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Short communication

The H region *HTBF* gene mediates terbinafine resistance in *Leishmania major*

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Leishmania spp. are the causative agent of leishmaniasis, a disease that has an estimated global prevalence of 12 million cases [1]. The disease has a broad spectrum of clinical presentations, from skin and subcutaneous lesions to visceral disease. The mainstream treatment for leishmaniasis consists of parenteral administration of pentavalent antimonials, which may result in dose-cumulative side effects. Amphotericin B is an even more toxic and less effective alternative. For the treatment of complex cases, inhibitors of the sterol biosynthesis pathway, such as terbinafine, offer an attractive possibility as they target parasite-specific physiological aspects [2].

The study of the ability of this organism to evade chemotherapy is important not only to understand different aspects of this protozoan's biology but also to help the design of effective treatments. Gene amplification is a common mechanism used for survival in *Leishmania* strains selected for resistance to drugs such as methotrexate [3]. *Leishmania major* selected in methotrexate, primaquine, or pentavalent antimonials derive resistance to these drugs through the amplification of a 48 kb locus in chromosome 23, the H region. P-glycoprotein A (*PGPA*) and Pteridine reductase 1 (*PTR1*) are the two genes related to drug resistance that have been identified within the H region. PGPA is an ATP-binding cassette which renders the parasite resistant to heavy metals (arsenite and antimonials) through a still unclear mechanism [4–6]. The alternative pteridine reductase PTR1 confers resistance to methotrexate, and has been shown to limit pathogenesis within the mammalian host [7–9]. *L. major* subject to drug pressure may become resistant to terbinafine through H region amplification, as observed in cell lines SF-R30 (selected in terbinafine) and PQ-R10 (selected in primaquine) [3]. Terbinafine is an antifungal drug that is capable of inhibiting squalene-epoxidase [10], a key enzyme in the biosynthesis of ergosterol, an essential component of the cell membrane. Terbinafine toxicity thus arises from ergosterol deficiency and squalene accumulation [11,12]. While the genes mediating resistance to other agents that induce H region amplification have been identified, terbinafine resistance has not been mapped yet.

The effects of terbinafine on the parasite growth are complex and were shown to elicit resistance. The identification of a terbinafine-resistance gene present within the H region took advantage of a strategy based upon gene inactivation through insertional mutagenesis and its correlation with loss of phenotype. Considering the 48 kb span of the H locus, the resistance phenotype was constrained to a smaller fragment in order to reduce the number of genes to be inactivated by the insertional mutagenesis protocol. The H region had already been divided into four fragments cloned into the shuttle vector pSNAR [13]. Terbinafine resistance was assessed using not only *L. major* transfectants bearing each of the four clones, but also the SF-R30 strain and the wild-type cell line LT252 as positive and negative controls, respectively. The fold resistance at EC_{50} for these lines is presented in Fig. 1B. Transfectants bearing the H region subfragments H1, H2, and H3 were not different from the wild-type cell line. However, the fold resistance exhibited by transfectant

Abbreviations: PCR, polymease chain reaction; GFP, green fluorescent protein; RTPCR, reverse transcriptase polymerase chain reaction; PFGE, pulse field gel electrophoresis; YIP, YPT interacting protein; PGPA, P-glycoprotein A; PTR1, Pteridine reductase 1; ORF, open reading frame; MOPS, 3-[*N*-morpholino] propane-sulfonic acid; HTBF, H region associated terbinafine resistance; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors

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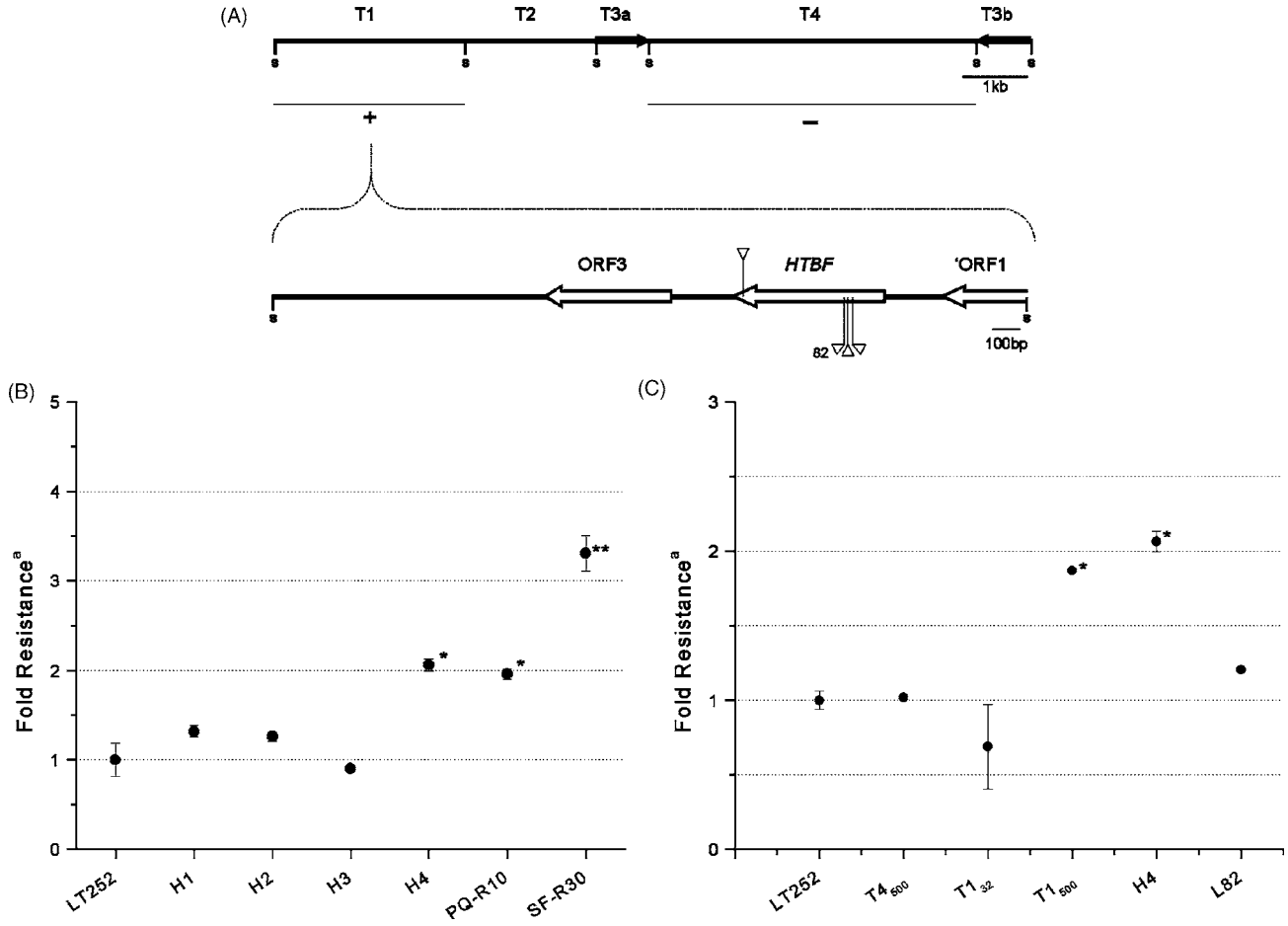

Fig. 1. Delimitation of the terbinafine-resistance gene within the H region. (A) *Sac*I fragments of H4 (T1, T2, and T4) were subcloned into pELHYG and tested for terbinafine resistance; plus and minus signs represent resistance and sensitivity, respectively; black arrows represent inverted repeats (T3a and T3b); S = *Sac*I site; sequencing of T1 revealed two complete open reading frames indicated by white arrows; the arrows on *HTBF* represent the placement of mariner transposon insertion events; the insertion named 82 was used to originate the L82 transfectant. (B and C) Fold resistance of *L. major* cell lines and transfectants at EC_{50} for terbinafine relative to that of the wild-type LT252, CC1 clone (MHOM/IR/84/LT 252); log-phase cells were seeded at a cell density of 10^5 cells ml^{-1} into drug-free medium as well as in media containing terbinafine ranging from 2 to 8 μg ml^{-1}; the drug-free culture growth was monitored daily until it reached late-log phase (0.8 to 1.0 × 10^7 cells ml^{-1}), when the terbinafine EC_{50} was determined as the concentration that decreased the cell growth rate by 50%; mean and S.D. are shown; *Values significantly higher than the wild-type value by Student's *t* test ($P < 0.05$); **Values significantly higher than all other lines and transfectants by Student's *t* test ($P < 0.05$). Terbinafine resistant cell lines were SF-R30 and PQ-R10 [3]; stable transfectants were initially selected on 32 μg ml^{-1} of hygromycin B and increased to 500 μg ml^{-1} in indicated transfectants by doubling steps; transfectants were named according to Clayton et al [27]; The fold-resistance is the ratio of terbinafine EC_{50}s for experimental and LT252 cells, measured in the same experiment[a].

H4 (LT*[pSNAR*^{8}*H4]*) was comparable to that of the resistant cell line PQ-R10 and significantly higher than that observed for wild-type cells ($P < 0.05$). Although fold resistance is typically low (about two-fold), it is consistently found in terbinafine resistant lines regardless of the selective force leading to H region amplification. The cell lines selected in terbinafine are an exception in which the fold resistance is significantly higher [3]. Different mechanisms might play a role in bringing about terbinafine resistance. In fact, *Leishmania* resistance to sterol biosynthesis inhibitors can be achieved by the expression of many loci [14], including the H locus, as shown in SF-R30 and PQ-R10 lines. Therefore, we cannot exclude the possibility that terbinafine selected cell lines, such as SF-R30, have developed different mechanisms of terbinafine resistance in addition to the amplification of the H locus.

In order to limit the locus that has imparted terbinafine resistance to *Leishmania*, the H4 fragment was restricted with *Sac*I, which generated five fragments (Fig. 1A). Two of the resulting fragments encompass the inverted repeats present in the right end of the H locus. The remaining fragments were subcloned into shuttle vectors pELHYG or pELHYGII and transfected into the parasite. *L. major* transfectants T1 (LT*[pELHYG*^{32}*T1]*) and T4 (LT*[pELHYGII*^{32}*T4]*) were isolated and kept under 32 μg ml^{-1} hygromycin B. The concentration of hygromycin B in culture was raised to 500 μg ml^{-1} in doubling steps so as to increase the plasmid copy number. The transfectants $T1_{500}$ (LT*[pELHYG*^{500}*T1]*) and $T4_{500}$

(LT*[pELHYGII*^{500}*T4]*) allowed the analysis of the effect of this phenomenon on the terbinafine-resistance phenotype. Control cell lines, as well as the different transfectants noted above, were used in a terbinafine resistance-assay (Fig. 1C). The fold resistance of transfectants $T4_{500}$ and $T1_{32}$ was not different from that observed for the wild-type cells. Nevertheless, the transfectant $T1_{500}$ exhibited a fold resistance that was significantly higher than that observed for wild-type cells and comparable to that exhibited by the transfectant H4. These results not only consistently linked the resistance phenotype to the H locus, but also narrowed the search for the resistance gene to only 2.8 kb contained in T1 fragment. Primer-island sequencing of the segment cloned into pELHYG revealed two complete open reading frames (ORFs) within the T1 locus (accession number AY227807). ORF2 and ORF3 had typical upstream and downstream putative AG *trans*-splicing acceptor sites and pyrimidine-rich tracks [15]. An incomplete open reading frame, ORF1, lacking a 5′-end was identified upstream of ORF2. Comparison with the public sequence databases revealed the homology of the 570 bp ORF2 to genes annotated in the human, worm, and yeast genomes. No significant sequence homology was found for 333 bp ORF1 and 360 bp ORF3.

Since transfection of segment T1 clearly rendered *L. major* resistant to terbinafine, this fragment was selected as a target for an insertional mutagenesis protocol. Inactivation of genes within T1 was carried out in vitro using the *mariner* transposition system [16]. Specific pairs of primers for each gene within T1 were used in a colony polymease chain reaction (PCR) protocol in order to identify useful insertion events. Primers LT007 and LT008 were selected to screen insertion events into ORF2, revealing 5 out of 96 events that had the transposable element inserted within the 570 bp ORF. One of the insertions disrupting ORF2, named 82 (Fig. 1A), was transfected into *L. major*. The transfectant obtained, L82 (LT*[pELHYG*^{500}*T1::/GFP***K82]*), was used in a terbinafine resistance-assay where $T1_{500}$, H4, and LT252 were the positive and negative controls, respectively. The transfectant L82, in which the insertion occurred 141 bp downstream of ORF2 ATG, presented a fold resistance that was not different from the wild-type, indicating the loss of the terbinafine resistance-phenotype (Fig. 1C). Therefore, the integration of the mariner element/GFP*K at one fourth of the putative gene length was enough to inactivate the terbinafine resistance associated to the 2.8 kb fragment T1. ORF2 was thus named H region associated terbinafine resistance (*HTBF*).

The BLAST analysis of *HTBF* revealed that its predicted amino acid sequence had significant homology to the YIP1 protein of *Saccharomyces cerevisiae*. The predicted HTBF and the yeast protein shared 23.9% identity at the amino acid level and there was 38.4% identity between this HTBF and the *Caenorhabditis elegans* homologue. Overall, the *Leishmania* HTBF shared 27.0% identity with the domain consensus of YIP1 protein from different organisms (COG5080). In the yeast, YIP1 interacts with YPT, a protein related to vesicle docking and trafficking [17]. YPT is a Rab/GTPase prenylated to a geranylgeranyl group that mediates attachment to membranes [18]. A pool of YPT exists in the cytosol, chaperoned by GDP dissociation inhibitor (GDI). Interaction with YIP1 promotes the dissociation of the YPT/GDI heterodimer, allowing YPT to reinsert into membranes [19]. A *YPT* homologue gene has already been identified in *L. major* and its product has been localized in the parasite Golgi apparatus [20].

The predicted HTBF is a 21 kDa protein containing four membrane-spanning domains, with both C and N terminus oriented toward the cytoplasmic face of the membrane. Kyte-Doolittle hydrophobicity plot analysis also indicated that HTBF is potentially an integral membrane protein. Southern blot analysis of pulse field gel electrophoresis (PFGE)-separated chromosomes, as well as *Sac*I digested genomic DNA, suggested that *HTBF* is present in one copy in the chromosome 23 of *L. major* (data not shown).

The transcription of *HTBF* was studied using Northern blot analysis of total RNA extracted from *Leishmania* cell lines LT252 and SF-R30, as well as from transfectants $T4_{500}$, $T1_{32}$, $T1_{500}$, and L82. The 570 bp *HTBF* probe recognized predominantly a 1.9 kb transcript in $T1_{32}$, $T1_{500}$, and SF-R30, which was absent in LT252 line or transfectant $T4_{500}$ (Fig. 2A). Although the basal level of the transcript was not detected, its expression is evident in the H region-amplified cell line and in the transfectants carrying the intact recombinant *HTBF*. Increased drug pressure led to higher transcript levels in $T1_{500}$ when compared to $T1_{32}$. A 5 kb transcript, which could correspond to read-through transcription across the circular episome [15], was also detected in transfectants carrying pELHYG-T1, but not in the SF-R30 line. As a consequence of the insertion into *HTBF*, the transcripts detected in transfectant L82 were 2 kb larger than those found in $T1_{500}$. *HTBF* transcripts were also detected in $T1_{500}$ and SF-R30 through a RT-PCR method (Fig. 2B). Being a more sensitive method, the RT-PCR also detected the basal level of transcript in the LT252 line and transfectant $T4_{500}$. As expected, a 2 kb larger band was also amplified from L82 total RNA. Reactions that did not include the reverse transcriptase confirmed the absence of contaminating DNA in the template samples (data not shown).

HTBF could mediate resistance to terbinafine through a general mechanism involving the increased formation and/or the redirection of vesicles. Studies on the mechanism of metal resistance in *Leishmania* mediated by the ABC transporter PGPA suggested that this protein acts on metal-thiol conjugates [21]. PGPA is associated to intracellular membranes in structures proposed to be part of exocytic and endocytic pathways in *Leishmania* [22]. These findings indicated that PGPA confers resistance to arsenite and antimonials by sequestration of metals into vesicles that could be exocytosed through the flagellar pocket. In this scenario, HTBF would improve the process of vesicle trafficking and/or docking. It is noteworthy that *L. tarentolae* H region-derived amplicons containing *PGPA* are likely

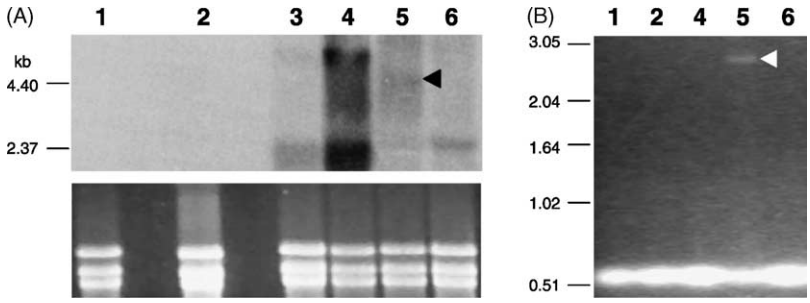

Fig. 2. Analyses of *HTBF* transcripts. (A) Northern analysis of total RNA in the cell lines LT252 (lane 1); SF-R30 (lane 6) and transfectants $T4_{500}$ (lane 2); $T1_{32}$ (lane 3); $T1_{500}$ (lane 4) and L82 (lane 5); the black arrowhead indicates the ~3.9 kb transcript in the transfectant bearing the transposon insertion into *HTBF*. Total RNA from promastigote forms was extracted using TRIzol® reagent (Gibco BRL) and manipulated as described elsewhere [28]. The *HTBF* probe was amplified by PCR with primers LT007 (5′-GCGCCCGGGCATATGCTCAACGAGGTGC) and LT008 (5′-CGCGGATCCTAAATACCAACCAGA). (B) RT-PCR amplification products using *HTBF* primers (LT007/LT008) and total RNA from LT252 (lane 1); $T4_{500}$ (lane 2); $T1_{500}$ (lane 4); L82 (lane 5); and SF-R30 (lane 6); the white arrowhead indicates the amplification product of 2.5 kb which represents the insertion of element/GFP*K into *HTBF*; reactions that did not include the reverse transcriptase confirmed the absence of contaminating DNA in the template samples (data not shown); reverse transcriptase-PCR was performed with Ready-To-Go™ RT-PCR Beads (Amersham Pharmacia Biotech) according to the manufacturer's specifications.

to carry the *HTBF* gene [23], which is located only 2.2 kb upstream of *PGPA* in *L. major*.

The terbinafine interference on the ergosterol biosynthesis may result in the use of exceptional sterols and cause membrane malfunctioning. It has been suggested that Ca^{2+}-dependent exocytosis may be essential in the maintenance of cell membrane integrity as it lowers surface tension [18,24]. In fact, proteolytic toxins, which specifically cleave soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE), also block exocytosis and inhibit membrane resealing. A calcium-dependant ergosterol mutant of *S. cerevisiae* had already been described [25] and studies of a temperature-sensitive yeast mutant of YPT revealed that rescue from growth arrest could be achieved by increasing extracellular Ca^{2+} [26]. These observations clearly indicate that increased external levels of calcium ions can rescue either mutants of ergosterol biosynthesis or vesicle-docking pathways by triggering the membrane repairing machinery. Thus, terbinafine interference in *Leishmania* ergosterol biosynthesis would be supposedly counteracted by the activation of the membrane repairing machinery through the enhanced expression of *HTBF*.

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