Arginase Plays a Pivotal Role in Polyamine Precursor Metabolism in Leishmania

CHARACTERIZATION OF GENE DELETION MUTANTS

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The polyamine pathway of protozoan parasites has been successfully targeted in anti-parasitic therapies and is significantly different from that of the mammalian host. To gain knowledge into the metabolic routes by which parasites synthesize polyamines and their precursors, the arginase gene was cloned from Leishmania mexicana, and Δarg null mutants were created by double targeted gene replacement and characterized. The ARG sequence exhibited significant homology to ARG proteins from other organisms and predicted a peroxisomal targeting signal (PTS-1) that steers proteins to the glycosome, an organelle unique to Leishmania and related parasites. ARG was subsequently demonstrated to be present in the glycosome, whereas the polyamine biosynthetic enzymes, in contrast, were shown to be cytosolic. The Δarg knockouts expressed no ARG activity, lacked an intracellular ornithine pool, and were auxotrophic for ornithine or polyamines. The ability of the Δarg null mutants to proliferate could be restored by pharmacological supplementation, either with low putrescine or high ornithine or spermidine concentrations, or by complementation with an arginase episome. Transfection of an arg construct lacking the PTS-1 directed the synthesis of an arg that mislocalized to the cytosol and notably also complemented the genetic lesion and restored polyamine prototrophy to the Δarg parasites. This molecular, biochemical, and genetic dissection of ARG function in L. mexicana promastigotes establishes: (i) that the enzyme is essential for parasite viability; (ii) that Leishmania, unlike mammalian cells, expresses only one ARG activity; (iii) that the sole vital function of ARG is to provide polyamine precursors for the parasite; and (iv) that ARG is present in the glycosome, but this subcellular milieu is not essential for its role in polyamine biosynthesis.

Leishmania is a genus of protozoan parasite that is the causative agent of leishmaniasis, a spectrum of devastating and potentially deadly diseases that affects ~12 million people worldwide. The parasite exhibits a digenetic life cycle in which the extracellular promastigote form resides in the phlebotomine sandfly vector, whereas the intracellular amastigote inhabits the phagolysosomes of macrophages from the infected mammalian host. Because there are no vaccines available to prevent leishmaniasis, chemotherapy offers the only avenue to combat the disease. Unfortunately, the current arsenal of drugs used to treat leishmaniasis is far from ideal, mainly because of toxicity and therapeutic unresponsiveness. Thus, the identification, characterization, and validation of novel therapeutic targets are urgently needed.

One biochemical pathway that has been successfully exploited for the treatment of parasitic disease is that for polyamine biosynthesis. Polyamines are ubiquitous organic cations found in virtually every eukaryotic cell and play critical roles in key cellular processes such as growth, differentiation, and macromolecular biosynthesis (1, 2). DL-α-Difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), the first enzyme in polyamine synthesis, is capable of eradicating Trypanosoma brucei infections in mice (3) and patients with late stage African sleeping sickness (4, 5). DFMO is also active against many other parasites (6, 7), including the promastigotes of Leishmania donovani (8). Interestingly, the selectivity of DFMO for the metabolic machinery of trypanosomes is not based on dissimilar DFMO binding affinities but rather to differences in ODC turnover rates between parasites and mammals (9–11). In addition, inhibitors of S-adenosylmethionine decarboxylase (ADOMETDC), the enzyme that provides aminopropyl moieties for spermidine and spermine synthesis, are also effective anti-trypanosomal agents (12, 13).

Leishmania sp. have served as particularly valuable model systems for dissecting metabolic pathways in protozoan parasites because of their ability to proliferate axenically in defined growth medium and the facility by which their genome is genetically tractable (14–17). All the genes of the polyamine pathway, ODC, ADOMETDC, and spermidine synthase

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2 The abbreviations used are: DFMO, DL-α-Difluoromethylornithine; ODC, ornithine decarboxylase; AdoMet, S-adenosylmethionine; dAdoMet, decarboxy-S-adenosylmethionine; ADOMETDC, S-adenosyl-methionine decarboxylase; SPDSYN, spermidine synthase; ARG, arginase; G418, Geneticin; PTS-1, peroxisomal targeting signal-1; GFP, green fluorescent protein; ORF, open reading frame; PBS, phosphate-buffered saline; AGM, agmatinase; HGPR, hypoxanthine-guanine phosphoribosyltransferase; DME-L, Dubecco’s modified Eagle-based medium specifically developed for growing Leishmania promastigotes; CS, chicken serum.

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(SPDsyn), have been cloned from L. donovani, and the creation of Δadc, Δadometdc, and Δspdsyn knockouts by targeted gene replacement has demonstrated the essential role of each of these enzymes in L. donovani promastigote proliferation and revealed significant dissimilarities between the polyamine biosynthetic pathways of this genus of protozoan parasite and the mammalian host (18-20). A plethora of molecular and biochemical studies on the polyamine biosynthetic pathway of T. brucei, L. donovani, and other parasites (21-25), little is known about the metabolic avenues by which polyamine precursors are produced. Ornithine, the amino acid from which polyamines are generated, is produced from arginine in mammalian cells by two genetically and biochemically distinct arginase (ARG) enzymes. An ARG activity has also been detected in Leishmania (26), and an arginase gene sequence from Leishmania amazonensis has been reported, although neither the gene nor protein was functionally characterized (27). ARG has also been touted as a potential antileishmanial drug target, because N-hydroxylarginine, an inhibitor of ARG that is produced by macrophages as an intermediate during the formation of nitric oxide, can reduce polyamine levels in Leishmania amastigotes and lowers parasite loads (28).

To initiate an investigation into the pathways by which polyamine precursors are synthesized and to begin a validation of ARG as a potential therapeutic target, we have cloned arginase from L. mexicana and generated Δarg knockouts by double-targeted gene replacement. The characterization of these gene deletion mutants reveals that the arginase is essential for promastigote viability and that the lethality of the null mutation could be conditionally bypassed by either low concentrations of putrescine, high concentrations of ornithine or spermidine, or episomal complementation. Furthermore, we have established that the L. mexicana ARG is localized to the glycosome, a unique organelle found exclusively in Leishmania and close relatives (29, 30), and that this localization is mediated by the peroxisomal targeting signal type 1 (PTS-1).

However, genetic complementation demonstrated that this glycosomal venue is not essential for ARG function, at least in promastigotes.

EXPERIMENTAL PROCEDURES

Materials, Chemicals, and Reagents—[14C]Glutamate (219 mM/µmol), [14C]putrescine (110 mM/µmol), and [14C]ornithine (53 mM/µmol) were purchased from Moravek Biochemicals Inc. (Brea, CA), whereas L-[guanido-14C]arginine (55 mM/µmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All restriction enzymes were bought from either Invitrogen Corp. or New Eng Lab. Biolabs, Inc. (Beverly, MA). Synthetic oligonucleotides were acquired from Invitrogen. Advantage HF 2 DNA polymerase was purchased from BD Bioscience, Taq polymerase was produced from either Invitrogen Corp. or New England Biolabs, Inc. (Cambridge, MA), and purchased from BD Bioscience, Pfu Turbo DNA polymerase was produced from either Invitrogen Corp. or New England Biolabs, Inc. (St. Louis, MO). All restriction enzymes were generously provided by Dr. Stephen M. Landfear (Oregon Health & Science University, Portland, OR). Parasites were cultivated in DME-L, a modified DME-L medium, prepared from Stratagene (La Jolla, CA), and purchased from BD Bioscience, Pfu Turbo DNA polymerase was acquired from either Invitrogen Corp. or New England Biolabs, Inc. (St. Louis, MO). All restriction enzymes were generously provided by Dr. Stephen M. Landfear (Oregon Health & Science University, Portland, OR). Parasites were cultivated in DME-L, a modified DME-L medium, prepared from Stratagene (La Jolla, CA), and purchased from BD Bioscience, Pfu Turbo DNA polymerase was performed at 570 and 600 nm on a Multitask Ascent plate reader (Thermo Labsystems, Vantaa, Finland). The percent reduction of dye was calculated according to a formula published in the manufacturer’s brochure. The greatest reduction was expressed as maximal concentration.

Construction of L. mexicana ARG—The arginase sequence from L. amazonensis (GenBank™ accession number AF038409) was used to design primers to amplify the ARG from L. mexicana genomic DNA using the PCR. Genomic DNA was isolated by standard protocols. The sense primer, 5′-GGGATCCATGGAAGCTGCAGTAAGTTC-3′, encompassed the initiation methionine codon (boldface type) and was preceded by a BamHI restriction site (underlined), whereas the antisense primer, 5′-TCTAGACTACAGCTTGGAGCTGTTGATCGGAG-3′, encoded the termination codon (boldface type) to which a BglIII site (underlined) was attached at the 5′-end. The arginase open reading frame (ORF) was amplified from 20 ng of L. mexicana DNA using a high fidelity polymerase (Advantage HF 2 DNA polymerase, BD Bioscience, Palo Alto, CA) and standard PCR conditions for amplifying sequence from genomic DNA (95°C for 5 min followed by 32 cycles of 95°C for 1 min, 55°C for 1 min, and 68°C for 2 min). The 1.0-kb-amplified DNA fragment was subcloned into the pCR® 2.1-TOPO™ vector of the TOPO TA Cloning™ kit (Invitrogen), and limited nucleotide sequencing confirmed the identity of the putative arginase fragment. The resulting plasmid was designated TOPO-arginase. The subcloned arginase fragment was then used to screen an L. mexicana cosmid library constructed by Dr. Scott M. Landfear (Oregon Health & Science University, Portland, OR) using high stringency conditions. Positive cosmids were subjected to two rounds of purification, and the cosmid DNA isolated by standard methods. The arginase locus within a positive cosmid was mapped by Southern blotting, and a ~7-kb BamHI fragment encompassing the arginase ORF was subcloned into pBlue-script (Stratagene, La Jolla, CA). The resulting plasmid was designated pBlue-script-arginase and submitted for nucleotide sequencing. The entire nucleotide sequences of the arginase ORF (both directions), and ~1 kb of each of the flanks was obtained.

Construction of pXG-GFP + 2′-ARG—To localize ARG, the gene was inserted into pXG-GFP + 2′, a leishmanial expression vector that conferred resistance to G418 and synthesized foreign protein as an NH2-terminus fusion with green fluorescent protein (GFP). The arginase ORF was excised from the TOPO TA-arginase vector with BamHI and ligated into the BamHI cut pXG-GFP + 2′ vector. Sequence analysis confirmed the correct orientation and reading frame of the arginase coding region. The resulting expression plasmid was designated pXG-GFP + 2′-arginase. This plasmid was then transfected into wild type L. mexicana using standard electroporation conditions (17, 18). Transfected parasites were selected in 20 µg/ml G418 and used for localization studies.

Fluorescence Microscopy—Lab-Tek® II Chambered Coverglass slides (Fisher Scientific) were coated with a 1:10 dilution of poly-L-lysine (Sigma-Aldrich Corp.) for 15 min. The chambers were rinsed with double-deionized water to remove excess poly-L-lysine and allowed to dry at room temperature. 1 × 105 Leishmania promastigotes were resuspended in 1.0 ml of phosphate-buffered saline (PBS), pipetted into the chamber, and allowed to attach for 15 min. Chambers were rinsed with 1.0 ml of PBS, and the parasites were stained with a Zeiss Axivert 200 inverted microscope and deconvolution performed using the constrained iterative method by Axiovision 3.1 software (Carl Zeiss Optical, Chesterfield, VA).

Immunofluorescence Microscopy—L. donovani promastigotes at a density of ~5 × 10^5/ml were pelleted by centrifugation at 3000 × g for 5 min and fixed with PBS supplemented with 4% paraformaldehyde and 0.1% glutaraldehyde for 30 min at room temperature. Parasites were then resuspended and incubated in a blocking solution consisting of 5% heat-inactivated goat serum (Gibco BRL), 0.1% Tween 20, and 0.1% Triton X-100, which also permeabilized the parasites, for 1 h at room temperature. Antibodies against the L. donovani ODC, SPDsyn, and ADOMETDC proteins (18–20) and purified antibodies to the L. donovani hypoxanthine-guanine phospho...
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Fig. 1. Multisequence alignment of phylogenetic diverse ARG proteins. A, ARG proteins were aligned according to the Feng-Doolittle algorithm (71). Sequences from L. mexicana ARG (LmARG), human ARG I (HsARG I), human ARG II (HsARG II), and Saccharomyces cerevisiae ARG (ScARG) are shown. Identical residues are highlighted in dark boxes, and similar amino acids are shown in shaded boxes. Residues that have been identified as strictly conserved among ARGs from different species are underlined. The PTS-1 of the L. mexicana ARG is indicated in boldface type, and the two amino acid differences between the L. mexicana and L. amazonensis ARG sequences are indicated by an asterisk. B, a restriction map of the arginase locus based on Southern blot analysis and nucleotide sequencing is depicted.

Ribosyltransferase (HGPRT) (35) were diluted 1:500 with blocking solution and incubated with the chamber slides to which wild type L. donovani (for ODC, SPDSSYN, and ADOMETDC localization) or L. mexicana transfected with pXG-GFP (for HGPRT detection) cells were affixed.

After incubation of fixed parasites with primary antibody for 1 h, all chamber slides were rinsed six times for 5 min with a PBS wash solution containing 0.1% Tween 20. Fixed cells were then incubated with secondary anti-rabbit antibody conjugated to Texas Red dye (Molecular Probes, Eugene, OR) that had been diluted 1:1000 in blocking solution containing 0.1% Tween 20. Fixed cells were then incubated with primary antibody for 1 h, all chamber slides were rinsed six times for 5 min with a PBS wash buffer. Chamber slides were washed in PBS and mounted with either Vectashield® mounting medium (Vector Laboratories, Inc., Burlingame, CA) or a solution of 50 m M Tris, pH 8.0, 8% n-propylgalact (Sigma-Aldrich Corp.), and 90% glycerol. Images shown in Fig. 2 (E and F) were obtained by Aurelie Snyder of the Oregon Health & Science University-Molecular Microbiology and Immunology Research Core Facility (www.ohsu.edu/core) with the Applied Precision Deltavision® image restoration system. Deconvolution was performed using the iterative constrained algorithm of Agard et al. (36), and additional image processing was performed on an Silicon Graphics Octane workstation. Images for Fig. 2 (A-D and G) were obtained by using a Zeiss Axiovert 200 inverted microscope and deconvolution accomplished using Axiovision 3.1 software (Carl Zeiss Optical, Chesterfield, VA).

Molecular Constructs for the Replacement of the Arginase Alleles—

The 5'- and 3'-flanking regions of the L. mexicana arginase ORF were identified from sequence analysis of the arginase locus and cloned into the appropriate sites within the pX63-HYG and pX63-PHLEO vectors. The 5'-flanking region was amplified by PCR with TopDNA polymerase (Promega Corp., Madison, WI), pBluescript-arginase as a template, the arginase script was amplified by PCR with the sense primer, 5'-TGCGCACACACAGATCTATATT-GTCGACCTTGCCAT-3', and the antisense primer, 5'-TAT-3'-GCAACTCCGAAACCAGATCTC-GTG-3', with SmaI and BglII to generate pX63-PHLEO-5'F that had been digested with SmaI, gel-purified, and ligated into SmaI-cut pX63-HYG and pX63-PHLEO vectors.

Because of the presence of a SmaI sites within the PHLEO coding region of pX63-PHLEO, a different cloning strategy was employed to insert the 3'-flanking region of arginase into pX63-PHLEO-5'F. A 720-bp PCR product was subcloned into the pCR® 2.1-TOPO® vector and excised with HindIII and SalI and inserted into pX63-HYG and pX63-PHLEO-5'F. To generate the 3' arginase flank for subcloning into pX63-HYG-5'F, a sense primer, 5'-GGGCTGTAAGCTTGGAGATACGCCCCGAGG-3', (HindIII site underlined), and the antisense primer, 5'-GGGATCCATACCTGACCTTTGCGACATG-3', (SalI site underlined). The resulting ~720-bp PCR product was first subcloned into the pCR® 2.1-TOPO® vector and then excised with HindIII and SalI and inserted into pX63-HYG and pX63-PHLEO-5'F that had been digested with HindIII and SalI. The resulting plasmids were designated pX63-HYG-5'F and pX63-PHLEO-5'F. To generate the 3' arginase flank for subcloning into pX63-HYG-5'F, a sense primer, 5'-GGGATCCATACCTGACCTTTGCGACATG-3', (SalI site underlined), and the antisense primer, 5'-GGGATCCATACCTGACCTTTGCGACATG-3', (SalI site underlined), were synthesized, and pBlue-Script-arginase was used as the template for PCR amplification. The ~500-bp PCR product was subcloned into the pCR® 2.1-TOPO® vector, excised with SmaI, gel-purified, and ligated into SmaI-cut pX63-HYG-5'F vector to generate the allele replacement vector pX63-HYG-Δarg.

The 5'- and 3'-flanking regions of arginase amplified by PCR using the sense primer, 5'-GGGATCCATACCTGACCTTTGCGACATG-3', and the antisense primer, 5'-GGGATCCATACCTGACCTTTGCGACATG-3', were cloned into pX63-PHLEO-5'F. A 1.0-kb sequence from the 3'-flanking region of arginase was amplified by PCR using the sense primer, 5'-TGCGCACACACAGATCTATATT-GTCGACCTTGCCAT-3', and the antisense primer, 5'-TAT-3'-GCAACTCCGAAACCAGATCTC-GTG-3', with BamHI and BglII to generate pX63-PHLEO-Δarg. The correct orientations of the 5'- and 3'-flanking regions within the gene-targeting
plasmids were confirmed by restriction mapping and limited nucleotide sequencing of the inserting sequence.

**Generation of Arginase Null Mutants**—The Δarg knockouts were generated by double-targeted gene replacement starting with wild type MNYC/BZ/62/M379 L. mexicana. pX63-HYG-Δarg and pX63-PHLEO-Δarg were digested with HindIII and BglII to liberate linear fragments, designated HYG-Δarg and PHLEO-Δarg, respectively, containing the drug resistance marker and the arginase flanking regions. HYG-Δarg and PHLEO-Δarg were isolated from agarose gels and then transfected into parasites using standard electroporation conditions for transfection of Leishmania promastigotes (17, 18). First, HYG-Δarg was transfected into wild type L. mexicana to create the arginase/Δarg heterozygote, and clones were isolated by selection on plates of semi-solid DME-L-CS medium containing 50 μg/ml hygromycin. The genotype of the arginase/Δarg heterozygote was then confirmed by Southern blot analysis using arginase flanking regions as probes. A heterozygous clone was then subjected to a second round of transfection with PHLEO-Δarg and Δarg null lines selected in liquid DME-L-CS media containing 50 μg/ml hygromycin, 50 μg/ml phleomycin, and 200 μM ornithine to circumvent potential ornithine auxotrophy. The Δarg genotype was verified by Southern blotting, and clones were isolated on semi-solid DME-L-CS plates containing 50 μg/ml hygromycin, 50 μg/ml phleomycin, and 200 μM ornithine. A total of 12 clones were picked and screened by Southern blotting using the arginase ORF as a probe. The gene replacements of two of the Δarg clones were evaluated in more detail by probing Southern blots with the arginase flanks.

**ARG Assays**—The ARG activities in wild type, knockout, and transfected parasites were determined using lysates prepared from 1 × 10^8 exponentially growing parasites that had been resuspended in 150 μl of 50 mM glycine/10 mM MnCl_2/1 mM dithiothreitol, pH 9.5. A protease inhibitor mixture (Roche Applied Science) was added, and the cells were homogenized by sonication for 10 s each. Protein concentrations in the resultant cell lysates were determined according to the Bradford method (37). ARG assays were initiated by the addition of 20 μl of parasite extract (2.5 mg of protein/ml) to an 80-μl reaction mixture containing 50 mM glycine/10 mM MnCl_2/1 mM dithiothreitol, pH 9.5, and 10 mM L-lysine-[^14]C]arginine (31.3 μCi/mmol). At various intervals, the enzyme reaction was terminated by the addition of 10-μl aliquots of the reaction mixture to 5 μl of glacial acetic acid. The [^14]C]urea product formed during the assay was detected by autoradiography and quantitated using a Beckman model LS 6500 liquid scintillation counter.

**Northern Blot Analysis**—The pXG-GFP-ΔargΔshl expression plasmid was linearized with the enzyme BglII and transfected into wild type L. mexicana parasites as reported (17, 18). Transfected parasites were selected in 20 μg/ml G418 in DME-L-CS medium supplemented with 200 μM putrescine and 200 μM ornithine. Transfectant lines harboring episomes encompassing the wild type and COOH-terminal deletion constructs were designated Δarg[pXG-arg-arginase] and Δarg[pXG-arginase-Δshl], respectively, according to the generally accepted genetic nomenclature for Leishmania (41).

**RESULTS**

**Isolation of the L. mexicana ARG**—The L. mexicana ARG was isolated from a cosmids library using a PCR product amplified from genomic DNA as a hybridization probe. Sequence analysis revealed a 987-bp ORF predicting a polypeptide of 329 amino acids and a molecular mass of ~36 kDa. The predicted amino acid sequence of the L. mexicana ARG exhibited 38.5%, 32.6%, and 55.8% amino acid sequence identities to the human ARG I, human ARG II, and Saccharomyces cerevisiae ARG proteins, respectively (Fig. 1A). The protein was virtually identical to its L. amazonensis equivalent, differing in only two amino acids proximal to the COOH terminus (V306D and C305R). A noteworthy feature of the leishmanial ARG protein is the deduced COOH-terminal tripeptide, Ser-Lys-Leu, the archetypal PTS-1 that can mediate the translocation of proteins into the glycosome (44–48). Southern blot analysis of the ARG locus revealed the gene to be single copy (data not shown).
and a restriction map was accordingly compiled (Fig. 1B).

Localization of ARG, ODC, ADOMETDC, and SPDSYN—To determine whether the *L. mexicana* ARG is indeed localized to the glycosomal compartment, a GFP-tagged arginase construct, pXG-GFP/H11001-arginase, was transfected into wild type *L. mexicana*. Analysis of the transfected line by direct fluorescence demonstrated a punctate staining pattern indicating ARG was constrained to an intracellular organelle (Fig. 2A). Co-localization experiments with antibodies against the *L. donovani* HGPRT, a known glycosomal marker (35), indicated that ARG and HGPRT inhabited the same subcellular milieu (Fig. 2, B and C). In contrast, immunofluorescence images obtained with *L. donovani* wild type parasites using monospecific antibodies to the ODC, ADOMETDC, and SPDSYN proteins (18–20) showed uniform diffuse staining patterns indicating that all three polyamine biosynthetic enzymes were localized to the *Leishmania* cytosol (Fig. 2, E–G).

Construction and Molecular Characterization of Δarg Knockouts—To evaluate the physiological role of ARG in intact *Leishmania*, each gene copy was sequentially replaced in *L. mexicana* with a drug resistance cassette lacking arginase coding sequences. The first arginase copy was eliminated with HYG/H9004-arg, and clonal arginase/arg heterozygotes were selected on plates containing 50 μg/ml hygromycin. The allelic replacement within the arginase/arg heterozygote was confirmed by Southern blotting (Fig. 3, A–C). A second round of transfection using the PHLEO-Δarg construct was then implemented to target the remaining wild type allele in the arginase/arg heterozygote so as to generate the Δarg knockout. Surprisingly, no parasites were recovered from semi-solid agar plates containing 50 μg/ml hygromycin and 50 μg/ml phleomycin, the selective agents for the drug resistance cassettes, and 200 μm ornithine. However, a batch culture of arginase/arg parasites transfected with PHLEO-Δarg, selected in liquid medium under identical conditions, was obtained. After confirming the loss of arginase in the liquid culture by Southern blotting, the uncloned population was plated on semi-solid media containing 50 μg/ml phleomycin, 50 μg/ml hygromycin, and 200 μm ornithine, and six colonies were selected for further analysis. The presence of hygromycin on the plates after the second round of transfection ensured that the PHLEO-Δarg construct had replaced the remaining wild type allele in the arginase/arg heterozygote (rather than eliminating the previously targeted allele), and ornithine was added to the selective medium to circumvent potential ornithine auxotrophy from arginase loss. After Southern blotting using the arginase ORF as a hybrid-
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Southern blot analyses of BamHI- and SacI-digested genomic DNA from wild type, arginase/arg, and Δarg2 and Δarg5 parasites hybridized to the arginase ORF revealed the expected ~5.0-kb band in the wild type and arginase/arg strains and clearly demonstrated the loss of all arginase coding sequences in the Δarg2 and Δarg5 null lines (Fig. 3A). Furthermore, blots hybridized to the arginase 3′ and 5′ flanking regions demonstrated the expected molecular rearrangements that occurred as a consequence of the homologous recombination events that gave rise to the heterozygous and homozygous knockout lines (Fig. 3, B and C). Replacement of a wild type arginase copy with either HYG-Δarg or PHLEO-Δarg created additional 3.6- and 3.4-kb SacI-BamHI fragments that hybridized to the 3′-flanking probe and extra 6.0-kb BamHI and 6.5-kb BamHI-SacI fragments that hybridized to the 5′-flank, respectively (Fig. 3, B and C). The loss of the remaining wild type arginase allele from the heterozygote is apparent after the second round of transfection with PHLEO-Δarg in both knockout lines. A schematic diagram of the expected sizes of the BamHI and SacI restriction fragments from the wild type and rearranged loci is also displayed (Fig. 3D).

ARG Activity Assays—To establish the phenotypic consequences of the gene replacements in Δarg parasites, ARG activities were measured in wild type, heterozygous, and knockout parasite extracts (Fig. 4). Whereas significant ARG activity was detected in both wild type and arginase/arg L. mexicana, the activity of ARG in the Δarg5 knockout was negligible. Somewhat surprisingly, the arginase/arg parasites had slightly higher ARG activity than their parental wild type strain, a trend that was consistently observed in several independent experiments.

Nutritional Requirements of the Δarg Null Mutants—To establish whether the Δarg knockouts required nutritional supplements to survive and proliferate, the abilities of wild type, arginase/arg, and Δarg L. mexicana to grow in medium lacking ornithine, the product of the ARG reaction, were evaluated. Although both wild type and arginase/arg heterozygotes grew at equivalent rates in unsupplemented medium, both the

Fig. 4. ARG activity in wild type and mutant parasites. The ARG activities in extracts of wild type (W), arginase/arg heterozygotes (△), and Δarg5 (■) parasites are portrayed. Values reported are those of triplicate samples, and the experimental procedure was repeated at least three times with essentially identical outcomes.

Fig. 5. Growth phenotype of genetically altered parasites. A, the abilities of wild type (○), arginase/arg (■), Δarg2 (△), and Δarg5 (◇) parasites to proliferate in DME-L-CS medium without ornithine or putrescine supplementation are shown. B, Δarg5 parasites were incubated in media containing either 200 μM ornithine (△), 500 μM ornithine (◇), 200 μM putrescine (○), or 200 μM ornithine plus 200 μM putrescine (●). Growth of arginase/arg heterozygotes (■) in media without supplementation is shown as the control. The inset depicts the long-term growth of the Δarg5 over 2 weeks. C, the Δarg5 parasites were incubated in various concentrations of ornithine (■), proline (△), glutamate (◇), ornithine plus proline (○), or ornithine plus glutamate (●). Parasite proliferation was evaluated after 5 days by measuring the ability of parasites to metabolize the dye alamarBlue™. The greatest reduction was expressed as 100% proliferation. D, Δarg5 parasites were grown in the presence of different levels of putrescine (■). E, Δarg5 parasites were grown in the presence of 200 μM putrescine (△), 1 mM spermidine (◇), 500 μM spermidine (○), or 200 μM spermidine (●). All growth experiments were performed at least three times with similar outcomes.
was repeated on two additional occasions with similar results. The experiment was measured in duplicate over a 5-min timeframe. The experiment of these two nonessential amino acids in mammalian cells (49), the abilities of glutamate and proline to rescue the Δarg knockouts was a differential capacity of the parasites to take up the two arginine downstream products. To test this conjecture, the ability of L. mexicana to take up the two positively charged compounds, each at a concentration of 20 μM was compared. At these ligand concentrations, the overall rate of putrescine uptake was ~2-fold greater than that of ornithine (Fig. 6A). The rates of putrescine and ornithine uptake into intact parasites were also compared as a function of ligand concentration (Fig. 6B). Apparent Kₘ values for putrescine and ornithine uptake were 33.5 ± 2.5 and 42.2 ± 8.8 μM, and V_max values were 373.2 ± 6.6 and 316.6 ± 14.4 nmol/min/10⁷ parasites, respectively.

Amino Acid, Polyamine, AdoMet, and dAdoMet Pool Analyses—To evaluate the metabolic consequences of an ARG deficiency in L. donovani promastigotes, ornithine and arginine pools were compared in wild type parasites and Δarg knockouts grown in the absence or presence of either putrescine or ornithine. Ornithine and arginine pools in wild type promastigotes were 130 and 57 nmol/10⁷ parasites, respectively (Fig. 7A). As expected, no ornithine could be detected in the Δarg null mutant grown in 200 μM putrescine, whereas arginine pools were 2-fold greater than in wild type parasites. Intracellular ornithine could be observed in knockouts supplemented with 200 μM or 1 mM ornithine, although the pools were much lower than those detected in wild type promastigotes. Low levels of arginine and ornithine were found in Δarg cells incubated without putrescine or ornithine for 4 days, although it should be noted that these cells were nutritionally compromised and not proliferating. Polyamine pools were also determined for these parasites. Although wild type parasites contained equivalent concentrations of both putrescine and spermidine, the knockouts grown in either 200 μM putrescine or 1 mM ornithine exhibited relatively low putrescine pools, whereas spermidine levels were similar to those in wild type parasites (Fig. 7B). Both polyamines were found at low levels in un-supplemented Δarg knockouts or Δarg parasites grown in 200 μM ornithine. Finally, AdoMet and dAdoMet pools were measured. AdoMet levels were not significantly different in the null mutants grown in either putrescine or ornithine than in wild type parasites, although dAdoMet levels were somewhat elevated over wild type pools (Fig. 7C). Un-supplemented Δarg cells displayed a low AdoMet content, but dAdoMet concentrations were still higher than those found in the wild type line.

Mislocalization Does Not Affect ARG Function in L. mexicana Promastigotes—The glycosomal localization of the L. mexicana ARG was established above using direct fluorescence (Fig. 2). To determine whether the glycosomal milieu is crucial to ARG function, the Δarg knockout was transfected with a mutant GFP-tagged arg construct, GFP-argΔski, in which the PTS-1 Δarg2 and Δarg5 knockout clones failed to thrive (Fig. 5A). In the absence of supplementation, the homozygous null mutants divided ~2–3 times after which they assumed a rounded morphology, arrested growth, and died within 2 weeks (data not shown).

To determine the nutritional requirements of the Δarg knockouts, parasites were initially incubated with 200 and 500 μM ornithine. These concentrations of ornithine enabled survival and restored the ability of the Δarg knockouts to multiply, although the growth rate was much slower than that exhibited by either wild type or arginase/arg knockout parasites (Fig. 5B). Indeed, maximal growth rate of the homogygous Δarg null mutants could only be rescued by concentrations of ornithine > 1.0 mM (Fig. 5C).

Because ornithine, the product of the ARG reaction, is both a precursor of glutamate and proline synthesis and also a metabolic product of these two nonessential amino acids in mammalian cells (49), the abilities of glutamate and proline to rescue the lethal Δarg mutation were evaluated. Neither glutamate nor proline, at concentrations up to 5 mM, could rescue the Δarg conditionally lethal phenotype or increase the rate of growth of the parasites in the absence or presence of equimolar quantities of ornithine (Fig. 5C).

Because ornithine is the immediate polyamine precursor, the effect of exogenous polyamine on the Δarg growth phenotype was also evaluated. Surprisingly, putrescine at a concentration of 200 μM, regardless of whether ornithine was present, was much more effective than equivalent concentrations of ornithine in enabling optimal growth of the Δarg parasites (Fig. 5B). Because the Δarg knockouts grew much better in 200 μM putrescine compared with the same concentration of ornithine, the levels of putrescine and ornithine that were required for optimal proliferation of Δarg parasites were determined (Fig. 5, C and D). Although a concentration of >1.0 mM ornithine was required to effect maximum parasite proliferation, optimal growth of Δarg parasites was achieved at 5–10 μM exogenous putrescine. Spermidine could also rescue the Δarg parasites (Fig. 5E). The concentrations of spermidine required to enable Δarg growth, however, were much higher than those needed for putrescine rescue.
was deleted. The \( \Delta \text{arg}[\text{pGFP-ARG}] \) and \( \Delta \text{arg}[\text{pGFP-arg\_skl}] \) transfectants expressed similar levels of ARG/arg protein as judged by Western blotting with anti-GFP antibody, as well as comparable levels of ARG activity (Fig. 8, A and B). The mislocalization of the GFP-arg\_skl fusion protein to the cytosol was then confirmed by direct fluorescence microscopy (Fig. 9).

Regardless of the location of the ARG/arg activity in the \( \Delta \text{arg} \) transfectants, both the \( \Delta \text{arg}[\text{pGFP-ARG}] \) and \( \Delta \text{arg}[\text{pGFP-arg\_skl}] \) lines were capable of growing in growth medium lacking ornithine and putrescine supplementation (Fig. 8C).

**DISCUSSION**

The creation and characterization of \( \Delta \text{arg} \) parasites by double-targeted gene replacement established that arginase is an essential gene in \( L. \text{mexicana} \) promastigotes. The \( \Delta \text{arg} \) line exhibited polyamine auxotrophy, and this auxotrophy could be
pharmacologically rescued by either putrescine, spermidine, or
ornithine supplementation or complemented with an episomal
copy of arginase. The ability of exogenous putrescine or sper-
midine to circumvent the lethality of the Δarg null mutation
demonstrates that the sole vital function of ARG, at least in
Leishmania promastigotes, is to provide the ornithine precur-
sor for polyamine synthesis. Whether ARG plays supplementary
but nonessential metabolic roles in promastigotes or has
additional vital functions in amastigotes remains to be
determined.

In mammalian cells, ornithine is also both a precursor and
product of proline and glutamate metabolism through a pyro-
line 5-carboxylate intermediate (49). Neither proline nor glu-
tamate, both of which are absent from DME-L medium and are
considered nonessential amino acids in Leishmania (50), how-
ever, affected the conditionally lethal growth phenotype of the
Δarg parasites in the absence or presence of ornithine. The
failure of proline or glutamate to promote growth of the Δarg
knockouts cannot be imputed to lack of amino acid entry into
the parasite, because both proline (51, 52) and glutamate (data
not shown) are efficiently taken up by Leishmania promasti-
gotes. Thus, the inability of proline and glutamate at 5 mM
concentrations to rescue the Δarg knockouts demonstrates that
ARG deficiency does not cause proline or glutamate auxotro-
phy. The fact that low concentrations of putrescine can com-
pletely rescue the conditionally lethal Δarg mutation estab-
lishes that ornithine is an unlikely precursor of either
nonessential amino acid. Moreover, it appears that neither
proline nor glutamate can substantially contribute to the orni-
theine pool for polyamine synthesis. Consistent with this latter
premise is a report that radiolabeled proline is not converted to
ornithine by L. donovani promastigotes (50). Cumulatively,
these data strongly support a singular function for ARG in
Leishmania promastigotes, that of supplying a source of
polyamines.

That the concentration of putrescine required to fully rescue
the growth impairment of the Δarg lesion was two orders of
magnitude lower than that of ornithine, the product of the ARG
reaction remains somewhat mysterious. This ~100-fold differ-
ence could not be ascribed to differential uptake rates of the
two compounds, because the rates of putrescine incorporation
were only ~2-fold greater than that of ornithine (Fig. 6). These
uptake experiments were performed in parallel at 20 μM li-
gand, a concentration of ornithine that does not enable growth
of the Δarg knockout but a concentration of putrescine that
fully circumvents the conditionally lethal mutation (Fig. 5).
The kinetic parameters, i.e. K_m and V_max values for ornithine
and putrescine uptake into intact L. mexicana promastigotes
were also similar (Fig. 6), although it should be noted that the
kinetic parameters for the uptake of either dibasic ligand may
reflect more than one transport system. However, pool meas-
urements revealed that ornithine levels in Δarg parasites prop-
agated in either 200 μM or 1 mM ornithine were only 1% and
15% of those in wild type cells with a concomitant diminution of
the putrescine pools (Fig. 7, A and B). These data imply that
ODC activity is reduced in Δarg parasites grown in ornithine.
Using the experimentally determined value for cell volume of a
Leishmania promastigote (53), ~11 fl per cell, the intracellular
ornithine level in Δarg parasites grown at suboptimal con-
centration of 200 μM is still ~12 mM, a value much greater than
the K_m value of 0.42 mM calculated for the L. donovani ODC
enzyme (54). Thus, the reduced apparent flux through ODC in
the Δarg cells cannot be explained by the low levels of ornithine
alone. It is possible that subcellular sequestering of the orni-
theine and putrescine pools in the parasite can somehow
contribute to the drastic differences observed for the amounts
of ornithine and putrescine that rescue the Δarg phenotype.

Interestingly, spermidine levels in Δarg cells grown in 1 mM
ornithine were normal, although they were markedly reduced in
Δarg parasites grown in 200 μM exogenous ornithine, a
suboptimal concentration for growth (Fig. 7B). Coupled with
the observation that Δarg parasites in 200 μM putrescine pro-
liferate rapidly and demonstrate normal spermidine but dimin-
ished intracellular putrescine pools, these data support the
previously drawn conclusion that spermidine is the vital poly-
amine for parasite viability and vitality (18–20). It is notewor-
thy that incubation of the Δarg mutants in ornithine or putres-
cine concentrations that allowed optimal proliferation did not
restore either corresponding intracellular pool to wild type
levels.
Arginase Knockouts in Leishmania mexicana

The ability to create a conditionally lethal phenotype at the arginase locus demonstrates that there exists only a single ARG activity in L. mexicana promastigotes. In contrast, mammalian cells express two genetically and biochemically distinct ARG isoforms, ARG I, a cytosolic enzyme that participates in the urea cycle, and ARG II, a mitochondrial protein that is speculated to be involved in proline, glutamate, and polyamine biosynthesis (49). The L. mexicana ARG appears to play a functional role more similar to ARG II, although the parasite protein exhibits a greater amino acid sequence identity to human ARG I than to ARG II (Fig. 1). Leishmania do not appear to express many of the urea cycle enzymes (55), and no sequences encoding urea cycle enzymes, other than for ARG and carbamoyl phosphate synthetase, an isofrom of which is required for pyrimidine biosynthesis, have been deposited in the emerging leishmanial genome sequencing data base. Skeletal muscle and several other tissues also accommodate yet another protein that converts arginine to ornithine, arginine-glycine amidinotransferase, an enzyme involved in creatine biosynthesis (56). Clearly, the conditionally lethal nature of the ARG knockouts establishes that Leishmania promastigotes have only a single avenue for ornithine biosynthesis. Interestingly, Trypanosoma cruzi, the causative agent of Chagas’ disease and a close evolutionary relative of Leishmania, appears to lack ARG (55). T. cruzi also lacks ODC (57) activity and is, therefore, an obligatory scavenger of host polyamines.

The L. mexicana ARG exhibits considerable homology to ARG enzymes from phylogenetically diverse organisms and contains all ten invariant sequences that are present in 21 different ARG proteins from phylogenetically diverse species (58, 59). According to the recently determined crystal structure of the rat ARG, most of these conserved residues are involved either directly in divalent cation coordination or in positioning other highly conserved residues for interaction with Mn2+ (60). The most striking structural feature of the L. mexicana ARG is the COOH-terminal tripeptide, Ser-Lys-Leu, which has now been genetically verified as the PTS-1 that serves as the topogenic signal for targeting ARG to the glycosome. No equivalent PTS-1 sequence is observed among 31 ARG family members that have been analyzed in detail (58, 59). Moreover, the L. donovani ODC (61), ADOMETDC (20), and SPDPSYN (19) polypeptides do not accommodate a PTS-1, and all three enzymes have now been immunolocalized to the cytosol (Fig. 2). Thus, the enzymes involved in the synthesis of polyamines, like those for purine and pyrimidine nucleotide synthesis (35, 62–64), are compartmentalized between the glycosome and cytosol in Leishmania. The rationale for the sequestering of ARG from the cytosolic ODC, SPDPSYN, and ADOMETDC is not apparent, but the glycosomal milieu is not essential for ARG function in promastigotes because of the ability of cytosolic arg (argashl) to complement ARG parasites (Fig. 8). Interestingly, arginine, the substrate of ARG, is an essential amino acid in Leishmania promastigotes (50). Thus, the polypeptide pathway in Leishmania promastigotes takes a rather circuitous route, originating with external arginine, which is then translocated across the parasite membrane through some yet undefined permeation mechanism, through the cytosol to the glycosome, after which the ornithine product of ARG is then released back into the cytosol for conversion to putrescine and spermidine, the two polyamines found in Leishmania (18–20, 26, 65, 66).

Although the polyamine auxotrophy of the ARG knockouts indicates that Leishmania only accommodate a single ARG activity, there is a second gene within the L. major genome database (AL121851) that encodes an ARG family member, one that exhibits high sequence homology with agmatinase (AGM) enzymes. Of note, this putative agmatinase sequence lacks an obvious PTS-1. AGM is present in prokaryotes and plants and is part of an alternative pathway by which polyamines are produced from arginine in these organisms. In the AGM pathway, arginine is decarboxylated by arginine decarboxylase to produce agmatine, which is then hydrolyzed to putrescine with the resultant liberation of a urea molecule. Recently, AGM has also been identified in mammalian cells (67, 68), although the existence of an arginine decarboxylase is still unresolved. The AGM pathway does not appear to contribute to the putrescine pool in Leishmania promastigotes, because Δodc (18) and Δarg promastigotes are putrescine auxotrophs. Furthermore, although ARG and AGM catalyze similar reactions and belong to the same enzyme superfamily (about 24% identity), the putative AGM does not appear to be a functional ARG, because the Δarg parasites displayed no residual ARG activity. The putative L. mexicana AGM has been recently cloned in this laboratory to assess its biochemical and physiological function in vitro and in vivo.

These genetic studies have demonstrated that ARG is an essential protein in Leishmania promastigotes. The existence of ARG inhibitors that can pharmacologically replicate a genetic deficiency in the enzyme, the unusual subcellular milieu of ARG, and the previously established biochemical disparities between the mammalian and leishmanial polyamine biosynthesis pathways, also imply that ARG could be a potential target for therapeutic manipulation of certain parasitic diseases. In the future, it will be critical to evaluate the phenotypic consequences of an ARG deficiency on the amastigote form of the parasite. Because these studies were undertaken in L. mexicana, an infectious strain (69, 70), infectivity studies in macrophages can now be initiated to determine the physiological role of ARG protein in the infectious form of the parasite. These studies will ultimately answer the fundamental issue of whether amastigotes depend upon endogenous polyamine synthesis or are able to scavenge polyamines within the phagolysosome where they reside.

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