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RNA editing and mitochondrial activity in promastigotes and amastigotes of *Leishmania donovani* ☆,☆☆

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ABSTRACT

Kinetoplast maxicircle DNA sequence organisation was investigated in *Leishmania donovani*, strain 1S LdBob. Gene arrangement in the coding (conserved) region of the maxicircle is collinear with that of most trypanosomatids, with individual genes showing 80–90% nucleotide identity to *Leishmania tarentolae*, strain UC. The notable exception was an integration of a full-size minicircle sequence in the ND1 gene coding region found in *L. donovani*. Editing patterns of the mitochondrial mRNAs investigated also followed *L. tarentolae* UC patterns, including productive editing of the components of respiratory complexes III–V, and ribosomal protein S12 (RPS12), as well as the lack of productive editing in five out of six pan-edited cryptogenes (ND3, ND8, ND9, G3, G4) found in these species. Several guide RNAs for the editing events were localised in minicircles and maxicircles in the locations that are conserved between the species. Mitochondrial activity, including rates of oxygen consumption, the presence and the levels of respiratory complexes and their individual subunits and the steady-state levels of several mitochondrial-encoded mRNAs were essentially the same in axenically grown amastigotes and in promastigotes of *L. donovani*. However, some modulation of mitochondrial activity between these developmental stages was suggested by the finding of an amastigote-specific component in complex IV, a down-regulation of mitochondrial RNA-binding proteins (MRP) and MRP-associated protein (MRP-AP) in amastigotes, and by variations in the levels of RPS12, ND3, ND9, G3 and G4 pre-edited transcripts.

1. Introduction

The life cycle of dixenous *Trypanosoma* and *Leishmania* spp. includes two types of hosts (vertebrate and invertebrate) which represent drastically different types of environments. Adaptations that evolved in *Trypanosoma brucei* to meet the challenges for survival and propagation in its hosts include well documented reversible changes in mitochondrial metabolism, and are accompanied by far less understood changes in mitochondrial gene activity, including editing of mRNA (recently reviewed by Hannaert et al., 2003; Lukeš et al., 2005; Stuart et al., 2005; Bringaud et al., 2006; Fenn and Matthews, 2007). Thus, while insect stage trypanosomes have a fully functional oxidative phosphorylation system, in bloodstream trypanosomes the cytochrome *c* oxidase and bc_1 complexes are no longer present and the function of ATP synthase functions to hydrolyse ATP in order to maintain a transmembrane potential (Clarkson et al., 1989; Bienen et al., 1991; Schnaufer et al., 2005; Vertommen et al., 2008). On the contrary, relatively little is known about changes in mitochondrial activity that might accompany differentiation of *Leishmania* during the insect (promastigote) and mammalian (intracellular amastigotes) stages of the life cycle. An early report indicated that promastigotes and lesion-derived amastigotes in *Leishmania mexicana* had similar oxygen uptake rates and sensitivity to inhibitors of the respiratory enzymes, indicating that oxidative phosphorylation was active in both developmental stages (Hart et al., 1981). The current view is generally that the metabolic changes between the stages of *Leishmania* are much less pronounced compared with *T. brucei* (Opperdoes and Coombs, 2007). Although a number of genes with stage-specific expression patterns were identified earlier (Bahr et al., 1993; Joshi et al., 1993; Charest and Matlashewski,1994; Kar et al., 2000; Nugent et al., 2004; Walker et al., 2006), the recent genome-wide transcriptome and proteome analyses revealed that only ~3.5% of genes demonstrate a stage-specific expression pattern (Holzer et al., 2006; Leifso et al., 2007; Morales et al., 2008).

There is still some controversy with respect to the presence and function of NADH dehydrogenase (complex I) in the respiratory chain at any stage in these organisms (Santhamma and Bhaduri,

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1995; Bermúdez et al., 1997; Opperdoes and Michels, 2008). The investigated nuclear and mitochondrial genomes of several *Leishmania* species encode subunits of this complex (Simpson et al., 1998; Hertz-Fowler et al., 2004; Peacock et al., 2007), although expression of these genes has not yet been verified. In spite of the presence of these genes, the complex could not be visualised by Blue Native gel analysis of mitochondrial lysate from promastigotes of *Leishmania amazonensis* and *Leishmania tarentolae* (Maslov et al., 2002). Moreover, complex I is not detected in *L. tarentolae* promastigotes by in gel activity staining or in vitro NADH–ubiquinone oxidoreductase activity measurements (A. Horváth, personal communication). Finally, a disruption of productive editing of several genes due to the loss of minicircle-encoded guide RNAs did not affect the viability of *L. tarentolae* cells in culture (Thiemann et al., 1994). These results suggest that, if this complex is indeed present, it is a small amount and/or is dispensable for proliferation of promastigotes, at least in culture. This also leaves open the possibility that the complex might be required in amastigotes or in some other life cycle stages, such as metacyclic promastigotes.

The mitochondrial genetic system, including RNA editing, has been well characterised only in one species of *Leishmania*, *L. tarentolae*, a parasite of geckos. Unfortunately, this organism has not been propagated as amastigotes, and that renders it unsuitable for investigation of mitochondrial adaptations during the life cycle. However, axenic cultivation of amastigotes of several human pathogenic species is possible by exposing the organisms to acidic pH and increased temperature (Bates, 1993). One such strain is a clonal derivative (LdBob) of the *Leishmania donovani* strain 1S (MHOM/SD/62/1S-$Cl2_D$) (Joshi et al., 1993; Goyard et al., 2003). This species represents an important agent of human visceral leishmaniasis in the Old World. In addition, a large collection of minicircle sequences from this and closely related species (*Leishmania infantum*, *Leishmania chagasi*) is already available in public databases and can be used to search for guide RNAs once the sequences of homologous pre-edited and edited maxicircle mRNA sequences are determined. The work presented below describes the initial characterisation of the mitochondrial genome and RNA editing of *L. donovani* 1S LdBob, as well as an investigation of the possibility of mitochondrial gene regulation during the life cycle of these parasites.

2. Materials and methods

2.1. Leishmania cultures and isolation of mitochondria

Promastigotes of *L. donovani* 1S clonal line LdBob were grown at 26 °C in M199 medium, while amastigotes of the same strain were cultivated at 37 °C with 5% CO_2 in the 'amastigote' medium as described previously (Goyard et al., 2003). Promastigotes of *L. tarentolae* UC strain were cultivated in brain heart infusion medium supplemented with 10 μg/ml hemin (Simpson and Braly, 1970). Mitochondria from all types of cells were isolated by hypotonic lysis followed by Renografin density gradient centrifugation (Braly et al., 1974). Typically 1–2 L cultures with cell densities of 20–40 $\times 10^6$ cell ml^{-1} were used to obtain ~0.5 g of isolated mitochondria (wet weight).

2.2. Measurement of respiration rate

Oxygen uptake by *L. donovani* cells was measured with a biological oxygen monitor, YSI 5300, equipped with the YSI 5331 oxygen probe. The rate (expressed as μmol of O_2 consumed per cell per min) was calculated by assuming the oxygen content of air-saturated Ringers solution of 0.227 mM at 28 °C or 0.2 mM at 37 °C (according to the YSI 5300 manual). KCN was used at 1 mM, and salicylhydroxamic acid (SHAM) at 0.1 mM. The cell concentration was 20–30 $\times 10^6$ ml^{-1} for promastigotes and 2–6 $\times 10^6$ ml^{-1} for amastigotes.

2.3. Protein electrophoretic and immunochemical procedures

Samples were analysed by single-dimension Tris–glycine SDS–polyacrylamide gels (Laemmli, 1970) and two-dimensional Blue Native/Tris–tricine SDS–polyacrylamide gels (Schägger et al., 1994). Gel loading was normalised by using 70 $\times 10^6$ cells or 50 μg of mitochondrial proteins, as appropriate. The resolved polypeptides were transferred onto nitrocellulose membranes by semi-dry blotting, as described previously (Horváth et al., 2000). After electrophoresis, the gels were stained either with Coomassie Brilliant Blue R250 (Sigma) or SYPRO Ruby (Molecular Probes). For autoradiography, dried gels were exposed to low energy screens and analysed by using the PhosphorImager (Molecular Dynamics). Antibodies used were described previously: mouse antibody against *L. tarentolae* Rieske iron–sulphur protein of cytochrome bc_1 (Nebohá čová et al., 2004), the rabbit antibodies against *L. tarentolae* subunit IV of cytochrome *c* oxidase (Maslov et al., 2002), *L. tarentolae* p18 protein (subunit b of mitochondrial ATP synthase) (Bringaud et al., 1995), *L. tarentolae* MRP1/2 complex (formerly named Ltp26/28 complex) (Aphasizhev et al., 2003). Rabbit polyclonal antibodies against *Leishmania major* adenylate kinase 2 (AK2) were provided by D. Nierlich (University of California – Los Angeles, Los Angeles, USA). Mouse antibody against the amastigote-specific protein A2 family (Zhang et al., 1996) was provided by S. Beverley (Washington University, Missouri, USA). Western blots were processed by using the SuperSignal West Pico chemiluminescent system (Pierce).

2.4. DNA isolation, cloning and sequence analysis procedures

Kinetoplast DNA was isolated by sedimentation through a CsCl cushion as described previously (Simpson and Berliner, 1974). The maxicircle component was purified after release from kinetoplast DNA networks by digestion with EcoRI followed by equilibrium centrifugation in CsCl-Hoechst 33258 gradients (Simpson, 1979). In the initial stage of the project, the maxicircle DNA was randomly sheared to fragments of average size of 3 kb using a GeneMachine HydroShear, a computer-controlled repetitive syringe-driven device with an occlusion of about 10 μm (Thorstenson et al., 1998). The sheared fragments were cloned into the pCR2.1 vector (Invitrogen) using standard procedures. Subsequently, oligonucleotide primers were designed based on the sequence conservation between *L. donovani* and *L. infantum* and used to amplify several missing regions of the maxicircle. Gaps between the contigs were joined using direct sequencing of the respective PCR products. The final sequence was assembled using the ContigExpress program of Vector NTI (Invitrogen). Both strands of the maxicircle DNA were sequenced. The sequences were analysed using Vector NTI programs. Guide RNA genes were searched for using the UWGCG program BESTFIT as previously described (Simpson et al., 1994; Maslov and Simpson, 2007).

2.5. RNA isolation and RT- PCR

Total cell RNA was isolated by Trizol extraction (Invitrogen) following the manufacturer's protocol. The same protocol was used for isolation of RNA from purified mitochondria. The isolated RNA was additionally treated with RNAse-free DNAse I (La Roche). cDNA was synthesised and amplified using SuperScript™ III One-Step reverse transcriptase (RT)-PCR System (Invitrogen). The amplification conditions included cDNA synthesis at 45 °C for 45 min, initial denaturation at 94 °C for 2 min, followed by 40 cy-