Endoplasmic reticulum anchored heme-oxygenase 1 faces the cytosol

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ABSTRACT

Heme-oxygenase 1 is an endoplasmic reticulum-anchored enzyme that breaks down heme into iron, carbon monoxide and biliverdin. Heme is a hydrophobic co-factor in many proteins, including hemoglobin. Free heme is highly cytotoxic and, therefore, both heme synthesis and breakdown are tightly regulated. During turnover of heme proteins, heme is released in the phago-lysosomal compartment or the cytosol. The subcellular location of the heme-oxygenase 1 active site has not been clarified. Using constructs of heme-oxygenase 1 with fluorescent proteins, and the endogenous heme-oxygenase 1 in two variations of protease protection assays, we determined that heme-oxygenase 1 is membrane-bound and faces the cytosol in non-activated macrophages *in vivo*. These findings imply that in quiescent macrophages, heme breakdown products are generated in the cytosol. This facilitates iron recycling to ferroportin for iron export and to ferritin for iron storage.

Key words: heme-oxygenase, tail-anchored proteins, fluorescence protease protection assay.

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Introduction

Heme is a highly hydrophobic molecule embedded in heme proteins and is used as a carrier and sensor of gases, as an electron donor and acceptor, and as a catalyst in many enzymatic reactions.[1] When released from hemoproteins, heme is degraded by heme-oxygenase (HO)1 or HO2. The subcellular location of heme protein degradation is not uniform. Nitric oxide synthase is digested by the proteasome and therefore its heme is released to the cytosol[2] while other heme proteins, such as hemoglobin, catalase and all the mitochondrial heme proteins release their heme into membrane-bound vesicles of the phago-endo-lysosomal system.[3]

HO1 and HO2 break down heme into iron, carbon monoxide (CO) and biliverdin, and this pathway is the main physiological heme degradation system.[4] Approximately 85% of all heme degraded in mammals comes from hemoglobin.[5] The fact that most heme targeted for breakdown is released in membrane-bound compartments implies that heme needs to be transported to the site of its degradation or the enzyme needs to be recruited to the site of heme release.

In recent years, several heme transporters have been cloned, including the heme importer HRG-1.[6] HRG-1 is expressed in the endo-lysosomal system[7] and is, therefore, a good candidate to transport heme released from hemoglobin to the cytosolic compartment. But little is known about the chaperones that can transport hydrophobic heme through the aqueous cytosol, and a full pathway for heme transport from the phagosome to the endoplasmic reticulum (ER)-bound HO1 still needs to be clarified.

The HOs have a single C-terminal transmembrane region[8] and are considered ER-anchored[9] due to their enrichment in microsomal fractions. HOs have no ER targeting sequence, and membrane anchoring can occur spontaneously into microsomal membranes but not into RBC membranes.[10] Studying microsomal rat liver fractions, it was suggested that the orientation of HO is not towards the microsomal lumen, as the major soluble (N-terminal) part was cleavable by trypsin from the microsomal fraction *in vitro*.[10] However, the orientation of HO has never been studied in intact cells.

The fluorescence protease protection assay (FPP)[11] and Western blotting were used to determine the subcellular localization and orientation of HO1 *in vivo*. The plasma membranes of cells expressing various conjugates of HO1 with fluorescent proteins were selectively permeabilized by digitonin *in vivo* and cytosolic soluble proteins leaked from these cells. Addition of protease-digested membrane-bound proteins abolished the fluorescence of the construct when the fluorescent protein was facing the cytosol. Fluorescence was protected in constructs where the fluorescent protein faced the lumen of a subcellular compartment. The cytosolic orientation of HO1 was determined with this method and confirmed by Western blot.

Design and Methods

Cloning of HO1-GFP, GFP-HO1 and CFP-HO1-GFP

Enhanced (E)-GFP and E-CFP was used for all cloning and is referred to as GFP and CFP, respectively. All GFP and CFP plasmids were a kind gift from Dr. Jennifer Lippincot-Schwartz (National Institutes of

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Health, USA). HO1 (EST; GenBank AA080006; IMAGE clone ID 536616) was obtained from the IMAGE Consortium through the American Type Culture Collection (ATCC). Details about the cloning procedure are described in the *Online Supplementary Appendix*.

Cell culture and transfection

J774.1 and RAW 264.7 cells were cultured at 37°C, 5% CO_2 and 95% humidity in RPMI 1640 medium (SIGMA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine (Biological Industries, Beit Ha'emek, Israel). J774.1 cells were grown to 50-70% confluency and transfected using the GeneJammer transfection reagent (Stratagene) according to the manufacturer's protocol. RAW cells were transfected by electroporation (*Online Supplementary Appendix*).

Immunofluorescence

Cells were grown on glass coverslips, fixed in 4% paraformaldehyde in PBS and labeled with anti HO1- (SPA-895 Stressgen) or anti Calnexin-antibody (SC 6465-R, Santa Cruz) (diluted 1:250 and 1:100, respectively, in 0.1% BSA and 10% FCS in PBS). Secondary antibody, Alexa chicken anti rabbit 647 (Invitrogen), was diluted 1:1000 in PBS with 0.1% BSA. Negative control tests were performed with secondary antibodies only.

Fluorescence protease protection (FPP) assay

Protein localization was determined by the FPP assay.[11,12] All incubations and washes were carried out in KHM-buffer (20 mM Hepes, 110 mM potassium-acetate and 2 mM MgCl, pH 7.3). The minimal effective digitonin concentration for plasma membrane permeabilization was determined for J774.1 and RAW264.7 cells expressing cytosolic soluble GFP, testing a range of 20-200 μM digitonin. Between 4-5×10^5 cells were incubated in 500 μL KHM with digitonin. Optimal digitonin concentration allowing permeabilization of the plasma membrane without affecting intracellular membranes was 60 μM for J774.1 and 140 μM for RAW264.7 cells. Cells were washed with KHM-buffer, incubated with 1.75 U/mL Proteinase-K for 90 s and washed again with KHM buffer.

Western blot

Cell pellets were lysed using Triton-lysis buffer: 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton-X-100 and protease inhibitor cocktail (Complete™ without EDTA, Roche). Of the postnuclear fraction, lysates originating from equal amounts of cells were separated by 14% SDS-PAGE as described.[13] Anti-GFP (Santa Cruz) 1:1000 and anti HO1 1:1000 were used.

Image visualization

Imaging and analysis were carried out on a Zeiss LSM 700 laser scanning confocal microscope and a Nikon eclipse 55i microscope.

Results and Discussion

To study the orientation of HO1 in the ER membrane, three fluorescent HO1 constructs, GFP-HO1, HO1-GFP and CFP-HO1-GFP were engineered (Figure 1A and E). To determine if the GFP constructs are localized to the ER-like endogenous HO1, the fusion constructs were transfected to J774.1 cells and co-localized with the ER protein Calnexin (Figure 1B). Also, the cellular distribution of the constructs appeared identical to endogenous HO1 (Figure 1B). In addition, we demonstrated that the constructs were membrane-bound just like endogenous HO1 by permeabilizing transfected cells with digitonin. Cells transfected with an unconjugated GFP construct, which is a soluble cytosolic protein, lost most of their fluorescence within 60 s of digitonin permeabilization (*data not shown*). In contrast, the fluorescence in the GFP-HO1 (Figure 1C) and in the HO1-GFP (*data not shown*) expressing cells was not affected by permeabilization. Detection of the fluorescent GFP signal throughout this experiment also provided a control to ensure that the loss of fluorescent signals in later experiments was not caused by photobleaching.

We used the FPP assay[11] to determine the orientation of HO1 in the ER membrane. Following permeabilization with digitonin and addition of Proteinase-K to the cell cultures *in vivo*, the fluorescence from the GFP-HO1 construct quickly decreased from 100% to 3±2% in 30 s (Figure 1D and *Online Supplementary Figure S1*), suggesting that the GFP moiety on the HO1 faces the cytoplasm and is accessible to Proteinase-K. In contrast, there was no change in fluorescence associated with HO1-GFP during the Proteinase-K treatment (Figure 1D) (decrease from 100% to 94±10% in 30 s), suggesting that the GFP of this construct faces the ER lumen where it is protected from the protease. To further support our findings, we also examined a CFP-HO1-GFP construct and found that the CFP degraded upon permeabilization while the GFP remained protected from the Proteinase-K (Figure 1E). According to these experiments, the active site of the enzyme is facing the cytosol. Similar experiments were also carried out with the macrophage cell-line RAW 264.7 and the same results were obtained (*data not shown*).

We corroborated these results with a biochemical protease protection (BPP) assay, analyzing native and transfected J774.1 cells after permeabilization and protease digestion by Western blot using anti-GFP and anti-HO1 antibodies. GFP has a molecular mass of 30 kD and that of endogenous HO1 is 32 kD; therefore, the molecular mass of the constructs is 62 kD. From the BPP assay, we concluded that the HO1-moiety of the HO1-GFP construct was cytosolic as it was digested in permeabilized cells that had been treated with Proteinase-K, while the GFP moiety was protected from Proteinase-K digest and was, therefore, located inside the ER membrane (Figure 2A). In contrast, in the GFP-HO1 construct, the GFP moiety is facing the cytosol and, after addition of digitonin and Proteinase-K, both GFP and HO1 were digested (Figure 2A). Endogenous HO1 in native J774.1 cells was also digested by Proteinase-K following digitonin permeabilization (Figure 2B). Taken together, the results obtained from both the FPP and BPP showed that both constructs are localized in a membrane compartment, most likely the ER, and that the active site of HO1 is facing the cytosol.

HO1 activity depends on its association with the ER-anchored NADPH-cytochrome c reductase (CPR)[14] that donates electrons to HO1 and to the many ER membrane-associated cytochrome p450s. CPR was shown to face the cytosol[15,16] and many cytochrome p450s were found on ER membranes facing the cytosol as well. This suggested that HO1 would have to face the cytosol to have direct access to its electron donor CPR. However, a cytochrome p450 was recently identified that faces the ER lumen and maintains enzymatic activity, suggesting that electron transfer from CPR can take place to either side of the ER membrane.[17] In addition to CPR, also biliverdin reductase (BVR), the enzyme that catalyzes the formation of biliru-

bin from biliverdin, is associated to HO1, and BVR is a soluble cytosolic protein. Therefore, the subcellular location of BVR and its association with HO1 is coherent with our finding of the cytosolic orientation of HO1.

HO1 has no leader-sequence for ER targeting and is localized mainly to the smooth and not the rough ER, indicating that it may be inserted post-translationally into the ER. The C-terminal transmembrane region contains 18 hydrophobic amino acids suggesting that HO1 is a tail-anchored (TA) protein. The fact that none of the GFP conjugates leaked from permeabilized cells indicated that they were membrane-bound. ER membrane anchoring is either spontaneous or may be facilitated by the GET complex or other proteins that facilitate TA insertion.[18] HO1 easily integrates

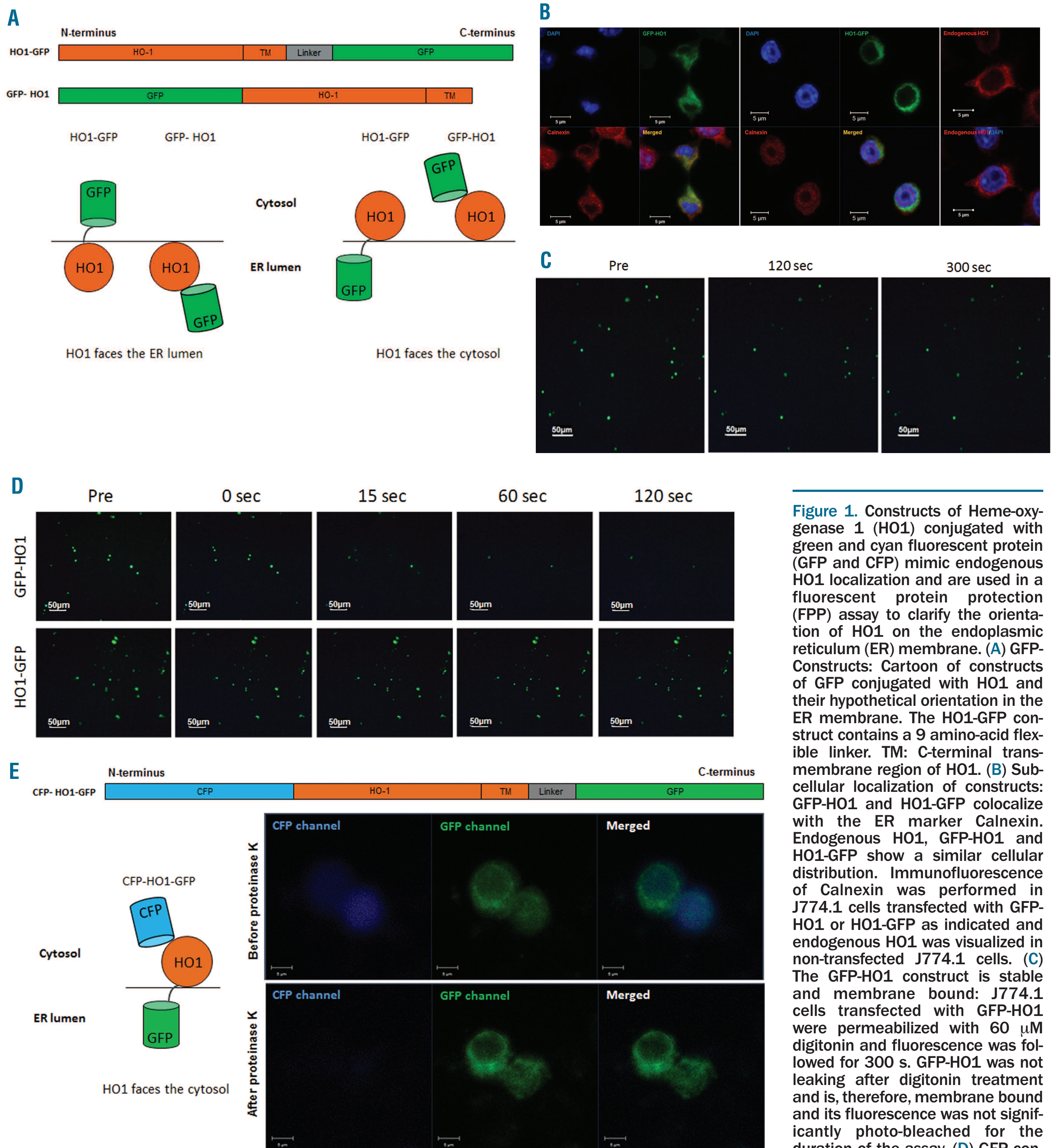

Figure 1. Constructs of Heme-oxygenase 1 (HO1) conjugated with green and cyan fluorescent protein (GFP and CFP) mimic endogenous HO1 localization and are used in a fluorescent protein protection (FPP) assay to clarify the orientation of HO1 on the endoplasmic reticulum (ER) membrane. (A) GFP-Constructs: Cartoon of constructs of GFP conjugated with HO1 and their hypothetical orientation in the ER membrane. The HO1-GFP construct contains a 9 amino-acid flexible linker. TM: C-terminal transmembrane region of HO1. (B) Subcellular localization of constructs: GFP-HO1 and HO1-GFP colocalize with the ER marker Calnexin. Endogenous HO1, GFP-HO1 and HO1-GFP show a similar cellular distribution. Immunofluorescence of Calnexin was performed in J774.1 cells transfected with GFP-HO1 or HO1-GFP as indicated and endogenous HO1 was visualized in non-transfected J774.1 cells. (C) The GFP-HO1 construct is stable and membrane bound: J774.1 cells transfected with GFP-HO1 were permeabilized with 60 μM digitonin and fluorescence was followed for 300 s. GFP-HO1 was not leaking after digitonin treatment and is, therefore, membrane bound and its fluorescence was not significantly photo-bleached for the duration of the assay. (D) GFP conjugated to the HO1 N-terminus faces the cytosol and GFP conjugated to the HO1 C-terminus faces a protected compartment: J774.1 cells transfected with GFP-HO1 or HO1-GFP as indicated were subjected to the FPP assay. Permeabilization with 60 μM digitonin was followed by Proteinase-K treatment, and the cells were imaged before addition of digitonin (Pre) and at indicated times after addition of Proteinase-K. (E) CFP-HO1-GFP construct supports finding that HO1 faces the cytosol: J774.1 cells transfected with a CFP-HO1-GFP were subjected to the FPP assay. Permeabilization with 60 μM digitonin was followed by Proteinase-K treatment, and cells were imaged before and after addition of Proteinase-K.

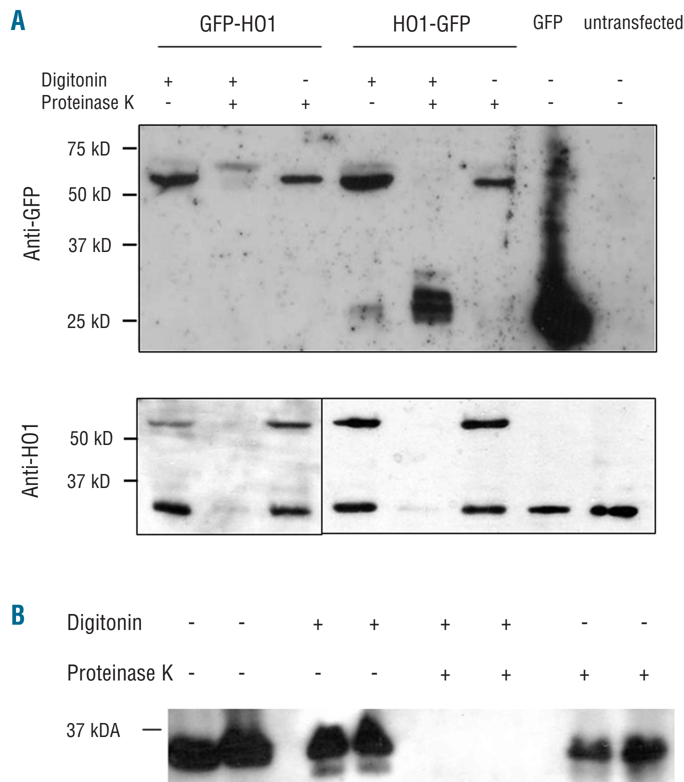

Figure 2. Western blot analysis of HO1 and GFP after a protease protection assay confirms the cytosolic orientation of endogenous HO1 and HO1 in both GFP constructs. (A) J774.1 cells transfected with GFP-HO1 or HO1-GFP as indicated, were subjected to 60 μM digitonin and 1.75 U/mL Proteinase-K. (Upper panel) Western blot of GFP demonstrates the complete digestion of the GFP-HO1 construct and the selective digestion of the unprotected HO1 in the HO1-GFP construct. (Lower panel) Western blot of HO1 on the same samples as in (A) demonstrates the complete digestion of HO1 of both constructs and endogenous HO1. (B) Non-transfected J774.1 cells were permeabilized with 60μM digitonin for 180 s followed by a Proteinase-K (1.75 U/mL) digest for 90 s. Following permeabilization and Proteinase-K treatment, Western blot of HO1 demonstrates complete digest of HO1.

into microsomal membranes *in vitro*, which may suggest that it is not depending on the GET complex or other cytosolic proteins for membrane insertion.

Spontaneous TA protein insertion may take place by binding of the protein to the water-bilayer interface and integration into the hydrocarbon region of the bilayer or by association of the TA protein with a non-polar region of a potential polytopic membrane protein that facilitates insertion.[19,20] Interestingly HO1 is hardly recruited to RBC membranes,[10] which may indicate the absence of such a membrane protein in red blood cells. It is likely that the GFP-HO1 construct is inserted with the same mechanism as the endogenous HO1 protein, because their C-terminal tails are identical. In contrast, the HO1-GFP and CFP-HO1-GFP construct depended on the addition of a flexible linker between the HO1 C-terminus and the GFP to be expressed and targeted to the ER membrane. The GFPs of these constructs are inserted into the ER-lumen and are protected in the protease-treated cells, although the mechanism of this insertion is not clear.

Heme is an essential molecule in most organisms. It is integrated into heme proteins as a functional moiety and is involved in many cellular processes. Very little is known about mammal heme chaperones involved in the transport of the highly hydrophobic heme from the site of its synthesis to the apoproteins that bind it, and from the degrading heme-proteins to the HOs. The source of heme for degradation by HO1 is likely exclusively from degrading heme-proteins.[21] Whether heme is transported to its degradation site or HO can traffic to another subcellular location in response to cellular stress is still not clear. So far, two examples of HO1 trafficking have been described. A soluble truncated HO1 can traffic to the nucleus[22] and HO1 is recruited to caveolae in pulmonary artery endothelial cells[23] and in macrophages after LPS stimulation where the released CO is involved in reducing inflammation.[24] HO1 trafficking after erythrophagocytosis is currently under investigation in our laboratory, and the discovery of the cytosolic orientation of the active site is an important piece in the puzzle of cellular HO1 trafficking.

Authorship and Disclosures

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