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Iron acquisition within host cells and the pathogenicity of *Leishmania*

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Summary

Iron is an essential cofactor for several enzymes and metabolic pathways, in both microbes and in their eukaryotic hosts. To avoid toxicity, iron acquisition is tightly regulated. This represents a particular challenge for pathogens that reside within the endocytic pathway of mammalian cells, because endosomes and lysosomes are gradually depleted in iron by host transporters. An important player in this process is Nramp1 (Slc11a1), a proton efflux pump that translocates Fe^{2+} and Mn^{2+} ions from macrophage lysosomes/phagolysosomes into the cytosol. Mutations in Nramp1 cause susceptibility to infection with the bacteria *Salmonella* and *Mycobacteria* and the protozoan *Leishmania*, indicating that an available pool of intraphagosomal iron is critical for the intracellular survival and replication of these pathogens. *Salmonella* and *Mycobacteria* are known to express iron transporter systems that effectively compete with host transporters for iron. Until recently, however, very little was known about the molecular strategy used by *Leishmania* for survival in the iron-poor environment of macrophage phagolysosomes. It is now clear that intracellular residence induces *Leishmania amazonensis* to express LIT1, a ZIP family membrane Fe^{2+} transporter that is required for intracellular growth and virulence.

Introduction

Leishmania spp. are intracellular protozoan parasites that replicate within phagolysosomes of host macrophages. They are the causative agents of human leishmaniasis, which currently threatens around 350 million people throughout the world. Depending on the species involved, the human pathology resulting from *Leishmania* infections can vary from self-healing cutaneous lesions to very severe visceralizing disease. If left untreated, the visceral form of leishmaniasis can have a fatality rate as high as 100% within 2 years. One remarkable feature of *Leishmania* is its capacity to thrive within acidified, hydrolase-rich phagolysosomes, compartments that are usually involved in the destruction of invading pathogens (Antoine *et al.*, 1998). Significant advances were made in our understanding of the role played by the host immune system in susceptibility to *Leishmania* infections (Sacks and Noben-Trauth, 2002; McMahon-Pratt and Alexander, 2004; Stanley and Engwerda, 2007). In contrast, little is known about the molecular strategies used by the parasites to survive and replicate within the hostile phagolysosomal environment. In particular, until recently it was not clear how *Leishmania* is equipped to deal with iron acquisition, which represents a key battleground for the survival of intracellular pathogens. Here we discuss the implications of a recent study identifying a ferrous iron transporter essential for *Leishmania* growth in macrophages.

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Iron acquisition is a tightly regulated process

In humans and other vertebrates, iron assimilated by mucosal cells from dietary components passes into the blood stream, where it is chelated by transferrin. Transferrins are single chain glycoproteins with two binding sites for Fe^{3+}, the ferric form of iron found in aerobic environments. At neutral pH transferrin binds Fe^{3+} atoms with high affinity, effectively ensuring that no free iron remains in circulation (Wessling-Resnick, 2000). After binding to specific cell surface receptors, the transferrin–Fe^{3+} complex (holotransferrin) is endocytosed. Endosomal acidification releases Fe^{3+} from transferrin, allowing its conversion into Fe^{2+} by endosomal reductases (Ohgami *et al.*, 2005) (Fig. 1). Ferrous iron (Fe^{2+}) is soluble in biological fluids, but can be very toxic because of the highly reactive hydroxyl radicals it generates in the presence of oxygen, by the Fenton reaction. For this reason, a number of mechanisms are in place for sensing the cellular iron concentration, and for responding appropriately by carefully modulating the uptake, storage and efflux of this metal (Wessling-Resnick, 2000).

Another reason for tightly regulating iron availability is related to the fact that this metal can also control important microbicidal responses, in a manner that has been often described as a double-edged sword (Weiss, 2002). Iron directly participates in the generation of toxic oxygen and nitrogen intermediates, by the Fenton reaction. However, production of nitric oxide by LPS/IFNγ-activated macrophages can downregulate the transferrin receptor and upregulate the synthesis of ferritin, the protein complex that chelates Fe^{3+} for storage in the cytosol. Both events are likely to restrict the access of pathogens to iron inside the endocytic compartment (Kim and Ponka, 2000). On the other hand, iron can also dampen the expression of inducible nitric oxide synthase by IFNγ-activated macrophages, favouring pathogen survival (Weiss *et al.*, 1994; Oexle *et al.*, 2003).

A major pathway for avoiding toxicity from intracellular iron accumulation is the translocation of Fe^{2+} into the cytosol, where it is safely stored in the oxidized Fe^{3+} form bound to ferritin. This function is in large part performed by the divalent cation efflux pump Nramp2 (Slc11a2/DMT1) (Gruenheid *et al.*, 1999; Picard *et al.*, 2000). Nramp2 deletion leads to postnatal lethality owing to a severe deficiency in intestinal iron absorption (Gunshin *et al.*, 2005), highlighting the central role of this endosome transporter in providing iron for essential metabolic pathways in the cytosol. Macrophages express an additional metal transporter, Nramp1 (Slc11a1), in their late endosomes/lysosomes. Nramp1 was initially proposed to mediate iron influx into phagolysosomes (Goswami *et al.*, 2001), but is now thought to function as a pH dependent divalent cation efflux pump, similar to Nramp2 (Jabado *et al.*, 2000). Interestingly, mutations in Nramp1 do not cause iron absorption phenotypes but severely impair the ability of phagocytes to inhibit the growth of intracellular pathogens such as *Salmonella*, *Mycobacteria* and *Leishmania* (Blackwell *et al.*, 2001; Forbes and Gros, 2001). The increased susceptibility to infection of $Nramp^{-/-}$ animals reinforces the view that transporters within the mammalian endocytic pathway can function in host defence, by restricting the access of pathogens to iron (Weinberg, 1992; Jabado *et al.*, 2000; Schaible and Kaufmann, 2004). It is now clear that an efficient iron uptake mechanism capable of competing with mammalian transporters for the same substrates is critical for the survival and replication of several intravacuolar pathogens.

How pathogens compete with the host for iron

Many bacteria internalize iron sequestered by siderophores, secreted molecules with high affinity for iron that effectively compete with transferrin and other host proteins for Fe^{3+} (Crosa and Walsh, 2002). Macrophages counter attack by secreting lipocalin 2, an antimicrobial peptide that captures iron-loaded bacterial siderophores, delivering it to receptors on mammalian cells for internalization (Flo *et al.*, 2004). In *Salmonella enterica* serovar

Typhimurium, a Gram-negative facultative intracellular bacterium, secreted siderophores that bind Fe^{3+} with high affinity are internalized after binding to outer membrane receptors (Hantke *et al*., 2003; Rabsch *et al*., 2003). However, for full virulence *Salmonella* depends not only on siderophore-mediated Fe^{3+} internalization systems, but also on Fe^{2+} transmembrane transporters. Genes encoding several ATP-dependent or proton-coupled Fe^{2+} transporters have been identified in *Salmonella*, such as *fepBCDG*, *sitA-D*, *feoABC*, *corAD* and the *Nramp* homologue *mntH* (Marquis and Gros, 2007). Mutations in *mntH* and *feoB* or *sitA-D* inhibit *Salmonella* replication within macrophages, and virulence in *Nramp1*$^{-/-}$ 129 mice (Janakiraman and Slauch, 2000; Boyer *et al.*, 2002). Thus, there is direct evidence that *Salmonella* has the molecular machinery required for competing with host cell transporters for Fe^{2+}. A similar strategy appears to be used by the intracellular bacterium *Mycobacteria*, which depends on siderophores for growth in macrophages, and responds to changes in iron availability by mounting a complex transcriptional response that regulates iron uptake and adjusts the transcription of iron storage genes (De Voss *et al.*, 2000; Rodriguez, 2006). Interestingly, *M. tuberculosis* also expresses a Nramp transporter homologue, named Mramp (Agranoff *et al.*, 1999). Mutants in this gene have no growth phenotype in macrophages or mice (Boechat *et al.*, 2002) and appear to accumulate similar amounts of iron in the phagosome (Agranoff *et al.*, 1999). This observation suggests that the function of Mramp is redundant with that of other ferrous iron transporters, as observed with the *Salmonella* Nramp homologue *dmntH*.

There is so far no evidence that protozoa can acquire Fe^{3+} through siderophores and siderophore receptors. A study using *Leishmania chagasi* showed that soluble molecules secreted by the parasites are not capable of removing iron from lactoferrin or transferrin (Wilson *et al.*, 1994). In trypanosomatid protozoa, receptor-mediated uptake of Fe^{3+} chelated to transferrin has been clearly demonstrated only in the African trypanosome, *Trypanosoma brucei* (Steverding *et al.*, 1995). This observation is consistent with the fact that African trypanosomes are exclusively extracellular in their mammalian host, and thus directly exposed to holotransferrin, the most abundant iron source in serum. Despite some suggestive evidence (Lima and Villalta, 1990; Voyiatzaki and Soteriadou, 1992; Borges *et al.*, 1998; Britigan *et al.*, 1998), it is still not clear whether a receptor-mediated transferrin uptake pathway is present in the two trypanosomatid parasites with an intracellular life style, *Trypanosoma cruzi* and *Leishmania* (Wilson and Britigan, 1998). After entering cells in lysosome-like vacuoles, *T. cruzi* escapes into the cytosol and replicates in direct contact with ferritin, the cytosolic Fe^{3+} carrier protein. In contrast, *Leishmania* spends its intracellular life cycle inside the endosomal pathway, where one of the major sources of iron is Fe^{3+} complexed to transferrin, or Fe^{2+} generated from Fe^{3+} by endosomal reductases (Fig. 1). As discussed below, such differences in environment may be reflected in specific mechanisms for iron acquisition in these parasites.

Iron uptake by *Leishmania* – how do they do it?

During phagocytosis macrophages generate an oxidative burst, which produces highly toxic reactive oxygen intermediates including superoxide anion (O_2^-) (Fang, 2004). The metalloenzyme superoxide dismutase (SOD) plays an important role in detoxifying O_2^-, by converting it into H_2O_2 and H_2O (Fridovich, 1978). Two genes (SODA and SODB), which show complete conservation of a signature sequence for SODs utilizing iron as an essential cofactor, were initially identified in *L. chagasi*. These genes complemented *Escherichia coli* SOD-deficient mutants, and their overexpression in *L. tropica* protected the parasites against the free radical generating agents paraquat and nitroprusside (Paramchuk *et al.*, 1997). Subsequent studies identified additional *Leishmania* SOD isoforms, and showed that *LcFeSODB1* is expressed at high levels in *L. chagasi* stationary phase promastigote and amastigote stages, while *LcFeSODB2* is more highly expressed in logarithmic growth promastigotes. Both *LcFeSODB1* and *LcFeSODB2* are targeted to glycosomes by the last three

amino acids of their carboxyl termini, suggesting that these proteins may act to protect glycosomal enzymes from O_2^- toxicity (Plewes *et al*., 2003). *Leishmania* SODA, on the other hand, was reported to localize to the parasite's mitochondria (Getachew and Gedamu, 2007). Null mutants of LcFeSODB1 could not be obtained, but a single-allele knockout showed decreased growth in the presence of paraquat, and significantly reduced survival within macrophages (Plewes *et al.*, 2003). Similar results were obtained with *L. tropica* (Ghosh *et al.*, 2003). It is now clear that SOD genes are present in the genome of several *Leishmania* species, including *L. donovani*, *L. tropica*, *L. aethiopica* (Genetu *et al.*, 2006) and *L. major* (Ivens *et al.*, 2005). In several of these studies, resistance to oxidative stress correlated with the ability of *Leishmania* to proliferate within macrophages, consistent with the fact that generation of reactive oxygen species is a major microbicidal mechanism in these cells.

The uptake of iron required for the critical antioxidant function of SOD and for other essential metabolic reactions poses a particular problem for *Leishmania*. Although exogenously added holotransferrin is able to reach parasite intracellular compartments and promote the growth of *Leishmania* within macrophages (Borges *et al.*, 1998), it is not clear whether there is sufficient Fe^{3+} complexed to transferrin available to the parasites under physiological conditions. Furthermore, as discussed above, the endosomal pathway of macrophages is actively depleted in Fe^{2+} by the divalent cation efflux pumps Nramp2 and Nramp1, so this metal is likely to be in very short supply inside *Leishmania* parasitophorous vacuoles. An important development in our understanding of how *Leishmania* acquires iron under these challenging conditions came from the observation that iron uptake in *L. chagasi* occurs preferentially in the reduced ferrous (Fe^{2+}) form. This finding was followed by the demonstration that *L. chagasi* expresses a NADPH-dependent iron reductase, capable of converting oxidized, ferric Fe^{3+} into the more soluble Fe^{2+} (Wilson *et al.*, 2002). There is extensive evidence that Fe^{3+} reduction is usually coupled to Fe^{2+} membrane transport in bacteria, yeast, plants and animal cells, so the discovery of a ferric reductase in *Leishmania* immediately suggested the potential existence of a ferrous iron transporter. As discussed below, our recent studies have identified such plasma membrane *Leishmania* transporter, LIT1, and shown that it is essential for intracellular replication and for virulence in animal models.

Identification and characterization of the *Leishmania amazonensis* ferrous iron transporter, LIT1

Leishmania major, *L. infantum* and *L. braziliensis* appear to encode in their genomes a single integral membrane ferric reductase, namely LmjF30.1610, LinJ30_V3.1630 and LbrM30_V2.1670 respectively (Ivens *et al.*, 2005; Peacock *et al.*, 2007). The predicted protein shows significant homology to the Fe^{3+} chelating reductase from *Arabidopsis thaliana* encoded by *FRO2* (Robinson *et al.*, 1999). The capability to reduce Fe^{3+} to Fe^{2+} is one of the main features of high affinity iron acquisition systems, so the presence of a putative ferric reductase across *Leishmania* species pointed to the probable existence of ferrous iron transporters. As discussed below, database searches followed by detailed analysis performed in our laboratory have identified LIT1, the first ferrous iron transporter to be detected in trypanosomatid protozoa.

The first gene encoding a Fe^{2+} transporter to be cloned from plants or animals was the *Arabidopsis thaliana IRT1*, which encodes an integral membrane protein with eight predicted transmembrane domains and potential metal binding sites rich in histidine residues (Eide *et al.*, 1996). Null mutants of *IRT1* in *Arabidopsis* have a severe growth defect in normal soil, which is rescued by exogenous iron addition (Vert *et al.*, 2002). Iron is the preferred metal substrate for IRT1, although cadmium, cobalt, manganese and zinc can also be transported (Eide *et al.*, 1996; Rogers *et al.*, 2000). This capacity for mediating the uptake of several transition metals is typical of ferrous iron transporters (Forbes and Gros, 2003), which are not

as highly specific for iron as the ferric iron transport systems (Kaplan, 2002). IRT1 belongs to the ZIP family of iron transporters, present not only in plants but also in yeast, *Drosophila*, *C. elegans* and humans (Guerinot, 2000). Most members of the ZIP transporter family are predicted to have eight transmembrane domains and a similar membrane topology, in which the amino- and carboxy-terminal ends are located on the extracellular side of the plasma membrane (Fig. 2). The length of different family members ranges 309–476 amino acids, the difference being due to a cytoplasmic variable region between transmembrane domains III and IV. The most conserved portion of ZIP family proteins is in transmembrane domain IV, predicted to form an amphipathic helix with a fully conserved histidine residue and an adjacent semi-polar residue, and thought to be essential components of the heavy metal binding site (Eng *et al.*, 1998; Guerinot, 2000).

Database searches revealed that *Leishmania* also expresses metal transporters of the ZIP family. The *L. major* genome sequence contains two identical genes in tandem, LmjF31.3060 and Lmj31.3070 (*LIT1-1* and *LIT1-2*) (Huynh *et al.*, 2006), which share 30% identity and 53.8% similarity with *Arabidopsis IRT1*. Two additional genes containing predicted ZIP metal permease domains were also detected: LmjF28. 1930 on chromosome 28 and LmjF33.3200 on chromosome 33. Both share reasonable similarity with putative zinc transporters from *Orysa sativa* and *Dictyostelium discoideum* respectively. At least one of these additional putative ZIP family metal permeases can also be found in the *T. cruzi* and *T. brucei* genomes. However, interestingly, only *LIT1-1 and LIT1-2* within the *Leishmania* genome have extensive similarity with the demonstrated *Arabidopsis* iron transporter, *IRT1*. This may reflect the specific need of *Leishmania* to compete with iron transporters present in the endocytic pathway of host cells. The *Leishmania* LIT1 protein is predicted to have a membrane topology similar to *Arabidopsis* IRT1, including the long intracellular variable loop region located between transmembrane domains III and IV (Huynh *et al.*, 2006). This region shows little conservation among ZIP family members, but *Leishmania* LIT1 also contains a conserved H×H×H motif that has been proposed as a potential metal binding domain (Gitan and Eide, 2000; Gitan *et al.*, 2003) (Fig. 2).

Complementation studies using an iron transport-deficient yeast strain, together with assays for direct uptake of ^{55}Fe by *Leishmania*, directly demonstrated that LIT1 is a divalent metal transporter with preference for iron. LIT1 is localized on the *Leishmania amazonensish* plasma membrane, but it is detected only on intracellular amastigotes residing for several hours within parasitophorous vacuoles. This apparently exclusively intracellular expression suggests that LIT1 may be upregulated under conditions of low iron availability. Supporting this hypothesis, LIT1 expression is accelerated in amastigotes replicating within Nramp1$^{+/+}$ macrophages (Huynh *et al.*, 2006), which are predicted to have lower levels of intraphagosomal iron when compared to Nramp1$^{-/-}$ macrophages. Thus, similar to what was shown for *Arabidopsis* IRT1 (Connolly *et al.*, 2002; Vert *et al.*, 2002) and other eukaryotic divalent cation transporters, expression of LIT1 transporter may be upregulated by iron deprivation. Regulation of gene expression in *Leishmania* occurs largely at the post-transcriptional level, consistent with the constitutive transcription of gene clusters into large polycystronic units that is typical of trypanosomatid genomes (Clayton, 2002; Ivens *et al.*, 2005). Studies of the mechanisms regulating amastigote-specific gene expression have implicated changes in mRNA stability (Charest *et al.*, 1996; Wu *et al.*, 2000) or translation efficiency (Zilka *et al.*, 2001; Boucher *et al.*, 2002). Future studies are needed to clarify the mechanism by which LIT1 expression is restricted to *Leishmania* intracellular amastigotes, and whether this process is triggered by the iron-poor environment of the parasitophorous vacuole. LIT1 is dispensable for *L. amazonensis* growth as promastigotes or amastigotes in axenic culture, suggesting the existence of alternative mechanisms for iron acquisition in the extracellular forms with access to a larger iron supply. This role may be fulfilled by the products of LmjF28.1330 and/or LmjF33.3200, the additional *L. major* genes with predicted ZIP metal permease domains.

Alternatively, haeme may be acquired through the haemoglobin endocytic receptor identified in *L. donovani* promastigotes (Sengupta *et al.*, 1999).

The *in tandem* localization of the two identical *LIT1* genes within a 5425 bp region of chromosome 31 allowed generation of a *L. amazonensis* null mutant lacking both *LIT1* copies. The LIT1$^{-/-}$ strain, although capable of normal growth and differentiation in axenic culture, lost the ability to replicate within macrophage parasitophorous vacuoles, and to induce cutaneous lesions in mice. Complementation of *LIT1*$^{-/-}$ with an episomal or integrated copy of *LIT1* restored virulence and the capacity for intracellular growth (Huynh *et al.*, 2006). Intriguingly, significant numbers of *LIT1*$^{-/-}$*L. amazonensis* persisted for several months in mouse tissues, without causing pathology (Huynh *et al.*, 2006). LIT1 null *Leishmania* strains may be useful for future studies of the mechanisms of *in vivo* persistence, which may involve infection of alternative cell types producing lower levels of reactive oxygen species.

Conclusion and perspectives

Major gaps still exist in our understanding of the biology of different *Leishmania* species, and how their properties correlate with the very different clinical forms of the disease. Before the overall process of *Leishmania* infection can be understood, and before the resulting pathology can be rationally treated, it will be necessary to understand how these parasites survive and replicate within acidified, hydrolase-rich phagolysosomes of macrophages. Our knowledge of the strategies used by *Leishmania* to survive within this harsh environment is still very limited. A significant insight into how *Leishmania* deals with the challenge of intracellular iron acquisition came from the identification of LIT1, a ferrous iron transporter that plays a critical role in intracellular growth and virulence of *L. amazonensis*. This transporter is expressed exactly when, and where it is needed to effectively compete with host iron transporters for this essential element. Identification of LIT1 has finally allowed *Leishmania* to be listed, along with *Salmonella* and *Mycobacteria*, as a pathogen known to possess molecular mechanisms for competing for iron inside the host endocytic pathway. Additional studies should now rapidly expand our knowledge of additional components in this critical pathway for *Leishmania* intracellular survival.

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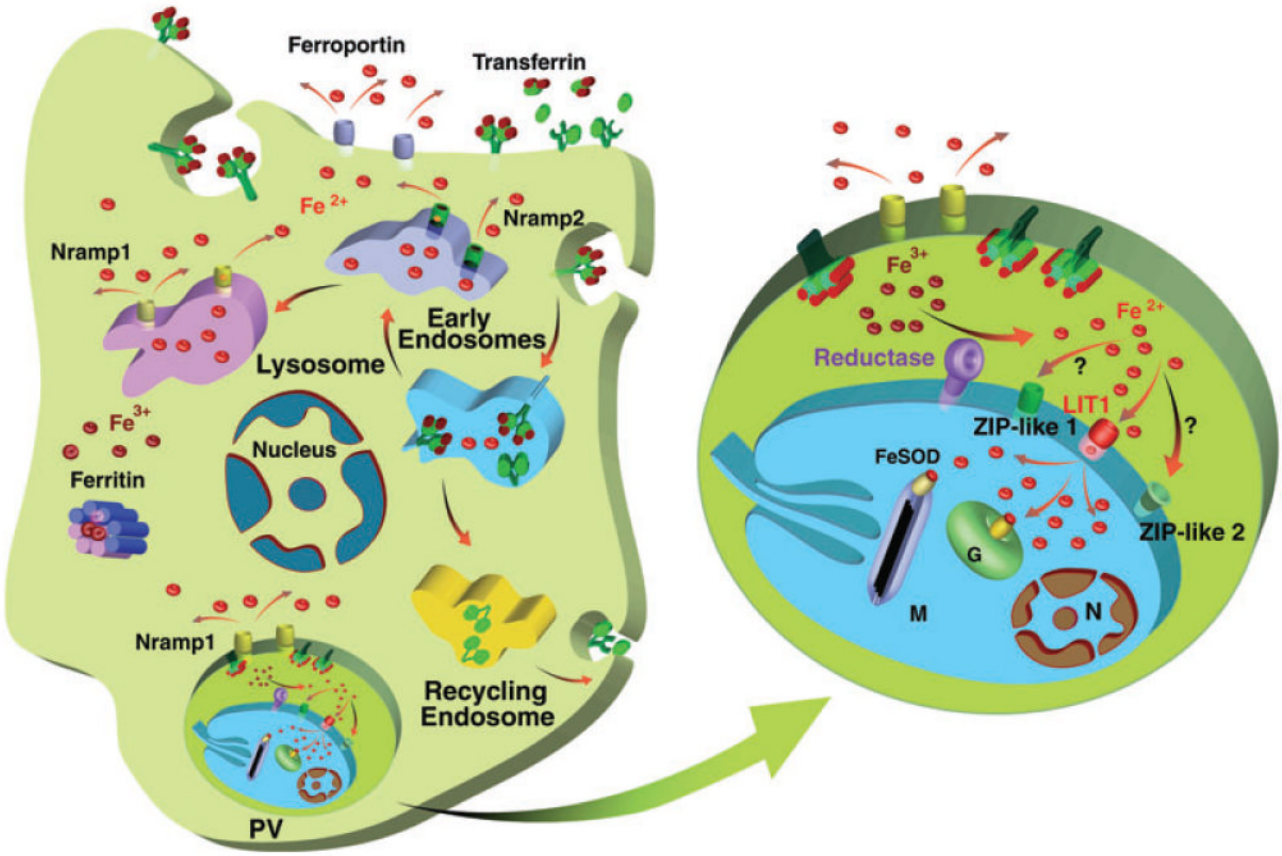

Fig. 1.

Diagram of iron import and export pathways in a *Leishmania*-infected macrophage. The enlarged parasitophorous vacuole (PV) shows an intracellular amastigote with the recently identified LIT1 transporter, the genome-encoded putative ZIP family transporters (ZIP-like 1 and 2), the putative reductase and the host divalent cation efflux pump Nramp1. G, glycosome; M, mitochondria, N, nucleus.

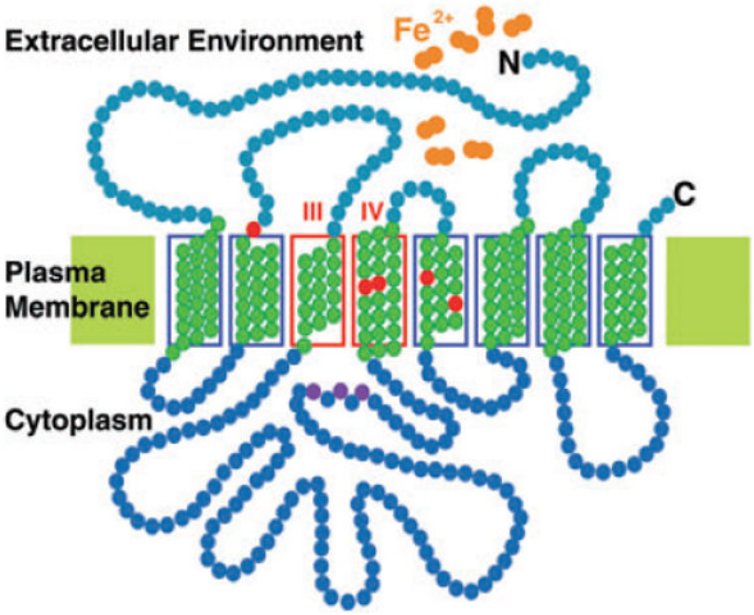

Fig. 2.

Topology of the LIT1 ferrous iron transporter on the plasma membrane of *Leishmania*. The amino acids shown in green form the transmembrane domains, the ones shaded in red correspond to conserved residues required for iron transport in *Arabidopsis* IRT1, and the ones in pink correspond to the conserved H×H×H motif present in the variable intracellular loop region. Transmembrane domains III and IV are indicated.