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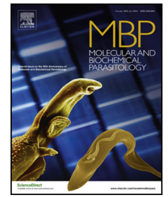

Short communication

Inducible protein stabilization system in *Leishmania mexicana*

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ABSTRACT

Targeted regulation of protein levels is an important tool to investigate the role of proteins essential for cell function and development. In recent years, methods based on the *Escherichia coli* dihydrofolate reductase destabilization domain (ecDHFR DD) have been established and used in various cell types. ecDHFR DD destabilizes the fused protein of interest and causes its degradation by proteasomes, unless it is stabilized by a specific ligand, trimethoprim. In this work we developed an inducible protein stabilization system in *Leishmania mexicana* based on ecDHFR DD.

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Leishmania are protozoan parasites causing leishmaniasis. They alternate between two main developmental stages. The flagellated extracellular promastigotes multiply in the gut of sandfly vectors, while the intracellular non-flagellated amastigotes reside inside the macrophages' phagolysosomes of vertebrates. Because *Leishmania* can evade the immune system and survive inside phagolysosomes, the complete immune response is usually triggered when the disease is already propagated [1,2]. Leishmaniases are classified into three clinical groups: cutaneous, muco-cutaneous and visceral, which is life-threatening if not treated. Although these parasites have received a considerable attention from researchers worldwide, there is no vaccine or universally applicable clinically effective drug available. In addition, cases of resistance may be a concern.

Numerous regulated gene expression systems have been developed to study biology of *Leishmania* parasites [3,4]. Most of them operate at the level of transcription and, because of this, are not well suited to study developmental regulation in these parasites [5]. A system based on inducible protein stabilization overcomes this limitation. Such a tool, utilizing the FK506-binding protein destabilizing domain (FKBP DD), was established for *L. major*, *L. braziliensis*, and *L. mexicana* [6,7]. An alternative system based on the *Escherichia coli* dihydrofolate reductase destabilizing domain (ecDHFR DD) was shown to be superior to FKBP DD [8]. Here, the ecDHFR destabilizes fused protein of interest and causes its degradation by 26S proteasomes, unless it is stabilized by a specific ligand, trimethoprim (TMP) [9,10]. This regulation is reversible, dose-dependent and provides an elegant strategy of "turning on and off" proteins, as needed. As such a system was successfully established in a related parasite, *Trypanosoma cruzi* [11], we decided to transpose it to *L. mexicana* strain MNYC/BZ/62/M379 (hereafter called *L. mexicana*), a causative agent of human cutaneous leishmaniasis. This species is of medical and biological importance with available genomic information [12]. It is relatively easy to cultivate and differentiate procyclic promastigotes of *L. mexicana* into metacyclic promastigotes and amastigotes *in vitro* [13], and to infect insects, macrophages and mice.

Log-phase grown *L. mexicana* procyclic promastigotes were transfected with linearized pLEXSY-hyg2 GFP-ecDHFR DD-HA construct encoding the Green Fluorescent Protein (GFP) fused to the HA_3-tagged ecDHFR DD on its C-terminus (Suppl. Materials and Methods and Suppl. Fig. 1). Transgenic cells, with this cassette integrated into the 18S rRNA locus, were selected in the presence of 100 μg/ml of hygromycin B and analyzed by fluorescence microscopy and Western blotting. To our surprise, in the absence of the stabilization ligand cells were brightly fluorescent (Suppl. Fig. 2A). The addition of either TMP or TMP-lactate for 24 h did not increase the fluorescent signal at 23 °C. We tried them both because the lactate salt of TMP was shown to work better in the *T. cruzi* system [11]. The integrity and correct integration of the construct were verified by sequencing. Of note, neither TMP, nor TMP-lactate influ-

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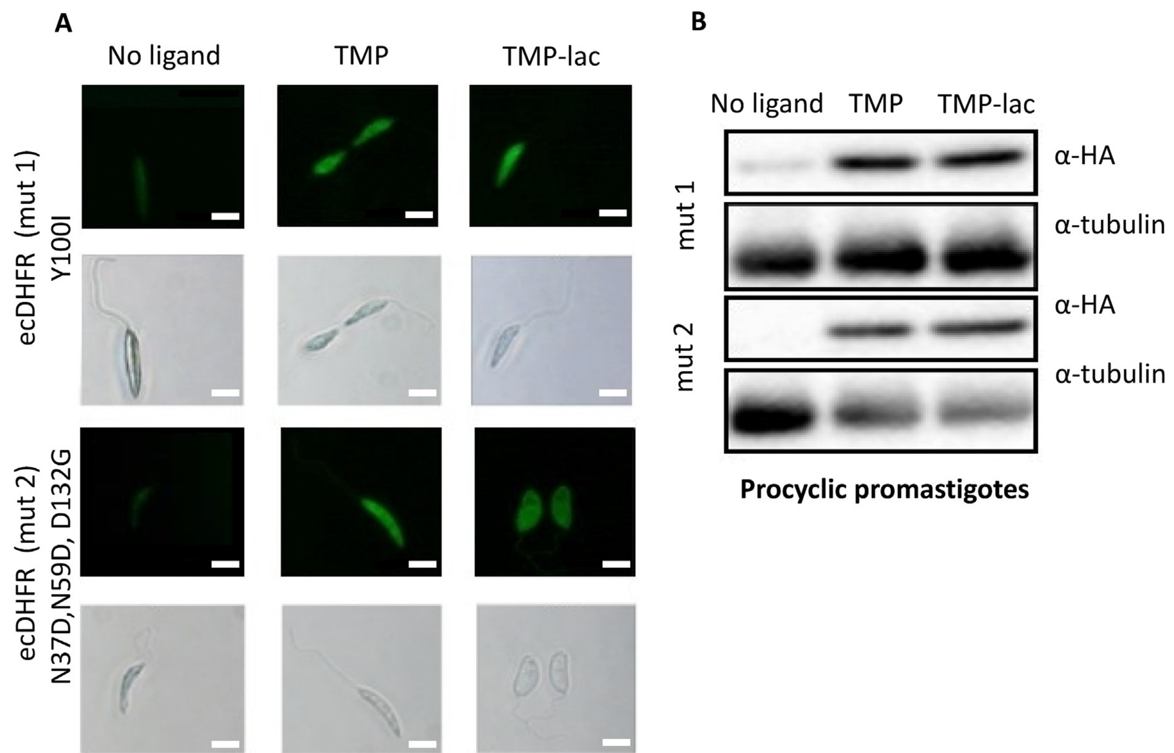

Fig. 1. Fluorescence and protein level of GFP-ecDHFR DD Y100I-HA (mut 1) and GFP-ecDHFR DD N37D, N59D, D132G-HA (mut 2) expressed in *L. mexicana* cells. A, Fluorescent and light microscopy of the not induced or induced with 10 μM of either TMP or TMP-lactate for 24 h cells. Scale bars are 5 μm. B, Western blotting showing levels of GFP-ecDHFR DD Y100I-HA (mut 1) and GFP-ecDHFR DD N37D, N59D, D132G-HA (mut 2) in L. *mexicana* procyclic promastigotes. Tubulin served as a loading control.

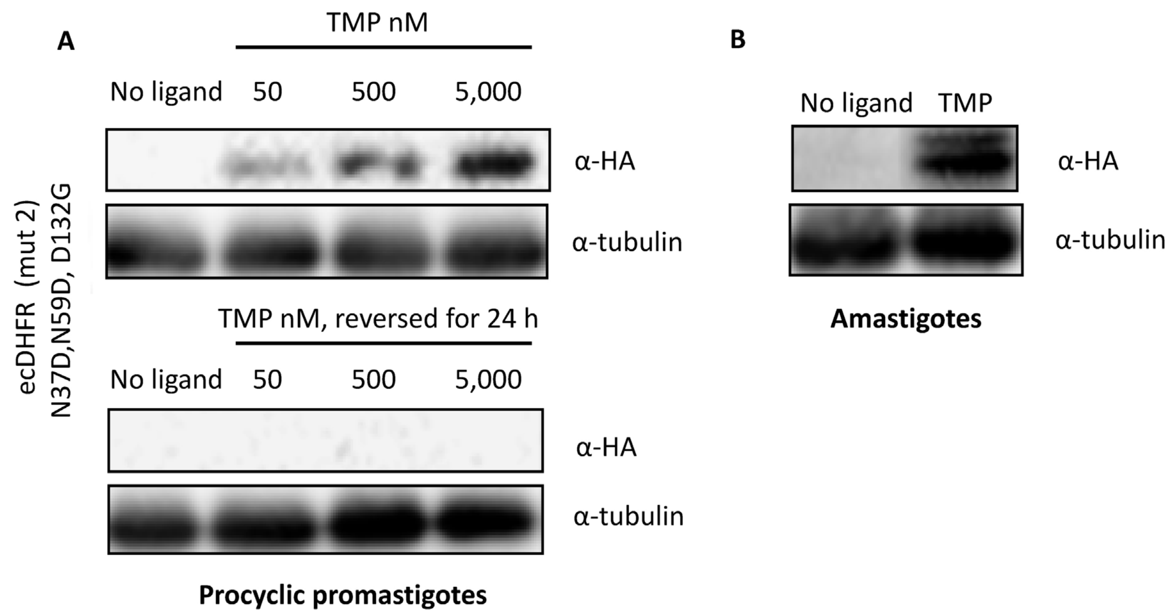

Fig. 2. Stabilization of GFP-ecDHFR DD N37D, N59D, D132G-HA (mut 2) expressed in *L. mexicana* cells. A, Western blotting showing levels of GFP-ecDHFR DD mut 2 upon titration of TMP (upper panel) and reversibility of the induction (lower panel). B, Western blotting showing levels of GFP-ecDHFR DD mut 2 in axenically differentiated *L. mexicana* amastigotes induced with 10 μM of TMP for 24 h. Tubulin served as a loading control.

enced growth of *Leishmania mexicana* at the concentrations used to stabilize proteins in our experiments (Suppl. Fig. 3). We concluded that the prototypical ecDHFR DD is not functional in *L. mexicana* reflecting the intrinsic differences between this species and *T. cruzi*.

To test whether a higher temperature would influence degradation of the ecDHFR DD-fused protein, the transgenic procyclic promastigotes were induced by TMP or TMP-lactate for 24 h at 23 °C and 37 °C and analyzed by Western blotting (Suppl. Fig. 2B). Confirming the fluorescence data, this experiment also demonstrated that GFP is not destabilized in the absence of a ligand and that addition of TMP or TMP-lactate has a marginal effect (Suppl. Fig. 2B, top 2 panels). Interestingly, the GFP-ecDHFR DD protein in procyclic promastigotes incubated at high temperature (37 °C) behaved the predicted way. It was degraded (albeit, not completely) in the absence of a ligand and stabilized by either TMP or TMP-lactate. Such a high temperature is detrimental for

Leishmania procyclic promastigotes and may potentially influence the protein abundance. Therefore, a similar experiment was carried out in axenically differentiated amastigotes cultivated at the normal (32 °C) and elevated temperature (37 °C) (Suppl. Fig. 2C). The observed pattern was the same as in the case of procyclic promastigotes at high temperature. We concluded that functionality of the ecDHFR DD is determined by its folding state and is temperature-dependent.

An incomplete ecDHFR DD-driven protein degradation in the absence of a ligand was also observed in other model organisms, such as *Saccharomyces cerevisiae* and *Caenorhabditis elegans* also grown at temperatures below 37 °C [8,14]. Two sets of mutations in ecDHFR DD – Y100I (mut1) and N37D, N59D, D132G (mut2) – were shown to facilitate correct folding of the destabilization domain and, subsequently, degradation of a protein of interest both at low and high temperatures in *C. elegans* [14]. These mutations were incorporated into the pLEXSY-hyg2 GFP-ecDHFR DD-HA (Suppl. Figs. 4 and 5) and tested in procyclic promastigotes of *L. mexicana* (Fig. 1). In both cases, the background level of GFP fluorescence without induction was dramatically decreased compared to the wild type ecDHFR (Suppl. Fig. 2A and B). The combination of 3 mutations N37D, N59D, D132G (mut2) was better than a single mutation Y100I (mut1) because of the lower level of the fusion protein in the absence of a ligand added. Both tested ligands (TMP and TMP-lactate) stabilized fusion proteins in a similar manner as confirmed by microscopy and Western blotting (Fig. 1). Further characterization was done with only one mutant (mut 2) and TMP.

The stabilization of GFP-ecDHFR DD N37D, N59D, D132G (mut2)-HA is dose-dependent in the range of concentrations analyzed and was facilitated by as little as 50 nM of TMP (Fig. 2A, upper panel). Protein stabilization is fully reversible. Incubation in the media with no TMP for 24 h results in complete destabilization of the target protein (Fig. 2A, lower panel). Induction is ligand-specific. No protein stabilization was detected when Rapamycin, a ligand for the FKBP DD was used (Suppl. Fig. 6). The system is not limited to procyclic promastigotes. When axenically differentiated amastigotes of *L. mexicana* bearing GFP-ecDHFR DD N37D, N59D, D132G (mut2)-HA were treated with TMP, a similar stabilization of the protein of interest was documented (Fig. 2B).

In summary, here we present an inducible protein stabilization system in human pathogen *Leishmania mexicana*. We believe that such a system can be widely used by the parasitology community to study effects of different proteins (including toxic ones) on biology, physiology and virulence of *Leishmania*. It is freely available from authors upon request.

Conflict of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2017.03.008.

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