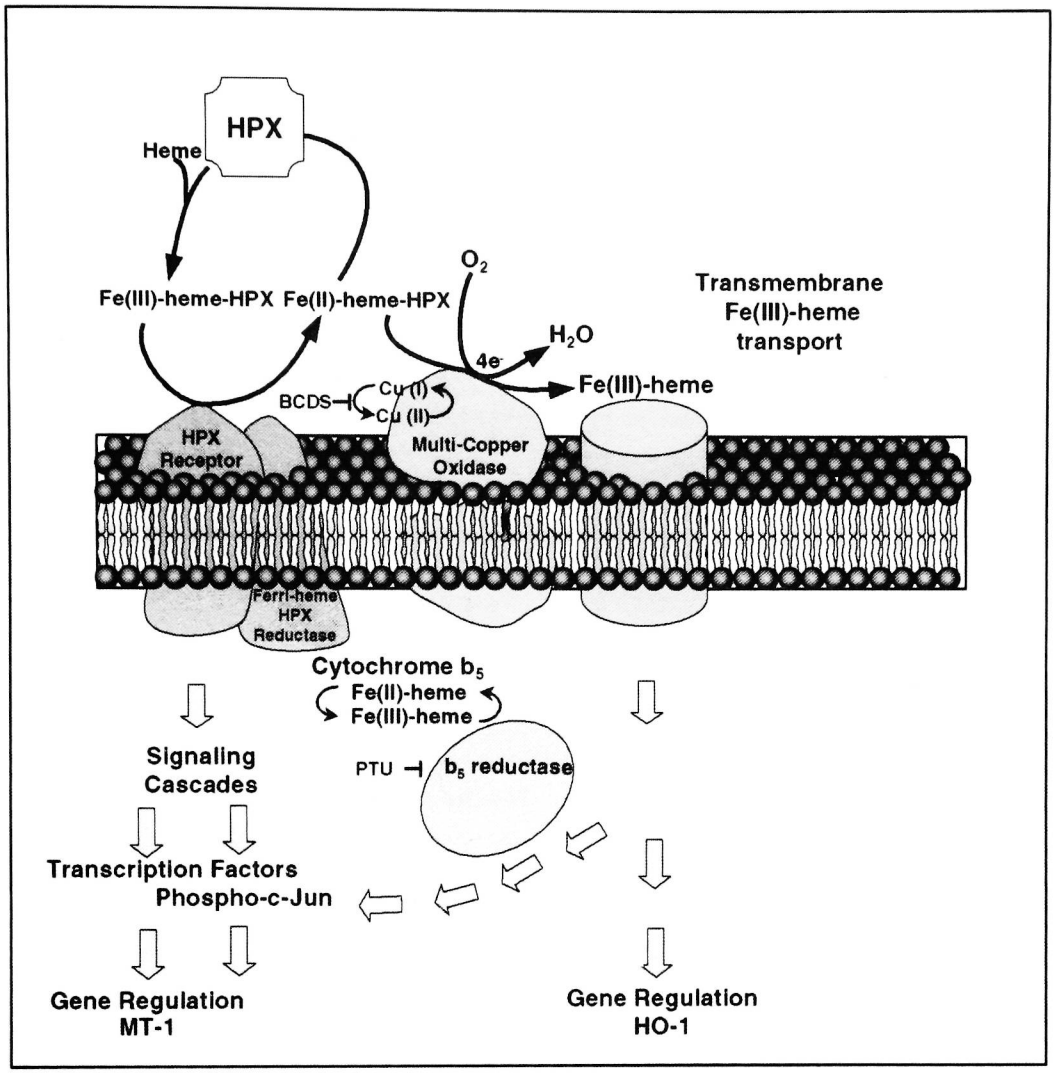

FIG. 8. A possible site for cytochrome b_5/b_5 reductase in redox processes that affect the response of HO-1 and MT-1 to heme–hemopexin. The participation of redox processes at the cell surface require a redox active metal in the porphyrin and can be distinguished from signaling events triggered directly via the hemopexin receptor. The events depicted, supported by data presented here or published previously, are: Cu(I) participates at the cell surface; the hemopexin receptor, a reductase and a copper-dependent protein are linked; ligand binding to the hemopexin receptor alone activates the JNK cascade; copper is needed but in different ways for HO-1 and MT-1 regulation by heme–hemopexin.

by heme–hemopexin. The data in Fig. 6 show that BCDS diminishes by 40–50% the levels of c-Jun phosphorylated within 10 min exposure to heme–hemopexin, consistent with a decrease in JNK activation. However, the phospho-c-Jun levels induced by CoPP–hemopexin, which also activates JNK (Eskew *et al.*, 1999), are not affected by BCDS. We draw two conclusions: first, that signaling to the JNK cascade occurs via ligand binding to the hemopexin receptor; and second, that the copper-dependent process is at a step requiring activation of the copper-dependent oxidase by a redox-active heme on receptor-bound hemopexin. This copper-dependent step leading to decreased phospho-c-Jun is not activated by CoPP–hemopexin–receptor interactions and may differ from that considered to be needed for heme transport. Alternatively, the activity of a c-Jun phosphatase may be increased.

Effect of BCDS on MT-1 regulation by CoPP–hemopexin

Evidence that copper is also involved in activation via the hemopexin receptor of one or more signaling pathway(s) for MT-1 mRNA regulation comes from additional experiments

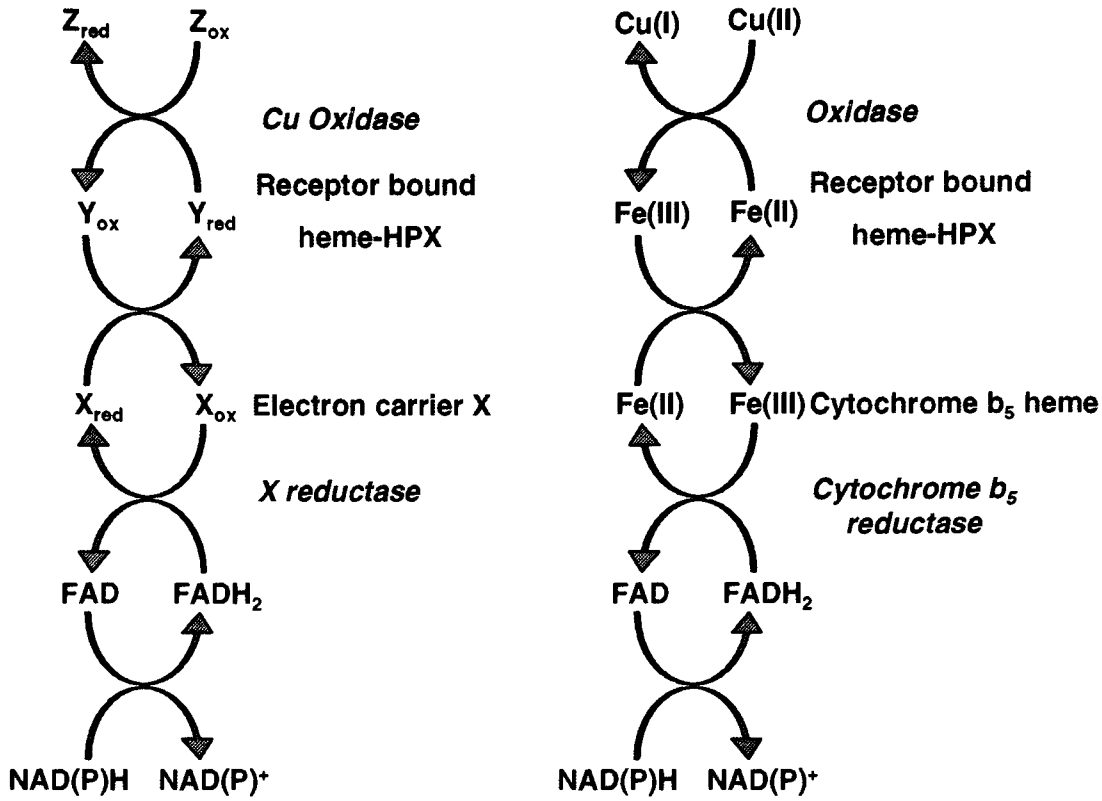

FIG. 9. Schematics of electron transfer pathways. A simple electron transfer pathway directly from cytochrome b_5 reductase to receptor-bound heme-hemopexin is shown on the right hand side of the figure. A more general pathway is shown on the left and additional redox partners (not shown) may also be involved. In the presence of the reductase inhibitor PTU, signals, which augment MT-1 mRNA induction in response to ligand binding to the hemopexin receptor, are proposed to come from an altered ratio of oxidized to reduced coenzyme on the b_5 reductase (RHS), or another reductase (LHS), or from increased levels of their electron acceptors. NAD^+ is one cofactor for cytochrome b_5 reductase and, as mentioned in the text, of $NADP^+$-supported activity

with CoPP–hemopexin. BCDS (25 μM) inhibits the induction of MT-1 mRNA by 10 μM CoPP–hemopexin (Fig. 7), although to a lesser extent than by heme–hemopexin. Thus, there is another role for copper in the hemopexin system that links surface effects with MT-1 gene regulation indicated by the data presented here.

DISCUSSION

The inhibition by BCDS of both MT-1 and HO-1 mRNA up-regulation by heme–hemopexin is shown here to be due to chelation of copper. BCDS and its copper complex are impermeable to cells. BCDS preferentially binds Cu(I), which is generated in an oxidizing, extracellular environment by an electron donor. Thus, the copper required in the gene regulatory pathway undergoes redox cycling in response to heme–hemopexin binding to its receptor and is either extracellular or at the plasma membrane (Fig. 1). In the model for the hemopexin system, a copper-dependent oxidase is one site of action of BCDS (Fig. 8 and Smith, 1999). Free heme and heme–hemopexin induce HO-1 but CoPP–hemopexin, a stable complex, does not (Smith *et al.*, 1993). Thus, heme uptake is needed for HO-1 regulation by heme–hemopexin (Smith, 1990).

Heme reduction is required for heme release from hemopexin and heme oxidation by the copper-dependent oxidase is then necessary for heme uptake into cells. The sites of action for BCDS appear different for HO-1 and MT-1 regulation. The inhibition by BCDS of HO-1 induction by heme–hemopexin is consistent with a role for the copper-dependent ferrous heme–hemopexin oxidase in HO-1 gene regulation, which we propose is linked to heme uptake. Whether BCDS decreases heme transport by hemopexin is not known.

A receptor-associated heme reductase is proposed to act in the surface events for the hemopexin system for heme release from hemopexin. Evidence for interaction between the iron and heme transport systems is indicated by the competition experiments in which the iron complexes inhibit regulation by heme–hemopexin. However, a specificity of regulatory responses upon activation of surface reductases apparently exists. This is conferred in part by the hemopexin receptor. Activation of surface reductases by ferric citrate and iron–transferrin (Inman *et al.*, 1994; Ekmekcioglu *et al.*, 1996; Yu and Wessling-Resnick, 1998) does not induce HO-1 or MT-1 regulation. Effects due to endocytosis are ruled out by the data from ferric citrate.

We have also used a cytochrome b_5 reductase inhibitor to probe predicted relationships between the oxidation state of members of surface redox processes leading to gene regulation. In the context of our working model for the hemopexin system, cytochrome b_5/cytochrome b_5 reductase is one component of the plasma membrane electron transfer processes that participates in surface events associated with the hemopexin system (Fig. 8). In maize roots, reduction of ferric citrate is carried out by a cytochrome b_5 reductase loosely associated with membranes and thought to act at the plasma membrane (Sparla *et al.*, 1997). In mammalian cells, one form of b_5/b_5 reductase is loosely associated with membranes and has a lower redox potential than the mitochondrial form. Cytochrome b_5 and its associated b_5 reductase could be linked directly with the heme–hemopexin–hemopexin receptor and another oxido-reductase or b_5 reductase alone may be involved. We further propose that transcriptional regulation is linked in part to the oxidation state of the electron carrier that accepts electrons from cytochrome b_5 reductase which is cytochrome b_5 or to other carriers in the electron pathway as depicted schematically in Fig. 9. Significantly, this is not the only requirement for specific gene regulation. One key molecule is the hemopexin receptor itself. The augmented induction of both MT-1 and HO-1 mRNAs when the b_5 reductase is inhibited requires that heme–hemopexin be bound to its receptor. PTU alone alters neither MT-1 nor HO-1 mRNA levels. When cytochrome b_5 reductase is inhibited by PTU, the electron acceptor of the reductase, *i.e.*, cytochrome b_5, remains oxidized. In addition, the flavin and NAD coenzymes of the reductase remain reduced, as does any the redox partner of (electron donor to) the reductase. (Both NAD and NADP-dependent cytochrome b_5 reductase activity has been reported). Heme–hemopexin would remain oxidized and heme would not be taken up into cells. On the basis of redox potentials, b_5/b_5 reductase would reduce heme–hemopexin in solution (redox potential of 0.65 mV), although receptor binding is anticipated to alter the redox potential of heme–hemopexin complexes.

Cobalt is far less redox active than iron, and CoPP–hemopexin does not generate carbonyl production as does heme–hemopexin and free heme (Eskew *et al.*, 1999). Thus, CoPP–hemopexin allows investigation of transcriptional activation from the receptor alone in the absence of tetrapyrrole transport (Smith *et al.*, 1993) and surface redox processes. The hemopexin receptor is the principal means of activation of signaling pathways that are currently being defined. One or more cascades lead to MT-1 gene transcription.

We also investigated whether copper plays a role at the cell surface in the activation of JNK signaling cascades when ligand binds to the surface hemopexin receptor and whether concomitant heme transport further influences cellular responses. Copper is shown here to be involved in activation of the JNK signaling cascade by heme–hemopexin. This is another important response of cells to the hemopexin heme transport system. JNK is not activated by free heme (Eskew *et al.*, 1999). The data presented here support one or more roles for copper in the activation of the JNK cascade that requires not only occupancy of the hemopexin receptor but also activation of the copper-dependent oxidase needed for heme transport (Fig. 8). Thus, the hemopexin receptor is linked to the heme–hemopexin reductase and with the copper-dependent oxidase.

Copper at the surface is also needed for additional signals from the hemopexin receptor, *i.e.*, from the data presented here to be distinct from the JNK cascade, for activation of MT-1 transcription. The nuclear translocation of a key regulatory factor of the MT-1 gene, MTF-1, is stimulated by both heme– and CoPP–he-

mopexin and inhibited by BCDS (Vanacore *et al.*, 2000). MTF-1 is a transcription factor that binds to metal-responsive elements in the MT-1 proximal promoter and is considered important for MT-1 regulation by a variety of structurally dissimilar inducers. The MREs are needed for the full response to heme–hemopexin in transient transfection assays of fusion gene constructs of the MT-1 proximal promoter (Ren and Smith, 1995).

Perspectives

The surface events leading to regulatory responses by heme–hemopexin are triggered before redox active iron is released by heme catabolism. We propose that Cu(I) participates in the initial surface events, consistent with the inhibition by BCDS. The participation of redox processes at the cell surface, requiring a redox active metal in the porphyrin, which is activated upon binding of heme–hemopexin to its receptor, can be distinguished from events triggered directly via the hemopexin receptor. The hemopexin receptor activates the JNK cascade. Signals that augment, for example, MT-1 mRNA induction in response to ligand binding to the hemopexin receptor are proposed to come from an altered ratio of oxidized to reduced enzyme on the b_5 reductase or from lack of reduction of cytochrome b_5 (*i.e.*, oxidation of cytochrome b_5; Fig. 9). Reduction of heme is needed to facilitate release from hemopexin needed for uptake. Effects on HO-1 regulation are considered to reflect changes in heme uptake, which is a principal, but not sole, requirement for HO regulation by the hemopexin system. A copper-dependent process plays a role in the pathway for c-Jun phosphorylation activated by the hemopexin receptor requiring heme release (*i.e.*, reduction) for transport. Another copper-dependent pathway is involved in MT-1 regulation with effects on MTF-1 (Vanacore *et al.*, 2000). Thus, this research points to important links between redox-cycling copper, heme homeostasis, and gene regulation.

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ABBREVIATIONS

BCA, bicinchoninic acid; BCDS, bathocuproinedisulfonate; $(BCDS)_2Cu(I)$, cuprous-bathocuproinedisulfonate complex; CoPP, Cobalt-protoporphyrin IX; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; heme, iron protoporphyrin IX; HO-1, heme oxygenase-1; HPX, hemopexin; JNK, c-Jun N-terminal kinase; MT-1, metallothionein-1; MTF-1, metallothionein transcription factor 1; PBS, phosphate buffered saline; PTU, propylthiouracil; PKC, protein kinase C; ROS, reactive oxygen species.

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