Heme Uptake by *Leishmania amazonensis* Is Mediated by the Transmembrane Protein LHR1

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Abstract

Trypanosomatid protozoan parasites lack a functional heme biosynthetic pathway, so must acquire heme from the environment to survive. However, the molecular pathway responsible for heme acquisition by these organisms is unknown. Here we show that *L. amazonensis* LHR1, a homolog of the *C. elegans* plasma membrane heme transporter HRG-4, functions in heme transport. Tagged LHR1 localized to the plasma membrane and to endocytic compartments, in both *L. amazonensis* and mammalian cells. Heme deprivation in *L. amazonensis* increased LHR1 transcript levels, promoted uptake of the fluorescent heme analog ZnMP, and increased the total intracellular heme content of promastigotes. Conversely, deletion of one LHR1 allele reduced ZnMP uptake and the intracellular heme pool by approximately 50%, indicating that LHR1 is a major heme importer in *L. amazonensis*. Viable parasites with correct replacement of both LHR1 alleles could not be obtained despite extensive attempts, suggesting that this gene is essential for the survival of promastigotes. Notably, LHR1 expression allowed *Saccharomyces cerevisiae* to import heme from the environment, and rescued growth of a strain deficient in heme biosynthesis. Syntenic genes with high sequence identity to LHR1 are present in the genomes of several species of *Leishmania* and also *Trypanosoma cruzi* and *Trypanosoma brucei*, indicating that therapeutic agents targeting this transporter could be effective against a broad group of trypanosomatid parasites that cause serious human disease.

Introduction

*Leishmania* spp. are protozoan parasites from the Trypanosomatidae family. In mammalian hosts *Leishmania* is an obligate intracellular parasite, replicating as amastigotes inside acidic phagolysosomes of macrophages. Disease caused by infection with *Leishmania* spp. has a severe impact on human populations throughout much of the tropics. The clinical manifestations range from self-healing cutaneous lesions to lethal visceralizing disease. In many regions of the world treatment of leishmaniasis still relies on toxic drugs such as pentavalent antimony, which requires high doses and a lengthy course of treatment [1,2]. Treatment failure is commonly observed with pentavalent antimonials, and alternative drugs are costly and not widely available in endemic areas. This situation, combined with the recent increase in *Leishmania* infections in urban areas [4,5,6], highlights the urgent need for identification of essential parasite molecular pathways that can be targeted by new drugs of lower cost and toxicity.

*Leishmania* species are uniquely dependent on the acquisition of heme from the environment. Heme is a metallolophorphyrin that serves as a prosthetic group for proteins that perform critical cellular functions such as oxidative metabolism, oxygen storage and transport, and signal transduction [7]. Unlike mammalian hosts which can synthesize heme [8], *Leishmania* and other trypanosomatid protozoa lack several enzymes in the heme biosynthetic pathway [9,10] and thus depend on an exogenous supply for survival. *Leishmania amazonensis* acquire exogenous heme as extracellular promastigotes and also as intracellular amastigotes replicating within macrophages [11]. The existence of a specific transporter or receptor for heme on the *Leishmania* plasma membrane has been speculated, based on reports showing high affinity heme binding to the cell surface of *L. amazonensis* promastigotes [12] and *L. infantum* axenic amastigotes [13], and specific uptake of the porphyrin heme analog MgPPIX in *L. donovani* [14]. However, the nature of the membrane-associated molecule(s) responsible for heme uptake by *Leishmania* has remained unknown. In this study, we identify *Leishmania* Heme Response-1 (*LHR1*), a *L. amazonensis* gene that shares homology with *HRG-4*, a *C. elegans* gene that encodes a plasma membrane heme importer [15]. We show that *LHR1* transcript levels increase during heme deprivation, and that the LHR1 protein localizes to the plasma membrane and endocytic compartments, promotes heme uptake, and regulates the intracellular pool of heme in the parasites. Our results identify LHR1 as a strong candidate for the elusive transmembrane transporter responsible for heme acquisition from the environment by *Leishmania.*
Author Summary

The biological activity of many proteins and enzymes requires heme, a large organic ring containing one iron atom at the center. It has been known for several decades that trypanosomatid protozoa lack several enzymes in the heme biosynthetic pathway. Therefore, unlike mammalian cells that can synthesize heme, these unicellular organisms must acquire heme from the environment. However, the mechanism by which this critical co-factor is transported into trypanosomatid parasites was unknown. In this study we identified LHR1, a trans-membrane protein from Leishmania amazonensis that mediates transport of extracellular heme into the parasites. Parasites partially deficient in LHR1 are impaired in heme import, and strains completely deficient do not survive. Genes highly similar to LHR1 are present in several species of trypanosomatid parasites that cause human disease, identifying this transporter as an important target for the development of anti-parasitic drugs.

Results

Identification of LHR1, a heme-responsive Leishmania gene

The presence of hemoproteins within Leishmania amazonensis in the absence of a functional heme biosynthetic pathway [11] suggested the existence of a membrane protein capable of importing heme from the medium. As a strategy to identify this molecule, we searched the TriTryp database (http://tritrypdb.org/tritrypdb/) for genes encoding transmembrane proteins with similarity to CeHRG-4, the prototypical heme transporter from another heme auxotroph, the nematode C. elegans [15] (WormBase Gene ID WBGene00009493). In addition to BLAST homology searches, we refined our approach by identifying predicted proteins similar to HRG-4 in size and in the number of putative transmembrane domains. This search strategy identified a single open reading frame HRG-4 in size and in the number of putative transmembrane domains. This search strategy identified a single open reading frame HRG-4 [15] (WormBase accession number CB275356). This gene, named Leishmania Heme Response-1 (LHR1) (Genbank accession number CB275356), shares 45% identity and 51% similarity with C. elegans HRG-4 [15] (Figure 2A). We amplified a 525-bp fragment from the L. amazonensis genome using nucleotide sequences from the TriTryp database, and amino acid sequence analysis confirmed the presence of the four predicted transmembrane domains also present in CeHRG-4. The predicted transmembrane topology suggests that the N- and C- termini of LHR1 are cytoplasmic, consistent with the proposed topology for CeHRG-4 [15], with extracellular exposure of the conserved histidine shown to be involved in heme uptake [16] (Figure 2B).

Given the relatively low sequence homology between Leishmania LHR1 and CeHRG-4, we first investigated whether LHR1 expression was influenced by heme availability. LHR1 transcript levels were elevated four fold within 15 h when L. amazonensis promastigotes were cultured in heme-deficient media, compared to heme replete conditions (Figure 2A). This result provided the first indication that LHR1 might be involved in heme homeostasis in Leishmania.

LHR1 promotes uptake of a heme analog and increases the heme content of Leishmania amazonensis promastigotes

Given the increase in LHR1 transcripts seen after cultivation in heme-deficient medium (Figure 2A), we investigated whether depriving L. amazonensis promastigotes of heme for 15 h led to a subsequent increase in the uptake of fluorescent zinc mesoporphyrin IX (ZnMP), a validated heme analog [17,18,19]. Low levels of ZnMP uptake were observed when the parasites were cultured in heme-containing medium, consistent with the low LHR1 transcript levels observed under these conditions. In contrast, the intracellular ZnMP fluorescence signal increased significantly after promastigotes were pre-incubated for 15 h in heme-deficient medium (Figure 2B). When maintained in regular heme-containing medium and then assayed for ZnMP uptake in the presence or absence of heme no intracellular signal was detected, showing that absence of heme during the 3–6 h period of the assay is not sufficient to promote ZnMP uptake (not shown). This result is consistent with our observations, which indicate that at least 12–15 h of heme deprivation is required to upregulate LHR1. The promastigotes remained fully viable after incubation in the absence of heme, as indicated by the viability indicator fluorescein diacetate (FDA) [20] (Figure 2B). Thus, culture conditions that upregulate LHR1 expression lead to a concomitant increase in heme uptake.

To directly examine the role of LHR1 in heme uptake by L. amazonensis, promastigote forms were transfected with an episomal expression plasmid carrying LHR1 tagged with 3xFLAG at the carboxyl terminus. Immunoblot analysis using monoclonal antibodies to the FLAG epitope detected two bands, one migrating at approximately 20 kDa corresponding to the predicted molecular mass of LHR1, and another band at >30 kDa (Figure 2C) that is likely to correspond to oligomers, as previously observed with C. elegans HRG-1 [15]. Uptake of the heme analog ZnMP by promastigotes transfected with LHR1-3xFLAG was measured by flow cytometry. Compared to untransfected parasites, LHR1-transfected promastigotes showed an enhanced fluorescence signal, reflecting an increased ZnMP uptake by the parasites. These values were further increased after pre-incubation of the parasites in heme-deficient medium for 15 h to upregulate LHR1 expression (Figure 2D). Importantly, LHR1 episomal expression also increased the total intracellular heme content in L. amazonensis promastigotes. The increased intracellular heme pool induced by LHR1 expression was observed in several independent experiments, performed with different numbers of promastigotes expressing LHR1-3xFLAG (Figure 3A,B), or GFP-LHR1 (Figure 4A, B).

LHR1 is targeted to the plasma membrane and lysosomal compartments in Leishmania and mammalian cells

To determine the sub-cellular localization of LHR1, L. amazonensis promastigotes transfected with GFP-LHR1 were cultured overnight in heme-deficient medium and then examined by confocal laser fluorescence microscopy. GFP-LHR1 was detected on the plasma membrane and in acidic intracellular compartments of promastigotes, as indicated by co-localization with lysotraker (Figure 4C). We also examined mouse macrophages infected for 24 h with axenic amastigotes expressing GFP-LHR1. The fluorescent chimeric protein was also localized at the plasma membrane of intracellular amastigotes, and in a large intracellular compartment that is likely to correspond to the megasome, the markedly enlarged lysosomal organelle typical of L. amazonensis amastigotes [21]. Parasites expressing GFP alone showed only diffuse cytosolic fluorescence, and no co-localization with lysotracker (Figure 4C, 5A). After ectopic expression in HeLa cells, GFP-LHR1 localized to the plasma membrane and lysosomes, which were identified by co-localization with fluorescent dextran chased for several hours into lysosomes (Figure 5B). Thus, in both L. amazonensis and mammalian cells, LHR1 is...
Figure 1. Identification and predicted topology of LHR1, a *Leishmania* transmembrane protein similar to the *C. elegans* heme importer HRG-4. (A) Amino acid alignment of *L. amazonensis* LHR1 and *C. elegans* HRG-4 (ClustalW PAM 250 Lasergene MegAlign software) shows that LHR1 also has four predicted transmembrane domains (TMD 1–4, boxed regions). Identical and conserved amino acids are highlighted in black.

![Amino acid alignment](image)

(B) Extracellular

![Extracellular view](image)

Intracellular

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LHR1 promotes heme uptake and functionally rescues a yeast strain defective in heme biosynthesis

To obtain direct evidence for the ability of LHR1 to transport heme across membranes and make it available for essential metabolic reactions in the cytosol, we performed heterologous expression in S. cerevisiae. This unicellular eukaryote utilizes exogenous heme very poorly, even when it lacks a heme biosynthetic pathway [22] [16]. The S. cerevisiae hem1Δ strain lacks δ-aminolevulinic acid synthase (ALAS), the first enzyme in the heme biosynthesis pathway. To grow, this strain requires supplementation of either δ-aminolevulinic acid (ALA), the product of ALAS, or excess hemin (≥10 μM) in the growth medium [23]. We found that hem1Δ requires 40-fold less hemin in the growth medium when transformed with either LHR1 or the C. elegans heme transporter CeHRG-4 (Figure 6A). We also used yeast assays to measure changes in regulatory intracellular pools of heme. LHR1 promotes heme uptake and functionally rescues a yeast strain defective in heme biosynthesis (Figure 2A), the level of ZnMP uptake over 2 h was reduced by ≈50% in LHR1/lhr1 when compared to wild type (Figure 2C).

The total intracellular heme content was also reduced by ≈50% in LHR1/lhr1 promastigotes, a phenotype that was partially restored by transfection of LHR1/lhr1 parasites with the LHR1-3xFLAG episomal plasmid (Figure 3D,E).

Repeated attempts were made to replace the second LHR1 allele with a NEO deletion construct without recovery of viable clones, suggesting that LHR1 is an essential gene in L. amazonensis. When attempts were made to delete the second LHR1 allele in LHR1/lhr1 parasites transfected with the LHR1-3xFLAG plasmid, the only viable promastigotes triple resistant to hygromycin, neomycin and nourseothricin (resistance conferred by the episomal LHR1-3xFLAG plasmid) that were recovered still had one endogenous LHR1 copy (data not shown). These results suggest that the levels of heme transport conferred by episomal expression of LHR1-3xFLAG are not sufficient to sustain promastigote growth, even when an abundant source of heme is provided in the culture medium. This observation is consistent with the incomplete rescue of the heme uptake and homeostasis phenotypes of LHR1/lhr1 L. amazonensis by LHR1-3xFLAG expression (Figure 8). Taken together, these results strongly suggest that LHR1 accounts for the majority of the heme transport activity of L. amazonensis.

Deletion of one LHR1 allele inhibits uptake of a heme analog and reduces the total heme content of Leishmania amazonensis promastigotes

To genetically examine the function of LHR1 in heme transport, we generated a LHR1 mutant in L. amazonensis using homologous recombination. The linearized HYG gene replacement construct was transfected into promastigotes, and genomic DNA from hygromycin B-resistant and wild type promastigote clones was isolated, digested with Xhol and analyzed by Southern blotting. Hybridization with the LHR1 probe detected a single DNA fragment of 6013 bp in genomic DNA from wild type and two independent hygromycin-resistant clones, as expected based on the presence of restriction sites for Xhol in the upstream and downstream genes, but not in LHR1 and HYG coding sequences (Figure 8A). In contrast, hybridization with the HYG probe detected a single band of 6,511 bp, consistent with the ≈6,500 bp band expected to be generated by substitution of LHR1 for HYG. These results demonstrated integration of HYG marker into the LHR1 locus, and replacement of a single LHR1 allele (Figure 8A).

The single knockout (LHR1/lhr1) mutant strain showed no obvious growth defect when cultivated in regular, hemin-containing medium, with or without transfection with LHR1-3xFLAG (Figure 8B). However, following incubation in heme-depleted medium, a condition that upregulates LHR1 expression (Figure 2A), the level of ZnMP uptake over 2 h was reduced by ≈50% in LHR1/lhr1 when compared to wild type (Figure 3C).

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Discussion

The existence of an essential pathway for acquisition of exogenous heme in Leishmania and other trypanosomatid protozoa was proposed decades ago [9], when it became clear that these organisms lack several enzymes of the heme biosynthetic pathway [10]. However, the molecule(s) responsible for this critical activity remained unknown. In this work we identify LHR1, a Leishmania gene upregulated under heme-deficient conditions that encodes a membrane protein able to promote heme uptake from the environment. Transfection of Leishmania with LHR1 promotes uptake of a heme analog and increases the total intracellular heme concentration in the parasites. A L. amazonensis single-allele LHR1 knockout strain shows reduced uptake of a heme analog and has a significantly smaller intracellular heme pool. Viable parasites lacking both chromosomal copies of LHR1 could not be isolated even when carrying episomal LHR1, suggesting that LHR1 performs a critical function that depends on levels of expression not achieved with the tagged LHR1-3xFLAG. Importantly, LHR1 functionally complemented a yeast strain deficient in heme biosynthesis, in both growth and heme-dependent gene expression assays. These results strongly support a role of LHR1 as a transporter and not a receptor for heme, because yeast cells lack an efficient heme import machinery [22] [16]. The efficiency of heme uptake from the environment may vary among Leishmania species, since Campos-Salinas et al. reported faster incorporation of Mg-PPi by L. donovani promastigotes [14] than what we observed with L. amazonensis. Future studies may provide evidence for the intriguing possibility that LHR1 is differentially expressed in visceral strains of Leishmania, a property that might be associated with their increased virulence and capacity to proliferate in internal organs.
Identification of a Leishmania Heme Transporter

A

Fold induction

+ Hemin

- Hemin

B

Phase

FDA

ZnMP

+ Hemin

T = 3 h

- Hemin

C

kDa

LHR1

LHR1-3XFLAG

T = 6 h

D

Cell Number

- ZnMP

+ ZnMP + Hemin

+ ZnMP - Hemin

% of Max

LHR1 over-expression

- ZnMP

+ ZnMP + Hemin

+ ZnMP - Hemin

% of Max

Fluorescence Intensity

Overlay

Overlay
LHR1 was identified based on its partial sequence identity and similarity to HRG-4, a gene encoding a plasma membrane heme transporter in the nematode *C. elegans*. HRG-4 was identified in a genetic screen designed to take advantage of the heme auxotrophy of *C. elegans* to identify heme-responsive genes [15]. LHR1 and CeHRG-4 [15] [16] have a similar molecular mass (~20 kDa), and four predicted transmembrane domains. One intriguing difference observed between CeHRG-4 and LHR1 is their subcellular localization. While HRG-4 is localized primarily on the plasma membrane, GFP-tagged LHR1 was detected on the plasma membrane and on endocytic compartments of *L. amazonensis*. In mammalian cells, GFP-LHR1 was also targeted to the plasma membrane and lysosomes, strongly suggesting that the large intracellular compartments containing LHR1 in *L. amazonensis* correspond to parasite lysosomal compartments. A morphometric and cytochemical study in *L. amazonensis* showed that during differentiation of promastigotes into amastigotes, the lysosomal vacuoles of promastigotes become a megasome, a very large compartment that can comprise up to ~5% of the total cell volume [21]. This stage-specific lysosomal pattern is very consistent with the intracellular localization of GFP-LHR1 in our study. In addition to the plasma membrane, GFP-LHR1 was observed in several intracellular vesicles in promastigotes and in one very large compartment in intracellular amastigotes.

The dual localization of LHR1 on the plasma membrane and on lysosomes raises interesting questions about the cellular site where heme is translocated into the cytosol. In yeast, LHR1 was targeted to the plasma membrane and promoted functional complementation of a strain incapable of synthesizing heme. However, earlier studies in *Leishmania donovani* showed that hemoglobin is internalized and degraded within parasite lysosomes [25], releasing heme that can then be transported into the cytosol to promote parasite growth. Interestingly, exogenously added hemin rescued the growth of a *L. donovani* strain defective in endocytic transport into lysosomes, indicating that heme translocation across the membrane can occur at both locations – the plasma membrane and the parasite lysosome [26]. The ATP-binding cassette protein LABC5G was also recently proposed to mediate the salvage of heme released after lysosomal degradation of internalized hemoglobin in *L. donovani*. This intracellular process for heme salvage from degraded hemoglobin was proposed to be distinct from the pathway directly promoting porphyrin transport into the parasites [14]. Additional studies should determine if LHR1 can also mediate the uptake of heme released from hemoglobin inside parasite lysosomes, or if it’s primary role is to transport heme from the environment directly across the plasma membrane.

LHR1 null strains could not be generated despite extensive attempts, suggesting that this transporter is essential for the survival of promastigote forms of *L. amazonensis*. Episomal expression of LHR1 increased the intracellular heme concentration of wild type and single knockout *L. amazonensis* promastigotes, but was not sufficient to allow recovery of viable parasites lacking both copies of the gene. This finding is likely related to the fact that episomal LHR1 expression failed to restore the intracellular heme concentration to the same levels observed in wild type parasites. Dysregulated expression and incomplete functional complementation is a frequent observation after episomal or integrated gene expression in *Leishmania* [27,28,29,30]. Incomplete restoration of heme acquisition in LHR1 double knockout parasites may result in the impairment of critical, essential roles played by hemoproteins in the parasites. For example, LFR1, the recently identified NADPH-dependent ferric iron reductase from *L. amazonensis*, contains a bis-heme motif that is essential for activity and required to allow iron acquisition through the ferrous iron transporter LIT1 [30]. Thus, deleting both copies of LHR1 may severely affect not only heme uptake, but also the ferrous iron acquisition process.

Searches of the TriTryp database indicate that highly syntenic, close homologs of *L. amazonensis* LHR1 (LmxM.24.2230) are present in the additional *Leishmania* species *L. major* (LmjF.24.2230), *L. braziliensis* (LhrM.24.2310) and *L. infantum* (LinJ.24.2320), and in the *Trypanosoma* species *T. brucei* (Tb427.08.6010, Tb927.8.6010), *T. brucei gambiens* (Tbg972.8.6030), *T. congolense* (TclL3000.8.5780), and *T. cruzi* (Tc00.104703511071.190). These trypanosomatid species are the causative agents of serious infectious diseases in humans (*L. infantum*, visceral leishmaniasis; *T. brucei gambiens*, sleeping sickness; *T. cruzi*, Chagas’ disease) or in livestock (*T. congolense* and *T. brucei brucei*, cattle Nagona). Given that the human genome does not include putative orthologs of *CeHRG-4* and LHR1 [15], our study suggests that LHR1 may represent a promising target for the development of new therapeutic drugs with a potentially broad impact in human health and quality of life.

Materials and Methods

Parasite culture

The *L. amazonensis* IFLA/BR/67/PH8 strain was provided by Dr. David Sacks (Laboratory of Parasitic Diseases, NIAID, NIH). Promastigotes were cultured at 26°C in promastigote growth medium: M199 (Gibco BRL) pH 7.4 supplemented with 10% heat-inactivated FBS, 5% penicillin-streptomycin, 0.1% hemin (25 mg/ml in 50% triethanolamine), 10 mM adenine (pH 7.5) and 5 mM L-Glutamine, as previously described [30]. Heme-depleted FBS was generated by treating heat inactivated FBS with 10 mM ascorbic acid for 4 h, followed by dialysis in PBS overnight and filter-sterilization. Heme depletion was verified by measuring the optical absorbance at 405 nm [31]. Parasite viability was assessed by fluorescent microscopy using fluorescein diacetate (FDA; Invitrogen) in combination with Propidium Iodide (PI; Sigma-Aldrich), as described in [20].

Identification of LHR1, plasmid construction and expression

A single open reading frame, LmjF.24.2230 (*L. major*), LmxM.24.2230 (*L. mexicana*), or LinJ.24.2320 (*L. infantum*) was
amazonsis. The PCR product was cloned into the pCR2.1-TOPO (Invitrogen) to generate a pCR-LHR1 plasmid, and the coding sequence was confirmed by sequencing. To construct a GFP-LHR1 gene fusion, pCR2.1-LHR1 digested with BamHI was cloned into pXGGFP2+ (courtesy of Dr. S. Beverley, Washington University), which drives expression in Leishmania of proteins fused to GFP at the N-terminus[32] to yield the pXG-GFP-LHR1 plasmid. After transfection, clones resistant to G418 (50 μg/ml) were isolated. To generate LHR1 tagged with 3xFLAG at the carboxyl-terminus, the primers 5’GGATCCCATATGCACAGTTCTCCTTCCTTTGAGC (forward) and 5’GGATCCATGAACGAGCGCAAGCG (reverse) (BamHI sites underlined, Ndel site in italics) were used to amplify the LHR1 ORF from pCR2.1-LHR1. The modified LHR1 ORF containing a Ndel site before the stop codon and flanked by BamHI restriction sites was cloned into pCR2.1 using TOPO PCR Cloning kit (Invitrogen) to generate plasmid pCR2.1-LHR1/Ndel-stop). A Ndel excised fragment of the 3xFLAG epitope tag (Sigma-Aldrich) was cloned into Ndel digested pCR2.1-LHR1/Ndel-stop) to generate pCR2.1-LHR1-3xFLAG. pCR2.1-LHR1-3xFLAG was digested with BamHI and cloned into pXG-SAT (courtesy of Dr. S. Beverley, Washington University) to yield the pXGSAT-LHR1-3xFLAG plasmid. After transfection and isolation of clones resistant to 50 μg/ml nourseothricin, LHR1-3xFLAG expression was detected by immunoblot[30]. Total protein extracts (50 μg) from wild type promastigotes expressing either LHR1 or LHR1-3xFLAG were separated on 15% SDS-PAGE, transferred to Immobilon membrane, blocked in 3% nonfat dry milk in TBS (50 mM Tris, 0.138 M NaCl, 2.7 mM KCl, pH 8.0) followed by detection with the mouse monoclonal antibody M2 that recognizes the FLAG epitope (Sigma-Aldrich).

The mammalian expression plasmid GFP-LHR1 was generated by amplifying GFP-LHR1 from pXG-GFP2+-LHR1 plasmid described above, with primers annealing to the start codon of GFP (forward) and to the stop codon of LHR1 (reverse). The resulting GFP-LHR1 fragment was cloned into pCR2.1-TOPO to create pCR2.1-kpol-GFP-LHR1-Xhol, double digested with kpol and Xhol, and the GFP-LHR1 fragment cloned into the kpol and Xhol sites of pShuttle-CMV[32] to yield pShuttle-CMV-GFP-LHR1.

LHR1 gene deletion

Gene deletion constructs containing hygromycin B phosphotransferase (HYG) or neomycin phosphotransferase (NEO) were based on the expression vectors pXG-HYG and pXG-NEO (courtesy of Dr. S. Beverley, Washington University, St. Louis, MO). A 1,000-bp flanking sequence upstream of L. amazonensis LHR1 was amplified using primers: 5’-GGATCGGCGACTGGTACAGG-3’ (UPLHR1 (forward)) and 5’-GGATCCGGCGGTTAAGGAAAATCGGAG-3’ (UPLHR1 (reverse)). A 2,500-bp flanking sequence downstream of LHR1 was amplified using primers: 5’-GGATCCGGCGGTTAAGGAAAATCGGAG-3’ (UPLHR1 (forward)) and 5’-CGCTTGAAGAATGGTACAGG-3’ (DOLHR1 (forward)) and 5’-CGCTTGAAGAATGGTACAGG-3’ (DOLHR1 (reverse)).

The upstream and downstream sequences were cloned into pCR2.1-TOPO vector (Invitrogen) creating the plasmid pCR2.1-UP-LHR1 and pCR2.1-DOWN-LHR1. The upstream region was excised from pCR2.1-UP-LHR1 plasmid with BamHI and cloned into pCR2.1-DOWN-LHR1 linearized with BamHI. The resulting plasmid pCR2.1-UP-LHR1-DOWN contained two SnaI sites at the junction of the upstream and downstream sequences. To generate the deletion constructs, the HYG and NEO ORFs were amplified using primers: 5’-GTATGGCATTTGGTTGATGAAAAAGGCTTGAAC-3’ (HYG) and 5’-GTATTTTTTTGGTTGATGAAAAAGGCTTGAAC-3’ (NEO).
Figure 4. GFP-LHR1 increases the heme content, and localizes to the plasma membrane and acidic endocytic compartments of promastigotes. (A) Absorption spectra of the hemochrome content of lysates of L. amazonensis promastigotes (4×10⁵) transfected with vector alone (GFP or 3xFLAG) or LHR1 (GFP-LHR1 or LHR1-3xFLAG), and grown in heme-containing medium. (B) Heme concentrations in (A) calculated based on the heme milimolar extinction coefficient of 20.7. Data are represented as the mean ± standard error of three independent experiments. * p = 0.020 (GFP-LHR1 vs. GFP), p = 0.031 (LHR1-3xFLAG vs. 3xFLAG) (two-tailed Student’s t test). (C) Spinning disk confocal microscopy images of live L. amazonensis promastigotes transfected with GFP vector alone or GFP-LHR1 and grown in heme-deficient medium. GFP-LHR1 is localized on the plasma membrane of promastigotes (arrowheads) and in intracellular compartments that colocalize with lysotracker (arrows). Bars = 3 μm. doi:10.1371/journal.ppat.1002795.g004
verse); LHR1: 5’GGATCCACCATGAACGAGCGCAAGCG (forward) and 5’ GGATCCCTATCGCGATGCACAGTTCT-CTTTTGAC (reverse), using the manufacturer’s protocol (Roche). The LHR1/Alhr1 single knockout strain was cultured in promastigote growth medium supplemented with 100 μg/ml hygromycin B. Transfection of LHR1/Dlhr1 with the LHR1-3xFLAG episomal expression plasmid was carried out as described above. The LHR1 gene deletion procedure was repeated using the NEO construct with or without prior transfection with LHR1-3xFLAG.

Fluorescence microscopy
Transfected L. amazonensis promastigote clones expressing GFP-LHR1 were selected in 50 μg/ml G418. To visualize LHR1, log phase promastigotes expressing GFP-LHR1 were imaged live on an UltraView Vox spinning disk confocal system (Perkin Elmer) equipped with an electron multiplier CCD camera (C9100-50; Hamamatsu). Images were acquired and processed using the Velocity software suite (PerkinElmer). For colocalization experiments, parasites were incubated with 1 μM Lysotracker red (InVitrogen) for 20 min at room temperature in serum-free M199 and washed twice before imaging. For LHR1 localization in the intracellular amastigote forms, bone marrow macrophages (prepared from C57BL/6 mice, as described in (25)) infected with L. amazonensis axenic amastigotes (differentiated from promastigotes transfected with the GFP vector, GFP-LHR1) were fixed with 2% PFA and treated with 0.1 mg/ml RNase A for 1 h. Samples were washed three times with PBS, stained for 1 min with 50 μg/ml PI, followed by three washes with PBS. Coverslips were mounted in ProLong (Molecular Probes, Invitrogen) and imaged on a confocal microscope (Leica TCS SP5 X) using the application suite software (Leica), followed by image processing with the Velocity software suite.

HeLa CCL-2 cells (HeLa 229) were seeded on 35-mm MatTek dishes (2.0 ml of 0.75×10⁵ cells/ml) 24 h before experiments in DMEM 10% FBS and 1% penicillin/streptomycin, and incubated at 37°C and 5% CO₂. The media was replaced with 250 μl OptiMEM I reduced serum and cells were transfected with Lipofectamine and 1 μg pShuttleCMV-GFP-LHR1, according to the manufacturer’s instructions (Invitrogen). To label lysosomes,
0.5 mg/ml Texas Red dextran (MW 10,000, Sigma-Aldrich) was added to cells followed by incubation at 37°C for 1 h, several PBS washes and a 2 h chase at 37°C, as previously described [34]. For live imaging, dishes were placed in an environmental chamber (LiveCell System; Pathology Devices, Inc.) at 37°C with 5% CO2 attached to an UltraView VoX spinning-disk confocal system (PerkinElmer) equipped with a CCD camera (C9100-50; Hamamatsu) and processed using Volocity software suite (PerkinElmer).

For imaging in S. cerevisiae, yeast transformants were cultivated to mid-log phase in 2% w/v raffinose SC (-Ura) medium supplemented with 0.4% w/v galactose and 250 μM ALA, and fixed with 4% formaldehyde for 1 h at room temperature. Immunofluorescence microscopy was performed as described elsewhere [35], and images were acquired using a DMIRE2 epifluorescence microscope (Leica) connected to a Retiga 1300 cooled Mono 12-bit camera.

Quantitative real time PCR

Log phase growth wild-type L. amazonensis promastigotes were washed twice with PBS, resuspended at 10⁶ parasites/ml in promastigote medium without hemin and 20% heme-depleted...
FBS, or regular promastigote medium with hemin and 20% untreated FBS. After 15 h at 26°C, a total of 10^8 promastigotes were collected. Three independent samples were used to isolate total RNA using Qiagen RNAeasy kit (Qiagen) according to the manufacturer’s instructions. cDNA synthesis was carried out using 1 μg of total RNA and Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. To quantify LHR1 transcript levels in each sample, 1 μl of the synthesized cDNA was amplified using LHR1 specific primers: sense 5’-CGCCTCGTACTTTTGTGGA-3’ and antisense 5’-CCTGAATCAATGAGGCCAAG-3’, and GAPDH specific primers: sense 5’-GAAGTACACGACCTTCTTC and antisense 5’-CGCTGATCACGACCTTCTTC as the reference gene. Quantitative real time PCR was performed using a BioRad iCyler iQ Real-Time PCR System (BioRad Laboratories) using the SYBR green fluorophore, according to the manufacturer’s instructions. All reactions were performed in triplicate, and no template DNA was included in each experiment as a negative control. The cycle threshold (Ct) value was determined, and the fold induction of LHR1 transcripts was calculated using the 2^(-ΔΔCt) method [36].

Measurement of total intracellular heme concentration

Heme (iron protoporphyrin IX) concentration was determined using the pyridine hemochrome method [37]. Log phase growth promastigotes cultivated in regular promastigote growth medium were collected by centrifugation, counted, washed once with PBS, resuspended in 1 ml of 1 mM Tris-HCl pH 8.0, and sonicated for 2 min in an ice water bath using a Branson digital sonifier at 30% power setting, in pulses of 5 seconds intercalated with 5 s of cooling. Aliquots of 840 μl were transferred to 13×100 mm glass tubes, 100 μl of 1 N NaOH was added followed by vortexing, and after 2 min 200 μl of pyridine solution (Sigma-Aldrich) was added to each sample followed by vortexing. Samples were then transferred to a 1 ml cuvette and a baseline absorbance spectrum was obtained. A few sodium dithionite crystals (2–3 mg) were added to the sample, and the reduced hemochrome absorbance spectrum between 500 and 600 nm was acquired after 1 min. Heme concentrations were calculated based on the millimolar extinction coefficient of 20.7, for the difference in absorption between the spectrum peak at 557 nm and the valley at 541 nm.

ZnMP uptake and quantitation

Zn(II) Mesoporphyrin IX (ZnMP) (Frontier Scientific) was dissolved in DMSO at 10 mM, and the uptake assay was performed as described [14], with modifications. Wild type and LHR1-3xFLAG-expressing L. amazonensis promastigotes (10^7 parasites per ml) were cultured in regular or heme-deicient medium (M199, 10% heme-depleted FBS, 1% Pen/Step) for 15 h. 2×10^9 parasites were collected by centrifugation, washed once with PBS and divided equally into regular promastigote growth

Figure 7. LHR1 promotes heme uptake in S. cerevisiae. Wild-type yeast transformed with yeast-optimized LHR1 (yLHR1) or the empty vector (vector) were grown in SC-Ura, 2% raffinose, 0.4% galactose medium and uptake of [55Fe] hemin was measured. Assays were performed with [55Fe] hemin at 1.2 μM and uptake was measured for the indicated number of minutes at 30°C. Assays were performed in triplicate and the experiment was replicated twice. The data represents the mean±/−SD of triplicate determinations.
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medium or heme-deficient medium containing 10 μM ZnMP for 3 or 6 h. The ZnMP uptake reaction was stopped by adding an equal volume of ice-cold 5% BSA in PBS, parasites was collected by centrifugation, washed, and the fluorescence intensity of ZnMP accumulation in a total of 3 × 10^6 parasites was measured by flow cytometry (BD FACSCanto, excitation at 488 nm and emission equal or greater than 670 nm) [15], or live-imaged in a Nikon E200 equipped with a DS-Fi1 camera and Digital Sight software. Flow cytometry data were analysed using FlowJo 6.3 software (Tree Star, Inc.).

**Yeast assays**

**Strains.** The DY1457 *S. cerevisiae* strains used were in the W303 background (W303 MAT_ura3-52 leu2-3,112 trp1-1 his3-11

![Figure 8. LHR1 is required for maintaining heme homeostasis in *L. amazonensis*. (A) Southern blot of genomic DNA from wild type (LHR1/LHR1) and heterozygous (LHR1/Δlhr1) promastigotes digested with XhoI and hybridized with LHR1 or HYG ORFs. Two independent hygromycin B resistant clones were used for genomic DNA isolation (LHR1/Δlhr1 C#1 and C#2). (B) Growth curves of LHR1/LHR1 and LHR1/Δlhr1 promastigotes expressing or not episomal LHR1-3xFLAG in promastigote growth medium containing hemin. (C) Flow cytometry quantification of ZnMP uptake by LHR1/LHR1 and LHR1/Δlhr1 promastigotes kept in heme-deficient medium during the 15 h pre-incubation and the 3 h assay. The numbers indicate the percentage of cells with fluorescence levels above the gate value (vertical line on horizontal bar, determined from measurements on parasites not incubated with ZnMP – grey shaded profile). (D) Absorption spectra of the hemochrome content of lysates of 4 × 10^6 LHR1/LHR1 (red), LHR1/Δlhr1 expressing LHR1-3xFLAG (blue) or LHR1/Δlhr1 (green) *L. amazonensis* promastigotes grown in heme-containing medium. (E) Heme concentrations in (D) calculated based on the heme millimolar extinction coefficient of 20.7. The results correspond to the mean±SD of triplicate determinations.

** p<0.0004; * p<0.022 (two-tailed Student’s t test).**

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Cells were maintained in YPD or appropriate synthetic complete (SC) media supplemented with 250 μM ALA (Frontier Scientific) [39].

Plasmid construction. pCeHRG-4 and LHRI (and HA-tagged versions) were cloned into the yeast vector pYes-DEST52 using BamHII and XbaI sites. The plasmid pYes-DEST52 was used as vector control.

Spot growth assay. The plasmids were transformed into the strain hem1A(6D) using the lithium acetate method [39]. Transformants were selected on 2% w/v glucose SC (-Ura) plates supplemented with 250 μM ALA. Five or six colonies of each transformation were picked and restreaked on 2% w/v raffinose SC (-Ura) plates supplemented with 250 μM ALA to deplete glucose for 48 h. Prior to spotting, cells were cultivated in 2% w/v raffinose SC (-Ura) medium for 18 h to deplete heme. Cells were then suspended in water to an OD600 of 0.2 (A600), 10 μl of ten-fold serial dilutions of each transformant were spotted onto 2% w/v raffinose SC (-Ura, -Trp) plates supplemented with either 0.4% w/v glucose and 250 μM ALA (positive control), or 0.4% w/v galactose and various concentrations of heme (no heme addition as negative control), and incubated at 30°C for 3 days before imaging.

β-galactosidase reporter assay. The plasmids for pCeHRG-4 or LHRI expression were co-transformed into the strain hem1A(6D) with pCYC1-LacZ. Transformant selection was performed as described above using SC auxotrophic medium supplemented with 250 μM ALA. Cells were depleted for heme in 2% w/v raffinose SC (-Ura, -Trp) medium for 12 h, and then were suspended in 10 ml 2% w/v raffinose SC (-Ura, -Trp) medium supplemented with 0.4% w/v galactose, and various concentration of heme to an OD600 of 0.1. Cells were cultivated at 30°C, 225 rpm for 12 h and subjected to the β-galactosidase assay as described elsewhere [35].

Fe-heme uptake. Heme uptake in yeast was measured as described [24] with the following modifications. S. cerevisiae strain W303 was transformed with pYesDEST52 (vector) or pyLHR1 (yLHR1). Transformants were grown in synthetic defined medium with 2% raffinose and 0.4% galactose and added to induction expression of yLHR1. Cells were harvested, washed, and resuspended in uptake buffer (phosphate-buffered saline, 5% glucose, 0.5% tween 20, 0.5% bovine serum albumin) at an A600 of 4.0. After pre-incubation at 30°C for 30 min, [55Fe] heme [40] was added at 1.2 μM and cells were incubated at 30°C for the indicated times. Cells were washed in assay buffer without glucose and retained [55Fe] heme was measured by scintillation counting.

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Author Contributions
Conceived and designed the experiments: CH XY DCM RLR OP CCP IH NWA. Performed the experiments: CH XY DCM RLR OP CCP IH NWA. Wrote the paper: CH IH NWA.

References
1. Alvar J, Aparicio P, Asensio A, Den Boer M, Canavate C, et al. (2008) The relationship between leishmaniasis and AIDS: the second 10 years. Clin Microbiol Rev 21: 334–359.
2. Markle WH, Makholm K (2004) Cutaneous leishmaniasis: recognition and treatment. Am Fam Physician 69: 1455–1460.
3. Sundar S, More DK, Singh MK, Sharma S, Sharma B et al. (2000) Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin Infect Dis 31: 1104–1107.
4. Jeronimo SM, Duggal P, Brau RF, Cheng C, Monteiro GR, et al. (2004) An emerging peri-urban pattern of infection with Leishmania chagasi, the protozoan causing visceral leishmaniasis in northeastern Brazil. Scand J Infect Dis 36: 484–490.
5. Darrouzet P (2001) The increase in risk factors for leishmaniasis worldwide. Trans R Soc Trop Med Hyg 95: 239–243.
6. Cortes S, Afonso MO, Alves-Pires C, Campino L (2007) Stray dogs and leishmaniasis in urban areas, Portugal. Emerg Infect Dis 13: 1431–1432.
7. Severance S, Hamza I (2009) Trafficking of heme and porphyrins in metazoans. Chem Rev 109: 4956–4961.
8. Schultz BJ, Chen C, Paw BH, Hamza I (2010) Iron and porphyrin trafficking in heme biogenesis. J Biol Chem 285: 26753–26759.
9. Chang KP, Chang CS, Sassa S, Tisseau D (1999) The role of the heme uptake system in the parasitophorous vacuole. J Biol Chem 274: 2758–2765.
10. Dutta S, Furuyama K, Sassa S, Chang KP (2000) Leishmania spp.: delta-aminolevulinate-inducible neogenesis of porphyria by genetic complementation vector control.
11. Rajagopal A, Rao AU, Amigo J, Tian M, Upadhyay SK, et al. (2008) Haem 1 is essential for parasite survival in the infected host. Embo J 17: 2619–2628.
29. Spath GF, Epstein L, Leader B, Singer SM, Avila HA, et al. (2000) Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite Leishmania major. Proc Natl Acad Sci U S A 97: 9258–9263.

30. Huynh C, Sacks DL, Andrews NW (2006) A Leishmania amazonensis ZIP family iron transporter is essential for parasite replication within macrophage phagolysosomes. J Exp Med 203: 2363–2375.

31. Sassa S, Nagai T (1996) The role of heme in gene expression. Int J Hematol 63: 167–178.

32. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, et al. (1998) A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 95: 2509–2514.

33. Medina-Acosta E, Cross GA (1993) Rapid isolation of DNA from trypanosomatid protozoa using a simple 'mini-prep' procedure. Mol Biochem Parasitol 59: 327–329.

34. Rodriguez A, Webster P, Ortego J, Andrews NW (1997) Lysosomes behave as Ca2+-regulated exocytic vesicles in fibroblasts and epithelial cells. J Cell Biol 137: 93–104.

35. Burke D, Dawson D, Stearns T, editors (2000) Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual. Cold Spring Harbor Laboratory Press. 205 pp.

36. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.

37. Paul KG, Theorell H, Akeson A (1953) The molar light absorption of pyridine ferroporphyrin (pyridine haemochromogen). Acta Chem Scand 7: 1204–1207.

38. Sherman F (1991) Getting started with yeast. Methods Enzymol 194: 3–21.

39. Ito H, Fukuda Y, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153: 163–168.

40. Galbraith RA, Sassa S, Kappas A (1983) Heme binding to murine erythroleukemia cells. Evidence for a heme receptor. J Biol Chem 260; 12190–12202.