Growth Phase Regulation of the Main Folate Transporter of *Leishmania infantum* and Its Role in Methotrexate Resistance*

Received for publication, August 12, 2004, and in revised form, September 13, 2004
Published, JBC Papers in Press, October 4, 2004, DOI 10.1074/jbc.M409264200

Dave Richard‡, Philippe Leprohon‡, Jolyne Drummelsmith§, and Marc Ouellette¶

*From the Centre de Recherche en Infectiologie du Centre de Recherche du Centre Hospitalier and Division de Microbiologie, Faculté de Médecine, Université Laval, Québec G1V 4G2, Canada*

The protozoan parasite *Leishmania* relies on the uptake of folate and pterin from the environment to meet its nutritional requirements. We show here that a novel gene (folate transporter 1 (FT1)) deleted in a *Leishmania infantum* methotrexate-resistant mutant corresponds to the main folate transporter (K$_m$, 410 nM). FT1 was established as the main folate transporter by both gene transfection and by targeted gene deletion. Modulation of the expression of FT1 by these manipulations altered the susceptibility of *Leishmania* cells to methotrexate. Folate transport was stage-regulated with higher activity in the logarithmic phase and less in the stationary phase. FT1 fused to green fluorescent protein led to the observation that FT1 was located in the plasma membrane in the logarithmic phase but was re-targeted to an intracellular organelle followed by a degradation of the protein in stationary phase. *Leishmania* has several folate transporters with different characteristics, and the growth stage-related activity of at least one transporter is regulated post-translationally.

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania* and can result in a variety of clinical symptoms with visceral infections that are usually fatal if left untreated (1). Even today, pentavalent antimonial agents are the mainstay for the control of this disease. However, resistance to this class of drugs is now prevalent in several endemic areas (2) demonstrating the urgent need for novel effective treatment alternatives. As a result of the effort to discover new drugs, miltefosine, an inhibitor of alkyl phospholipid metabolism, has recently been approved for the treatment of visceral leishmaniasis in India (3). Unfortunately the ease with which miltefosine-resistant *Leishmania* mutants can be generated in *vitro* suggests a strong potential for the appearance of such mutants in the field (4); thus there is still a need for the development of effective new therapies against this parasite.

The folate biosynthesis pathway has been exploited extensively for the development of a variety of drugs. Folates are made of a pterin moiety conjugated to para-aminobenzoic acid and glutamic acid. Reduced folates are key cofactors in the biosynthesis of thymidylate, and inhibitors of this pathway act primarily at the level of dihydrofolate reductase, the enzyme responsible for supplying the cell with reduced folates (for reviews, see Refs. 5 and 6). Antifolates are widely used in chemotherapy but have not been successful yet against infections caused by *Leishmania*. Nevertheless many distinct features in the folate metabolism of this organism have been identified, some of which could prove to be useful therapeutic targets (7, 8). A number of lead antifolate molecules were indeed shown to be active against *Leishmania* (for a review, see Ref. 9).

Our understanding of pterin and folate metabolism and transport in *Leishmania* is due in large part to studies of resistance mechanisms to the model antifolate drug methotrexate (MTX) (for reviews, see Refs. 7 and 8). Resistance to MTX in *Leishmania* can be conferred by several genes but is also usually correlated with a reduced accumulation of the drug (10–12). This reduced uptake of MTX is paralleled by a marked decrease in folate uptake, suggesting that the expression of a common folate/MTX transporter is strongly down-regulated in some MTX-resistant *Leishmania*. Since *Leishmania* is a folate auxotroph, mutants with markedly reduced folate uptake must compensate for this decrease so the parasite can meet its folate requirements. In *Leishmania tarentolae*, the mechanism of compensation involves the overexpression of the biopterin transporter 1 (BT1), which transports folates but not MTX to meet the folate requirements of the cell (13). BT1 is a biopterin transporter (13, 14) that is part of a family of transmembrane proteins. Fourteen putative proteins belonging to this family are part of the *Leishmania* genome (www.genedb.org). This family has members in a variety of organisms, including the related parasite *Trypanosoma brucei* as well as the plant *Arabidopsis thaliana* and the cyanobacteria *Synechocystis* and *Synechococcus*. A second member of this family in *Leishmania*, the folate transporter 5 (FT5), was recently investigated and shown to be a very high affinity and low capacity folate/MTX transporter (15). However, FT5 does not correspond to the main *Leishmania* folate transporter.

Folate transport in *Leishmania* is known to be regulated with the growth stage of the parasite with high activity in the logarithmic stage of the parasite and residual activity in the stationary phase (16, 17). Here we present our functional studies of the folate transporter 1 (FT1), a transporter belonging to...
the BT1 family. Molecular and biochemical analyses indicated that it corresponds to the primary high affinity folate/MTX transporter of *Leishmania* cells. Modulation of the expression of FT1 changed antifolate susceptibility. The FT1 protein was located in the plasma membrane in the logarithmic growth phase of the parasite, but the protein was retargeted intracellularly when the parasite reached the stationary growth phase.

**EXPERIMENTAL PROCEDURES**

**Leishmania Growth**—The *Leishmania infantum* and the *L. tarentolae* TarII wild-type cells and the mutant MTX1000.6 cell lines have been described previously (10, 18). *L. infantum* and *L. tarentolae* cells were grown in SDM-79 medium supplemented with 10 and 5% heat-inactivated fetal bovine serum and 5 mg/ml hemin, respectively. *Leishmania* promastigotes were transfected by electroporation as reported previously (19). In this work, we also transfected the FT1 gene in TarII MTX1000.6 and generated an *L. infantum* line with one FT1 allele disrupted (LiFT1DKO). In the latter cell line we also transfected an intact episomal copy of the FT1 gene.

**DNA Manipulations**—Total DNA was isolated using DNAzol reagent (Invi- trogen). Southern hybridization was performed following standard procedures (18). A cosmid derived from a *L. tarentolae* genomic library using a 60-bp PCR probe (see Ref. 15) and the *L. infantum* FT1 probe were obtained by PCR. The primer sequences used were 5′-ATG-GCC-GCC-CCC-CGG-3′ and 5′-CTA-TGG-TGG-ATG-GCC-3′ for the *L. tarentolae* and *L. infantum* cosmid DNA library using 3000 V Biochemicals. Transport studies were carried out using various concentrations of radioactive aminopterin (10 nM to 1 μM) to determine the apparent affinity constant (*Kₐ*). The quantity of accumulated radioactivity was normalized with *Leishmania* cell numbers. To measure folate and MTX transport, the uptake of cells incubated on ice was subtracted.

**Confluent Microscopy**—Cells were immobilized with 2% paraformaldehyde and mounted on microscope slides with coverslips. Samples were viewed with an Olympus FV300 confocal scanning laser system installed on an Olympus IX-70 inverted microscope with an argon laser. Visualization of the fluorophore was achieved using a 488 nm excitation filter and 510/530 nm emission filter. Samples were scanned for green fluorescence (AF488) using an argon laser with a 10× objective (numerical aperture, 1.40) and 20× objective (numerical aperture, 1.40) zoom. Images of 1024 × 1024 pixels were obtained using Olympus Fluoview 300 software and processed using Adobe Photoshop software.

**Western Blots**—Protein electrophoresis, protein transfer, and reactions with polyclonal anti-GFP antibodies (Molecular Probes) were performed following standard procedures (25).

**RESULTS**

**Gene Deletion of Members of the BT1 Family in Methotrexate-resistant *L. infantum*—*Leishmania* cells selected for MTX resistance often exhibit a defect in the transport of MTX and folate (10–12). The proteins responsible for the transport of pterins and folates in *Leishmania* are starting to be known and correspond to a novel family of membrane proteins known as the BT1 family (pfam.wustl.edu). BT1 is a high affinity (*Kₐ*, 4.9 μM) biotin transporter (13, 14). The completed *L. major* genome (www.genedb.org) suggests the presence of 14 proteins putatively part of the BT1 family. Some of these genes can be deleted in cells with reduced folate/MTX transport (15, 26). To identify the primary folate/MTX transporter we generated *L. infantum* MTX-resistant mutants. One of these mutants, called LiMTX1000.1, had a profound defect in MTX accumulation (Fig. 1A) that was paralleled by a decrease in folate accumulation (Fig. 1A). To test whether deletion of members of the BT1 family correlated with a defect in MTX/folate accumulation, we hybridized the total DNA of *Leishmania* cells to a probe derived from the *L. tarentolae* FT1 gene. This probe is known to recognize several members of the BT1 gene family (15). As expected, several hybridizing bands were observed within the MTX-susceptible strains; some of these bands might contain more than one homologue, but at least three of these bands were deleted or rearranged in LiMTX1000.1 (Fig. 1B, lane 2). Consequent to gene rearrangements, a new hybridizing band of 3 kb was observed in the mutant. To test whether one of the deleted bands could correspond to the main folate transporter, we screened a *L. infantum* cosmid DNA library using the same probe derived from the FT1 gene. Several cosmids were obtained and further characterized. One of them contained a 2.3-kb NheI-NheI fragment that appeared to be deleted in the LiMTX1000.1 mutant (Fig. 1B). The nucleotide sequence of this fragment and further sequencing on the cosmid revealed an open reading frame of about 2.1 kb resulting in a predicted protein of 708 amino acids (Fig. 2). The predicted protein encoded by this open reading frame is 80 and 59% identical to the *L. tarentolae* FT3 and FT5, respectively, and 41% identical to the *Leishmania mexicana* BT1 (Fig. 2). Homologues of these proteins are found in the kinetoplastid parasites *Leishmania* spp., *T. brucei*, and *Trypanosoma cruzi*; in the
apicomplexan parasites *Plasmodium falciparum*, *Toxoplasma gondii*, and *Eimeria tenella*; in *A. thaliana*; and in the cyanobacteria *Synechocystis* and *Synechococcus*. In the now completed *Leishmania major* genome project a total of 14 putative pteridine transporters have been found on chromosomes 4, 6, 10, and 19, and in *Leishmania donovani* a series of six putative pteridine transporter genes (*FT1* to *FT6*) were similar to the gene sequenced. Sequence comparison and ClustalW analysis (data not shown) indicated that the open reading frame (GenBank™ accession number AY577522) is closely related to a cluster of *L. donovani* sequences with the highest identity with *FT1* (99%) (GenBank™ accession number AF084469), and we thus retained the name *FT1* for the isolated *L. infantum* gene. This family of putative pteridine transporters is predicted to contain 12 putative transmembrane domains but otherwise is not closely related to other families of transport proteins, including proteins known to transport folates in other organisms.

**FT1 Is the Main *L. infantum* Folate Transporter**—The *L. infantum* gene *FT1* was cloned into an expression vector and transfected in the *L. tarentolae* MTX1000.6 mutant in which folate and MTX uptake are greatly impaired (10, 15). [3H]MTX uptake measurements were done for this *L. infantum* mutant cell line under the conditions tested, we observed an irremovable accumulation of either folate or MTX was seen in the transfectant and compared with the mutant. While no measurable accumulation of either folate or MTX was seen in the wild-type cells (Fig. 3), the level of accumulation in the *L. infantum* wild-type; 2, *L. infantum* MTX1000.6 transfectant compared with levels found in the *L. infantum* null mutant (see below). The *HYG* targeting cassette also led to the loss of a *NheI* site, thus generating an 8.3-kb *NheI* fragment when hybridized to the FT1 probe (Fig. 4B, lane 3). Since *Leishmania* is a diploid organism, one remaining intact *FT1* allele needs to be disrupted. The *HYG* targeting cassette was thus transfected into the *FT1-NEO*-single knock-out strain. Transfectants growing in the presence of both G418 and hygromycin B were analyzed by Southern blot. The integration of the *HYG* cassette also led to the loss of a *NheI* site, thus generating an 8.3-kb *NheI* fragment when hybridized to the FT1 probe (Fig. 4B, lane 3). The integrations of the selectable markers were confirmed by hybridization with *NEO* and *HYG* probes. The *NEO* marker was present in both the *FT1* single and double knock-out mutant cells (Fig. 4C, lanes 2 and 3, respectively), while the *HYG* probe only hybridized, as expected, to the DNA isolated from the *FT1* knock-out mutant (Fig. 4D, lane 3). The integration of the inactivation cassettes was further confirmed by PCR using specific *NEO* and *HYG* internal primers along with a primer downstream of the *FT1* gene but outside the inactivation cassette (Fig. 4A, primer 10). As expected, no amplicons were seen in wild-type cells (Fig. 4E, lanes 1 and 2) or in a single knock-out *NEO* transfectant (Fig. 4E, lane 3) while using the *HYG* primer. In the double *NEO* and *HYG* transfectant, amplified fragments of the expected size were obtained (Fig. 4E, lanes 5 and 6), confirming that both inactivation cassettes integrated within the desired locus. The integration of both *NEO* and *HYG* markers in the *FT1* gene would suggest that there are no more intact copies of the gene. However, a 2.3-kb *NheI*-NheI fragment of the size of an intact *FT1* allele still hybridized to an *FT1* probe in the *FT1* knock-out mutant. The intensity of the hybridizing band was lower in the DKO mutant, suggesting that indeed *FT1* was inactivated and that other homologous genes comigrated with *FT1* as was previously seen in the *FT5* null mutant (15). The functional analysis of the mutant indeed suggested that we were dealing with an *FT1* null mutant (see below).

An important decrease in the transport of folate acid and MTX was observed in the *FT1* knock-out mutant (Fig. 5, A and B). The *FT1* knock-out mutant still retained ~25% of transport activities for both substrates. This residual transport could be due to the
presence of FT5 previously described in L. tarentolae (15) or to other members of the BT1 family. To confirm that this reduction in folic acid and MTX transport was due to the inactivation of FT1, we transfected the FT1 DKO mutant with pSP/H9251 BLE/H9251 FT1. Transport experiments with this transfectant showed a reversion in the level of transport of both substrates to wild-type levels (Fig. 5, A and B), confirming that the observed decrease in transport was indeed due to inactivation of FT1.

**FT1 and Its Role in Antifolate Resistance**—The mutant TarII MTX1000.6 is highly resistant to MTX and is associated with a marked reduction in the uptake of the drug resulting from the deletion of several genes belonging to the BT1 family (15). Introduction of LiFT1 in this mutant increased the transport of both folate and MTX (Fig. 3), and the FT1 transfectant was found to be much more sensitive to MTX than the mutant, even reverting to the sensitivity level of wild-type cells (Fig. 6A). Deletion of members of the BT1 family is correlated with MTX resistance (Ref. 15 and Fig. 1), thus an FT1 null mutant would be expected to become resistant to the drug. The FT1 DKO mutant strain showed an EC50 more than 20 times higher than the susceptible parental cells, while the FT1 SKO exhibited an intermediate increase in MTX resistance (Fig. 6B). Introduction of an episomal copy of FT1 in the FT1 DKO mutant restored the sensitivity found in wild-type cells (Fig. 6B).

**FT1 Is a Membrane Protein Specifically Expressed in Logarithmic Phase Parasites**—To determine the cellular localization of the FT1 protein, we generated an FT1 protein fused to GFP.
at its C-terminal end. The FT1-GFP fusion was functional since upon transfection in the mutant TarII MTX1000.6 it led to levels of folate transport similar to those of the unfused protein (Figs. 7B and 3). Confocal microscopy analysis of the FT1-GFP-transfected cells, analyzed in the early logarithmic phase of growth, revealed plasma membrane localization of the hybrid protein (Fig. 8A, panel 1). Folate uptake in L. major is dependent on growth phase with maximal activity in the logarithmic phase and residual activity in the stationary phase (16, 17). Analysis of folate uptake in L. tarentolae wild-type cells showed a similar growth phase regulation (Fig. 7A). Most interestingly, the same type of regulation was seen in MTX1000.6 cells transfected with FT1-GFP where substrate accumulation decreased when the parasites went from logarithmic to stationary phase (Fig. 7B).

Our functional FT1-GFP fusion allowed us to study its cellular fate through the growth phases. The protein was localized to the plasma membrane during early logarithmic phase (24 h) where folate uptake was higher (Figs. 7B and 8A, panel 1). As the parasites reached late logarithmic phase (48 h), the transporter could still be found at the membrane; however, a portion of the fluorescence appeared to have a distinct localization (Fig. 8A, panel 2). When L. tarentolae entered the stationary phase (72 h), the accumulation of folate was reduced by 75% compared with the early logarithmic phase (Fig. 7B). The majority of the FT1-GFP fusion protein in early stationary phase parasites no longer localized to the plasma membrane and instead appeared to have been redirected intracellularly (Fig. 8A, panel 3). By late stationary phase (96 h) the fluorescence pattern had resolved into two distinct spots, one near the flagellar pocket and one close to the nucleus of the parasite (Fig. 8A, panel 4). To ascertain whether not all GFP fusion chimeras relocalize during the growth phase of the parasite, we studied the localization of a GFP fusion with the ATP-binding cassette transporter PGP3 (23). Here it was found to localize to an intracellular organelle near the flagellar pocket. Analysis of the PGP3-GFP transfectants revealed no difference in localization of the fusion protein between early logarithmic and late stationary phases of the parasite (Fig. 8A, panels 5 and 6).

To verify whether the retargeting of FT1-GFP resulted in its degradation, we probed whole-cell protein extracts of parasites isolated at different phases of growth with an anti-GFP antibody. The theoretical size of the FT1-GFP protein is 106 kDa, and Western blot analysis of proteins isolated from logarithmic phase showed no protein degradation (Fig. 8B, lanes 1–3). The FT1-GFP protein decreased in extracts isolated from cells in stationary phase and residual activity in the stationary phase (16, 17). This could be due to a specific degradation of the FT1 protein leading to the generation of a protease-resistant GFP, a phenomenon that has already been observed with two other Leishmania proteins (27, 28). Indeed this band had the same size as the GFP protein detected in Leishmania cells (Fig. 8B, lane 5). Probing of extracts isolated from a GFP transfectant and a PGP3-GFP transfectant in both logarithmic and stationary phase showed no protein degradation (Fig. 8B, lanes 5–8). These results are consistent with folate transport in Leishmania being growth phase-regulated by redistribution of the FT1 protein from the plasma membrane to an intracellular compartment.

**DISCUSSION**

Leishmania is a pterin and folate auxotroph and needs to import these essential substrates from the external medium. The proteins involved in the import of these molecules are becoming known. BT1 is a bioppterin transporter (13, 14) whose
gene is often amplified in field strains, suggesting that biotin uptake is important for Leishmania growth. Inactivation of BT1 leads to numerous interesting phenotypes including increased MTX susceptibility, increased metacyclogenesis, and reduced survival in animal models (13, 29–31). Recently we characterized FT5, a high affinity albeit low capacity folate/MTX transporter. The FT5 gene is part of a large multigene family, some members of which are often deleted in MTX-resistant mutants. In these mutants, transport of both folic acid and MTX is severely abrogated. The Leishmania genome comprises 14 open reading frames that could be part of the BT1 family. The functions of most proteins in this family are unknown, but as FT5 is not the primary folate transporter (15), this suggests that another member of the BT1 family is. Several Leishmania strains selected for MTX resistance and exhibiting markedly decreased MTX transport always have several members of the FT family deleted or rearranged (Refs. 15 and 26 and Fig. 1B). We were able to isolate and partially characterize FT1 from L. infantum by looking for genes with restriction fragments of the same size as the fragment deleted in the mutant. FT1 corresponded to a high affinity (Table I) and relatively high capacity folate transporter (Figs. 3 and 5) with kinetic properties comparable to L. infantum wild-type cells. FT1 activity accounted for more than 75% of all folate transport in Leishmania (Fig. 5). The remaining transport was possibly due to FT5 or other BT1 family transporters. Leishmania cycles between sand flies and host macrophages, and through its life cycle, the parasite may encounter environments with variable essential nutrient concentrations. For example, to take advantage of the high folate concentrations in the insect midgut where intensive Leishmania replication occurs, the parasite could have evolved a high capacity transporter like FT1. In conditions where the nutrient is scarce, the higher affinity transporter FT5 may play a more prominent role. Other BT1 homologues could have the ability to transport different types of pteridines, or perhaps multiple genes are required because differential expression occurs at discrete periods during the parasite life cycle (17). Folate transport and the proteins involved have been extensively studied in mammalian systems (for a review, see Ref. 32). Folate transport activities have been reported in Xenopus (33) and in bacteria (34, 35), but the gene products responsible for these activities have not yet been isolated, and work on FT5 and FT1 represents the first detailed analyses of non-mammalian folate transporters.

The study presented here showed that FT1 is an important MTX resistance gene. Transfection of this gene into the highly MTX-resistant mutant L. tarentolae MTX1000.6 completely eliminated this resistance by restoring MTX uptake (Fig. 6A). Inactivation of FT1 led to MTX resistance (Fig. 6B), although these strains were not as resistant as highly resistant mutants.
This can be easily explained since more than one transporter gene is usually deleted in mutant cells (Ref. 15 and Fig. 1, panel 6). This can be easily explained since more than one transporter and often several resistance mechanisms co-exist in the same parasite.

Further experiments are required to determine whether the stage-specific regulation of the folate transporter FT1 occurs by a similar mechanism.

In conclusion, we functionally characterized a novel gene product in Leishmania that corresponds to the primary high affinity folate transporter. We provided evidence that the stage-specific regulation of folate transport consists, at least in part, of a growth stage-dependent degradation of the FT1 protein. It will be interesting to find whether this type of regulation extends to other members of the B1 family and to other Leishmania proteins.
Singh, A. K., Olivier, M., and Ouellette, M. (2002) Infect. Immun. 70, 62–68
32. Matherly, L. H., and Goldman, D. I. (2003) Vitam. Horm. 66, 403–456
33. Lo, R. S., Said, H. M., Unger, T. F., Hollander, D., and Miledi, R. (1991) Proc. R. Soc. Lond. B Biol. Sci. 246, 161–165
34. Kumar, H. P., Tsuji, J. M., and Henderson, G. B. (1987) J. Biol. Chem. 262, 7171–7179
35. Shane, B., and Stokstad, E. L. (1975) J. Biol. Chem. 250, 2243–2253
36. Guimond, C., Trudel, N., Brochu, C., Marquis, N., El Fadili, A., Peytavi, R., Briand, G., Richard, D., Messier, N., Papadopolou, B., Corbeil, J., Bergeron, M. G., Legare, D., and Ouellette, M. (2003) Nucleic Acids Res. 31, 5886–5896
37. Coderre, J. A., Beverley, S. M., Schimke, R. T., and Santi, D. V. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2132–2136
38. Meek, T. D., Garvey, E. P., and Santi, D. V. (1985) Biochemistry 24, 678–686
39. Ghedin, E., Debrabant, A., Engel, J. C., and Dwyer, D. M. (2001) Traffic 2, 175–188
40. Hicke, L., and Dunn, R. (2003) Annu. Rev. Cell Dev. Biol. 19, 141–172
Growth Phase Regulation of the Main Folate Transporter of *Leishmania infantum* and Its Role in Methotrexate Resistance

Dave Richard, Philippe Leprohon, Jolyne Drummelsmith and Marc Ouellette

*J. Biol. Chem.* 2004, 279:54494-54501. doi: 10.1074/jbc.M409264200 originally published online October 4, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409264200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 15 of which can be accessed free at http://www.jbc.org/content/279/52/54494.full.html#ref-list-1