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Functional characterization of nucleoside transporter gene replacements in *Leishmania donovani*

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Abstract

Leishmania donovani express two nucleoside transporters of non-overlapping ligand selectivity. To evaluate the physiological role of nucleoside transporters in *L. donovani*, homozygous null mutants of the genes encoding the LdNT1 adenosine–pyrimidine nucleoside transporter and the LdNT2 inosine–guanosine transporter were created singly and in combination by single targeted gene replacement followed by selection for loss-of-heterozygosity. The mutant alleles were verified by Southern blotting, and the effects of gene replacement on transport phenotype were evaluated by rapid sampling transport measurements and by drug resistance profiles. The *Δldnt1*, *Δldnt2*, and *Δldnt1*/*Δldnt2* mutants were all capable of proliferation in defined culture medium supplemented with any of a spectrum of purine nucleobases or nucleosides, except that a *Δldnt2* lesion conferred an inability to efficiently salvage exogenous xanthosine, a newly discovered ligand of LdNT2. Each of the three knockout strains was viable as promastigotes and axenic amastigotes and capable of maintaining an infection in J774 and bone marrow-derived murine macrophages. These genetic studies demonstrate: (1) that *L. donovani* promastigotes, axenic amastigotes, and tissue amastigotes are viable in the absence of nucleoside transport; (2) that nucleoside transporters are not essential for sustaining an infection in mammalian host cells; (3) that the phagolysosome of macrophages is likely to contain purines that are not LdNT1 or LdNT2 ligands, i.e., nucleobases. Furthermore, the *Δldnt1*, *Δldnt2*, and *Δldnt1*/*Δldnt2* knockouts offer a unique genetically defined null background for the biochemical and genetic characterization of nucleoside transporter genes and cDNAs from phylogenetically diverse species and of genetically manipulated *LdNT1* and *LdNT2* constructs.

Keywords: Transport; Transporters; Purines; Pyrimidines; Infectivity

1. Introduction

Leishmania donovani is the etiologic agent of visceral leishmaniasis, a devastating and often fatal disease. *Leishmania* species are digenetic protozoan parasites in which the extracellular, flagellated promastigote exists in the phlebotomine sandfly vector, and the intracellular amastigote resides within the phagolysosome of macrophages and reticuloendothelial cells of the mammalian host. The current arsenal of drugs used in the treatment of leishmaniasis – or for that matter any parasitic disease – is far from ideal, and the need for more efficacious and selective anti-leishmanial drugs that exploit fundamental biochemical differences between parasite and host is acute.

Perhaps the most striking metabolic discrepancy between parasites and their mammalian hosts is the disparate mechanisms by which they generate purine nucleotides. Whereas mammalian cells synthesize purine nucleotides from amino acids and 1-carbon compounds, all of the protozoan parasites studied to date are incapable of *de novo* synthesis of the purine ring [1]. Thus, each genus of parasite expresses a unique complement of purine salvage enzymes that enable the parasite to scavenge preformed purines from the host milieu. The initial step in purine acquisition by the parasite involves the translocation of host purines across the parasite surface membrane, a process that is mediated by nucleoside and nucleobase transporters.

Nucleoside permeation into *L. donovani* is carried out by two high affinity transporters with non-overlapping ligand specificity, LdNT1 and LdNT2. LdNT1 transports adenosine and pyrimidine nucleosides, whereas LdNT2 is selective for ino-

Abbreviations: ENT, equilibrative nucleoside transporter; LOH, loss-of-heterozygosity; PCR, polymerase chain reaction; FBS, fetal bovine serum; UTR, untranslated region; ORF, open reading frame; PBS, phosphate buffered saline; PRT, phosphoribosyltransferase

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sine and guanosine [2,3]. Mutants genetically deficient in either LdNT1 (TUBA5) or LdNT2 (FBD5) activity have been created by negative selection, and the genes for these permeases were subsequently cloned from a cosmid library by functional rescue of these nucleoside-transport-deficient *Leishmania* [2–4]. Based on their primary structures and predicted membrane topologies, LdNT1 and LdNT2 belong to the equilibrative nucleoside transporter (ENT) family, although subsequent electrophysiological investigations revealed each to be an electrogenic proton symporter [5].

The TUBA5 and FBD5 parasites were originally derived after mutagenesis and selection in tubercidin, an adenosine analog, or formycin B, an inosine isomer, respectively [4]. Southern blot analyses revealed no gross gene rearrangements at the *LdNT1* and *LdNT2* loci in the nucleoside transport-deficient *L. donovani*, and Northern blot analyses indicated that the *LdNT1* and *LdNT2* transcripts were expressed at normal levels in TUBA5 and FBD5 cells, respectively [2,3]. Subsequent sequence analyses of the mutant alleles revealed the basis of the genetic deficiencies to be either point mutations within *LdNT1* and *LdNT2* that cripple the permeation mechanisms of the encoded permeases or a nonsense mutation within one *ldnt2* allele [6,7]. Because of the potential for residual nucleoside transporter activity in TUBA5 and FBD5 parasites and the possibility that functionally incompetent transporters are generated or accumulate in some subcellular milieu within the nucleoside transport-deficient lines, the TUBA5 and FBD5 cells do not provide an optimal null background for transfection studies with nucleoside transporter genes or cDNAs.

Thus, to assess the consequences of a complete deficiency in LdNT1 and LdNT2 activity on the ability of *L. donovani* to transport, salvage, and proliferate in various purines, *Δldnt1*, *Δldnt2*, and *Δldnt1*/*Δldnt2* knockouts were isolated by a combination of targeted gene replacement and selection for loss-of-heterozygosity (LOH). Mutants lacking *LdNT1* could not transport adenosine but were capable of growing on all purine nucleosides and nucleobases tested. Mutants possessing a *Δldnt2* lesion failed to transport inosine and xanthosine but were capable of robust growth on inosine and guanosine but not xanthosine. All three knockout strains were capable of transforming into axenic amastigotes and establishing an infection in murine macrophages. These studies establish that nucleoside transport is not essential for the viability of either parasite life cycle stage, intimates that purine nutrition within the intracellular milieu of the macrophage does not require nucleosides, and provides an ideal vehicle for future structure-function studies on LdNT1 and LdNT2 and for the expression of heterologous transporters genes and cDNAs within a genetic background completely devoid of nucleoside transport mechanisms.

2. Materials and methods

2.1. Chemicals, materials, and reagents

[^{3}H]inosine (38.4 Ci/mmol) and [^{3}H]xanthosine (8.6 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA), [^{3}H]adenosine (40 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [α-^{32}P]dCTP (3000 Ci/mmol) was supplied by MP Biomedicals (Irvine, CA). All restriction and DNA modifying enzymes were bought from either Invitrogen (Carlsbad, CA), Gibco-BRL Life Technologies Inc. (Gaithersburg, MD), or New England Biolabs, Inc. (Beverly, MA), and the polymerase chain reaction (PCR) protocols were performed using the Advantage^{TM} HF-2 PCR kit from BD Biosciences (Franklin Lakes, NJ). Synthetic oligonucleotides were acquired from Invitrogen. The pX63-HYG [8] and pX63-PHLEO [9] leishmanial vectors that harbor genes conferring hygromycin and phleomycin resistance, respectively, were generous gifts from Dr. Stephen M. Beverley (Washington University School of Medicine). Hygromycin, tubercidin, and formycin B were obtained from Sigma–Aldrich (St. Louis, MO). All other materials, chemicals, and reagents were of the highest purity commercially available.

2.2. Parasite cell culture

The *L. donovani* LdBob strain [10] was provided by Dr. Stephen M. Beverley (Washington University, St. Louis, MO). LdBob promastigotes were routinely cultured at 26 °C in purine-replete M199-based medium [10] or in Dulbecco's modified Eagle-*Leishmania* (DME-L) medium [11] in which the bovine serum albumin was replaced with 10% dialyzed fetal bovine serum (FBS), 1 mM glutamine, 1× RPMI 1640 vitamin mix, 10 μM folate, and 100 μM xanthine. Once isolated, the *Δldnt1* and *Δldnt2* strains were continuously cultured in 50 μg/ml hygromycin and 50 μg/ml phleomycin, respectively. The *Δldnt1*/*Δldnt2* double knockout was maintained in both 50 μg/ml hygromycin and 50 μg/ml phleomycin. Single cell cloning of promastigotes was accomplished in semi-solid medium on plates as described [11], and axenic amastigotes were grown at 37 °C as reported [10].

2.3. Oligonucleotide primers

Primers used in the PCR-based amplification of 5′ and 3′ flanking segments of the *L. donovani LdNT1* and *LdNT2* loci are designated by the flank (either 5′ or 3′), the targeted locus, and orientation. The restriction sites in the PCR primers that were employed in the subcloning of the amplified products into vectors are underlined:

- 5′-*LdNT1*-sense, 5′-AAGCTTGAGGTGCCCGCGATTGTTG-3′;
- 5′-*LdNT1*-antisense, 5′-GTCGACGGAGAATGGGAGAAGAGAG-3′;
- 3′-*LdNT1*-sense, 5′-TCTAGAGGATCATCGTAGCGGCGTC;
- 3′-*LdNT1*-antisense, 5′-AGATCTAGCAGCGGGGCACAAGGTG;
- 5′-*LdNT2*-sense, 5′-CATTAAGCTTCCCTACTTGCCTTGCTG-3′;
- 5′-*LdNT2*-antisense, 5′-TTCAAGCTTAGTTTTAATCAGTCAGAGTAACTCAGTAAG-3′;