Genetic Analysis of Purine Metabolism in Leishmania donovani*

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To dissect the contributions of hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenine phosphoribosyltransferase (APRT), and adenosine kinase (AK) to purine salvage in Leishmania donovani, null mutants genetically deficient in HGPRT and/or APRT were generated by targeted gene replacement in wild type cells and preexisting mutant strains lacking either APRT or AK activity. These knockouts were obtained either by double targeted gene replacement or by single gene replacement followed by negative selection for loss-of-heterozygosity. Genotypes were confirmed by Southern blotting and the resultant phenotypes evaluated by enzymatic assay, resistance to cytotoxic drugs, ability to incorporate radiolabeled purine bases, and growth on various purine sources. All mutant strains could propagate in defined growth medium containing any single purine source and could metabolize exogenous [3H]hypoxanthine to the nucleotide level. The surprising ability of mutant L. donovani lacking HGPRT, APRT, and/or AK to incorporate and grow in hypoxanthine could be attributed to the ability of the parasite xanthine phosphoribosyltransferase enzyme to salvage hypoxanthine. These genetic studies indicate that HGPRT, APRT, and AK, individually or in any combination, are not essential for the survival and growth of the promastigote stage of L. donovani and intimate an important, if not crucial, role for xanthine phosphoribosyltransferase in purine salvage.

Protozoan parasites cause a variety of devastating and often fatal diseases in humans and their domestic animals. The treatment and control of parasitic diseases, however, is severely compromised by the dearth of effective and selective antiparasitic therapies. Many of the current antiparasitic drugs cause severe toxicity in the host, a predilection that can be attributed to lack of target specificity. Moreover, these drugs are potentially mutagenic and/or carcinogenic, they often require protracted courses with multiple drug administrations, and therapeutic unresponsiveness and drug resistance have exacerbated the necessity for new and improved antiparasitic agents.

The institution of a rational therapeutic regimen for the treatment and prevention of parasitic diseases hinges upon exploitation of fundamental biochemical disparities between parasite and host. Perhaps the most striking metabolic discrepancy between parasites and humans is the purine pathway. Whereas mammalian cells can synthesize the purine heterocycle de novo, all protozoan parasites studied thus far are auxotrophic for purines (1). As a consequence, each genus of parasite has evolved a unique complement of purine salvage enzymes that enable the organism to scavenge host purines. Unique features of the purine salvage pathway of Leishmania and Trypanosoma constitute the basis for the susceptibility of these genera to several pyrazolopyrimidine analogs of naturally occurring purine bases and nucleosides (2, 3). The intact parasites efficiently metabolize these analogs to the nucleotide level, whereas mammalian cells are essentially incapable of these metabolic transformations. One of these pyrazolopyrimidines, allopurinol (4-hydroxypyrazolo[3,4]pyrimidine, HPP),1 a drug that is nontoxic to humans and is widely used in the treatment of hyperuricemia and gout (4), has demonstrated significant therapeutic efficacy in patients with either cutaneous leishmaniasis (5) or chronic Chagas disease (6).

Leishmania donovani, the causative agent of visceral leishmaniasis, is a digenetic parasite that exists as an extracellular promastigote within the insect vector, members of the phlebotomine sandfly family, and as a nonmotile intracellular amastigote within the phagolysosome of macrophages and other cells of the reticuloendothelial system of the mammalian host. L. donovani expresses a number of enzymes capable of converting preformed purines directly to nucleotides. These enzymes include hypoxanthine-guanine phosphoribosyltransferase (HGPRT; IMP:pyrophosphate phosphoribosyltransferase; EC 2.4.2.8), adenine phosphoribosyltransferase (APRT; AMP:pyrophosphate phosphoribosyltransferase; EC 2.4.2.7), xanthine phosphoribosyltransferase (XPR; XMP:pyrophosphate phosphoribosyltransferase; EC 2.4.2.22), and adenosine kinase (AK). Leishmania also contain a plethora of purine interconversion enzymes including nucleosidases, phosphorylases, deaminases, and IMP branchpoint enzymes (1). Overall, the purine pathway is divagating and intricate, and this metabolic complexity and the apparent overall diploid nature of the parasite (7–9) has hindered a thorough characterization of the purine pathway in the parasite by straightforward biochemical and genetic approaches.

The ability of Leishmania to undergo a high rate of homologous gene replacement (7, 10–12) and to take up foreign DNAs after transfection (13, 14) now permits an assessment of specific gene function by targeted gene replacement and facilitates the genetic dissection of complex metabolic pathways such as that for purine acquisition. As both the HGPRT (15) and APRT (16) genes and their flanking regions have been isolated, we have examined the relative contributions of HGPRT, APRT,

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1 The abbreviations used are: HPP, allopurinol; AK, adenosine kinase; APP, 4-aminopyrazolopyrimidine; APRT, adenine phosphoribosyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; HGXPRT, hypoxanthine-guanine-xanthine phosphoribosyltransferase; kb, kilobase pair(s); LOH, loss-of-heterozygosity; PCR, polymerase chain reaction; PRT, phosphoribosyltransferase; XPR, xanthine phosphoribosyltransferase.
and AK to purine salvage in \textit{L. donovani} promastigotes by sequentially eliminating each HGPRT and APRT allele from wild type cells and from preexisting mutants lacking either APRT (8) or AK (17) activity by homologous gene replacement and/or direct selection. These strains were created either by replacing each allele sequentially with independent drug resistance markers or by disrupting the first allele with one drug resistance marker and selecting for loss-of-heterozygosity (LOH) with drug pressure to obtain the homozygous null mutant. The new alleles in the heterozygotes and homoyzogotes were examined by Southern blotting, and the resultant phenotypes of the mutant strains have been evaluated for enzymatic activities, ability to take up purines, drug resistance profiles, and growth properties.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—[\textsuperscript{3}H]Hypoxanthine, [\textsuperscript{3}H]Citidine, [\textsuperscript{3}H]Adenine, and [\textsuperscript{3}H]Xanthine, all at 56 mCi/mmol, and [\textsuperscript{3}H]Adenosine at 32.5 mCi/mmol were obtained from Moravek Biochemicals (Brea, CA). HPLC grade [\textsuperscript{3}H]dCTP (3000 Ci/mmol) and [\textsuperscript{3}H]dATP (1320 Ci/mmol) were bought from Du Pont-NEN. All restriction and DNA modifying enzymes were acquired from either New England Biolabs, Inc. or Life Technologies, Inc., and Thermus flavus DNA polymerase was purchased from Epicentre Technologies (Madison, WI). The pX63-NEO and pX63-HYG drug resistance cassettes were furnished by Dr. Stephen Beverley (Harvard Medical School). The sources of all other chemicals and reagents were of the highest quality commercially available.

**Cell Culture**—Promastigotes of the Sudanese 1S strain of \textit{L. donovani} were grown in DME-L culture medium (18). Unless otherwise specified, DME-L contains 100 \mu M xanthine as a purine source. Clonal lines of \textit{L. donovani} were isolated as colonies on semi-solid DME-L medium containing appropriate selective agent as indicated (18). Initial recipient strains for the transfection experiments reported here included the wild type DI700 line and its two clonal derivatives, TUBA2 and APPB2A3 cells, as the potential loci for the complete genetic basis for the APRT deficiency in this strain has not been characterized. The \textit{HGPRT} activity in \textit{L. donovani} promastigotes was measured by the method described previously (17). To obtain parasites in which the wild type allele in the heterozygous recipient had been specifically disrupted by homologous recombination of the second targeting construct. The second protocol for selecting homozygous null mutants at either the HGPRT or APRT loci was to select for LOH by plating in semi-solid agarose to which a toxic substrate of the encoded gene product had been added. Specific selective conditions were 1–3 mM HPP to obtain \textit{Δaprt} clones and 100 \mu M APP to obtain \textit{Δaprt} clones. Selections for LOH at the HGPRT and APRT loci included G418 and/or hygromycin, as specified, in the medium to retain drug resistance cassettes that had been previously integrated at the appropriate locus/loci by targeted gene replacement.

All \textit{L. donovani} transformants that had integrated copies of either \textit{X63-HYG} or \textit{NEO-Δaprt} into the relevant loci were maintained continuously under selective pressure in the drugs for which they contained resistance markers. In addition, knockout cell lines created by single targeted gene replacement as described above were also grown perpetually in the agent in which they were selected, i.e. 2 mM HPP or 100 \mu M APP as appropriate.

**Enzyme Assays**—Exponentially growing \textit{L. donovani} were harvested in \textit{DME-L} medium, 5 mM MgCl\textsubscript{2}, 1 mM dithiothreitol and lysed either by sonication or by three rounds of freeze-thawing. HGPRT (22), APRT (22), and AK (17) activities were measured in the supernatants as reported.

**Radiolabel Incorporation Assays**—The rate of \textsuperscript{[3]H]}hypoxanthine incorporation into phosphorylated metabolites in the absence or presence of excess nonradiolabeled purines was ascertained at room temperature using a slight modification of the DE-52 filter disk method outlined by Iovannisci et al. (17). \textsuperscript{10} \mu M parasites were harvested by centrifugation, washed with phosphate-buffered saline, and resuspended in 1.0 ml of modified DME-L growth medium lacking the hemin and xanthine components prior to the addition of radiolabel and purine additives. [\textsuperscript{3}H]Hypoxanthine and nonradiolabeled purines were present at 1.8 \mu M and 100 \mu M, respectively, in the uptake assays. The assay was terminated by removing 100-\mu l aliquots of (10\textsuperscript{5}) cells, diluting them in ice cold phosphate-buffered saline, and subjecting them to centrifugation. The pelleted cells were washed twice in 1.0 ml of phosphate-buffered saline, lysed in 1% Triton X-100, and blotted onto a 1.5-cm\textsuperscript{2} piece of DE-51 impregnated paper. The disks were washed as described (17).

**RESULTS**

**Derivation of Mutant Strains by Gene Replacement**—A flow chart describing the derivation and lineages of all of the mutant strains that were created as a result of targeted gene replacement and/or direct selection in cytotoxic substrates is depicted in Fig. 1. To obtain \textit{Δaprt} clones, the three parental cell lines were isolated on agarose gels, and purified using the GeneClean Kit (Intermountain Scientific Corp., Bountiful, UT). Designation of the targeting DNAs originated from the plasmid nomenclature without the initial letter, e.g. X63-HYG-\textit{Δaprt} from pX63-HYG-\textit{Δaprt}. All transfected cells were grown for 24 h under nonselective conditions prior to the initiation of drug pressure.

**Gene Replacement**—Heterozygous loci were disrupted by the polymerase chain reaction (PCR) have been reported previously (21). The construction and authentication of the pX63-NEO-\textit{Δaprt} and pX63-HYG-\textit{Δaprt} plasmids employed in the allelic replacements of the HGPRT and APRT loci, respectively, have also been described (21). The pX63-NEO-\textit{Δaprt} contains a 1.7-kb 5' and a 1.8-kb 3' flanking region from the \textit{L. donovani} \textit{HGPRT} encoding the neomycin phosphotransferase (\textit{NEO}) gene, while the pX63-HYG-\textit{Δaprt} includes a 1.4-kb 5' and a 1.5-kb 3' flank of the \textit{L. donovani} APRT circumscribing the hygromycin phosphotransferase (\textit{HYG}) marker. To generate a \textit{HYG} construct for replacing the HGPRT locus, the 1.7-kb 5' and 1.8-kb 3' flanks were employed for making pX63-NEO-\textit{Δaprt} were reamplified using the PCR conditions described by Hwang et al. (21) and ligated into the appropriate restriction sites within pX63-HYG. The pX63-HYG-\textit{Δaprt} construct was verified for identity and orientation by limited restriction mapping and nucleotide sequencing.

**Transfection**—Parasites were transfected using electroporation conditions reported previously (11, 21). All targeting constructs were cleaved from their plasmids by digestion with HindIII and BglII just prior to electroporation, incubated on ice, and purified using the GeneClean Kit (Intermountain Scientific Corp., Bountiful, UT). Designation of the targeting DNAs originated from the plasmid nomenclature without the initial letter, e.g. X63-HYG-\textit{Δaprt} from pX63-HYG-\textit{Δaprt}. All transfected cells were grown for 24 h under nonselective conditions prior to the initiation of drug pressure.

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single wild type \( HGPRT \) allele was disrupted by targeted gene replacement approaches were obtained from DI700 (DI700:H\(^{+/+}\) \( A^{+/+} \)) and TUBA2 (TUBA2:H\(^{+/+}\) \( A^{+/+} \)) cells with X63-NEO-\(\Delta \)hgprt as the targeting vector and from APPB2A3 (APPB2A3: \( H^{+/+} \)) cells with X63-HYG-\(\Delta \)hgprt. Homozygous null mutants (\(\Delta \)hgprt) were then obtained from the three hgprt/\( HGPRT \) lines by two independent strategies, either by targeting with the appropriate construct containing the second drug resistance marker (DI700:H\(^{+/+}\) \( A^{+/+} \), TUBA2:H\(^{+/+}\) \( A^{+/+} \), and APPB2A3:H\(^{+/+}\)) or by direct negative selection (DI700: \( H^{+/+}\) \( A^{+/+} \) and TUBA2:H\(^{+/+}\) \( A^{+/+} \)) for LOH (see Fig. 1). Nomenclature for all the \( \Delta \)hgprt cell lines shown in Fig. 1 conforms to that employed for the heterozygotes.

The ability to generate \( \Delta \)hgprt null mutants after only a single round of transfection permitted the creation of the \( L. \) donovani cell lines in which both wild type alleles of the \( HGPRT \) and \( APRT \) loci had been displaced, each with a single targeting construct. Cell lines heterozygous at the \( APRT \) locus (aprt \( \)/ \( APRT \)) were thus created from the DI700:H\(^{+/+}\) \( A^{+/+} \) and TUBA2:H\(^{+/+}\) \( A^{+/+} \) cell lines, as well as from the parental TUBA2:H\(^{+/+}\) \( A^{+/+} \) strain by targeted gene replacement using the X63-HYG-\( \Delta \)aprt construct. These derivatives were designated DI700:H\(^{+/+}\) \( A^{+/+} \), TUBA2:H\(^{+/+}\) \( A^{+/+} \), and TUBA2: H\(^{+/+}\) \( A^{+/+} \), respectively (Fig. 1). \( \Delta \)aprt clonal progeny, DI700: H\(^{+/+}\) \( A^{+/+} \), TUBA2:H\(^{+/+}\) \( A^{+/+} \), and TUBA2:H\(^{+/+}\) \( A^{+/+} \), were then selected for LOH from the three aprt/\( APRT \) heterozygotes in 100 \( \mu \)M APP (Fig. 1).

**Southern Blot Analysis**—Southern blot analysis verified the existence of the new alleles that had been created after either double or single targeted gene replacement. The maps of the genomic loci for \( HGPRT \) and \( APRT \) and the novel alleles created by homologous recombination of X63-NEO-\( \Delta \)hgprt into the \( HGPRT \) locus and X63-HYG-\( \Delta \)aprt into the \( APRT \) locus have been reported previously (21). The X63-HYG-\( \Delta \)hgprt construct and its rearranged allele after insertion into the chromosome are displayed in Fig. 2. The maps of the \( HGPRT \) and \( APRT \) loci are also included in Fig. 2 to show the location of the probes employed in the Southern blotting experiments. The expected size differences of the wild type and disrupted \( HGPRT \) alleles in strains that were generated or employed for these studies can be visualized in Fig. 3. The interrupted \( HGPRT \) alleles could be distinguished from the wild type allele by an altered EcoRI restriction pattern (Fig. 3). After cleavage of genomic DNA with EcoRI and hybridization to probe B derived from the 3’-flanking region of \( HGPRT \) (see Fig. 2), only the anticipated 4.6-kb EcoRI fragment from the wild type allele was discerned in the three parental, i.e., DI700:H\(^{+/+}\) \( A^{+/+} \), APPB2A3:H\(^{+/+} \), and TUBA2:H\(^{+/+}\) \( A^{+/+} \), strains in which the \( HGPRT \) locus was intact (see Fig. 3, probe B). Although the \( HGPRT \) was originally isolated as a 3.5-kb EcoRI fragment (see “Experimental Procedures”), the 4.6-kb EcoRI band is the expected size of the wild type \( HGPRT \) allele, as the 3’ EcoRI restriction site of the cloned 3.5-kb fragment originates from the cosmids. In contrast, bands of 6.4 and 6.2 kb were observed in those strains in which an \( HGPRT \) allele had been targeted by either X63-NEO-\( \Delta \)hgprt or X63-HYG-\( \Delta \)hgprt, respectively (Fig. 3). Cell lines heterozygous for \( \text{HGPRT}^{+/+} \) or \( \text{HGPRT}^{-/-} \) retained the 4.6-kb EcoRI fragment derived from the wild type locus, while \( \text{HGPRT} \) null mutants (\( \text{HGPRT}^{-/-} \) or \( \text{HGPRT}^{+/+} \)) lacked the wild type 4.6-kb EcoRI restriction fragment and exhibited only the expected 6.4- and 6.2-kb displacements by X63-NEO-\( \Delta \)hgprt and X63-HYG-\( \Delta \)hgprt, respectively (Fig. 3, probe B). The interruption of the wild type \( HGPRT \) allele was verified by hybridization of genomic DNA to probe A corresponding to the coding region of \( HGPRT \) (see Fig. 2). The 4.6-kb EcoRI fragment was detected only in cell lines that were wild type or heterozygous at the \( HGPRT \) locus, i.e. the \( \text{HGPRT}^{+/+} \), \( \text{HGPRT}^{-/-} \), and \( \text{HGPRT}^{+/+} \) strains and not in \( \Delta \)hgprt null mutants that were derived by either double targeted (\( \text{HGPRT}^{+/+} \) or single targeted (\( \text{HGPRT}^{-/-} \)) gene replacement (Fig. 3, probe A).

It should be noted that after targeting wild type cells with X63-NEO-\( \Delta \)hgprt or X63-HYG-\( \Delta \)hgprt, 23 of 24 clones isolated and analyzed by Southern blotting displayed only simple gene replacements, whereas 12 of 15 colonies isolated from hgprt/ \( \text{HGPRT} \) heterozygotes targeted with X63-HYG-\( \Delta \)hgprt exhibited complex genetic events other than simple gene replacements. These enigmatic genetic alterations in the anomalous cell lines were not analyzed in detail, and the reasons for the large discrepancies in the frequency by which various genetic events were observed during each round of transfection is unknown. However, genetic events that do not involve simple allelic replacements after homologous recombination of an extrachromosomal fragment have been observed previously in *Leishmania* spp. (7, 12, 23).
targeted by X63-HYG-Daprt was also validated by Southern blot analysis (Fig. 4). aprt/APRT heterozygotes and Δaprt homozygous knockouts were only derived from the DI700 and TUBA2 lines (see Fig. 1), as APPB2A3 cells already lacked APRT activity (8). Digestion of genomic DNA with SalI and BamHI and hybridization to probe D derived from the 5’-flanking region of APRT (Fig. 2) revealed the presence of the 3.5-kb SalI/BamHI restriction fragment from the wild type allele in all A+/+ and A−/− cells (Fig. 4, probe D). The aprt/APRT heterozygotes contained an additional 1.4-kb SalI band (see Fig. 2) derived from the new allele created by integration of X63-HYG-Daprt into the APRT locus. Hybridization of DNA from all of the presumptive Δaprt null mutants to probe D revealed only the 1.4-kb band expected from the integration of the drug resistance cassette into both alleles of the APRT locus (Fig. 4, probe D). Genetic disruption of wild type APRT alleles was confirmed by hybridization of the same blot to probe C encompassing most of the APRT coding region (Fig. 4, probe C). APRT coding sequences existed only in cells containing one or two wild type APRT copies, whereas the knockout lines TUBA2:H+/+A−/− and TUBA2:H−/+A+/+ failed to hybridize to probe C (Fig. 4, probe C). The Southern blots of aprt/APRT and Δaprt derivatives of DI700 cells hybridized to probes C and D have been published previously (21); thus Southern blots of only ak TUBA2 and its derivatives are depicted in Fig. 4.

Enzyme Assays—The phenotypic consequences of HPRT and APRT replacements were evaluated in most cell lines by direct enzymatic assay. These data paralleled those already reported for the DI700:H+/+A+/+, DI700:H−/+A−/−, DI700:H+/+A+/+, DI700:H−/+A−/−, and DI700:H+/+A+/+ (21). A representative HPRT enzyme assay is presented for the DI700:H+/+A+/+, DI700:H−/+A−/−, DI700:H+/+A+/+, DI700:H−/+A−/−, TUBA2:H+/+A+/+, TUBA2:H−/+A−/−, TUBA2:H+/+A+/+, and APPB2A3:H+/+ cell lines in Fig. 5A. All null mutants created by single and double gene targeting strategies expressed extremely low activities of HPRT activity, as expected, whereas the DI700:H+/+A+/+ heterozygote expressed an intermediate level of HPRT activity as compared with the strains that are wild type at the HPRT locus. APRT deficiencies were established for the TUBA2:H−/+A+/+ and APPB2A3:H−/+ strains, whereas DI700:H+/+A−/− and TUBA2:H+/+A+/+ expressed equivalent wild type APRT activity (Fig. 5B). An absolute deficiency in APRT could not be established definitively in any cell line expressing HPRT activity because of the high rate of substrate deamination catalyzed by adenine deaminase activity present in Leishmania parasites (24). All TUBA2-derived
cell lines exhibited the expected AK deficiency of the TUBA2 clone (data not shown), while DI700 cells expressed 6.2 nmol of AK activity/min/mg of protein.

**Drug Resistance Phenotypes**—As *L. donovani* HGPRT is known to catalyze the phosphoribosylation of HPP (2, 3, 15), a potential “lead” compound for the treatment of leishmaniasis, the effects of HGPRT deficiency on HPP sensitivity were evaluated in a variety of cell lines. All parental cell lines exhibited an EC<sub>50</sub> (effective concentration of drug that inhibits growth by 50%) for HPP of <10 μM and all Δhgprt null mutants tested exhibited EC<sub>50</sub> values for HPP of >2 mM (data not shown). All Δhgprt lines exhibited cross-resistance to 4-thiopurinol, another pyrazolopyrimidine HGPRT substrate (2, 3). Similarly, cells that were wild type at the APRT locus exhibited EC<sub>50</sub> values for APP of <1.0 μM, whereas the EC<sub>50</sub> values for all Δaprt lines were greater than 100 μM. All hgprt/HGPRT and aprt/APRT heterozygotes displayed intermediate EC<sub>50</sub> values for HPP (<100 μM) and APP (<1.0 μM), respectively. EC<sub>50</sub> values for wild type, heterozygous, and homozygous cell lines at both the HGPRT and APRT loci are similar to those published previously (21). No meaningful variations in HPP riboside sensitivity were observed among wild type and mutant cell lines (data not shown), consistent with the proposed non-phosphoribosylation-based mechanism for incorporation of this nucleoside analog into *Leishmania* parasites (25).

**Growth of Mutant Cell Lines in Purines**—The ability of *L. donovani* to be propagated in completely defined growth medium enables an assessment of their ability to grow under conditions in which the purine source is varied. No differences in the rate or extent of growth were observed among *L. donovani* strains when the exclusive source of purine in the culture medium was hypoxanthine, guanine, adenine, xanthine, inosine, guanosine, or adenosine.

**Radiolabeled Hypoxanthine Incorporation Assay**—The capability of Δhgprt cells to grow in hypoxanthine was somewhat surprising in view of the fact that prokaryotic (26) and mammalian (27) cells lacking HGPRT activity cannot proliferate when exogenous hypoxanthine is the sole source of purine for nucleotide synthesis. As a consequence, the abilities of wild type and Δhgprt cells to incorporate [14C]hypoxanthine into phosphorylated metabolites was compared. All cell lines tested, including DI700:H<sup>+/+</sup>A<sup>+/+</sup>, DI700:H<sup>+/+</sup>A<sup>+/+</sup>, DI700:H<sup>+/+</sup>A<sup>+/+</sup>, TUBA2:H<sup>+/+</sup>A<sup>+/+</sup>, TUBA2:H<sup>+/+</sup>A<sup>+/+</sup>, and APPB2A3:H<sup>b/h</sup>, could efficiently incorporate [14C]hypoxanthine into their purine pool, intimating an alternative mechanism for hypoxan-
Incorporation of [14C]hypoxanthine into these cell lines was compared. 100 thine in the culture medium selectively interfered with the cell lines (data not shown).

Hypoxanthine incorporation rates into any of the *L. donovani* concentrations of either hypoxanthine, adenine, adenosine, in-thine conversion to the nucleotide level in *D. m* [14C]hypoxanthine (panel A) or of DI700:H\(^{\text{+/f}}\) A\(^{+/f}\) (C), TUBA2:H\(^{+/f}\) A\(^{+/f}\) (C), TUBA2: H\(^{+/f}\) A\(^{+/f}\) (C), and APPB2A3: H\(^{+/f}\) (X) to phosphoribosylate 20 μM [14C]hypoxanthine (panel A) or of DI700:H\(^{+/f}\) A\(^{+/f}\) (C), TUBA2:H\(^{+/f}\) A\(^{+/f}\) (C), TUBA2: H\(^{+/f}\) A\(^{+/f}\) (C), and APPB2A3: H\(^{+/f}\) (X) to convert 20 μM [14C]adenine to the nucleotide level (panel B) were determined as described under "Experimental Procedures."

Fig. 5. HGPRT and APRT assays. The abilities of lysates of DI700: H\(^{+/f}\) A\(^{+/f}\) (C), DI700: H\(^{+/f}\) A\(^{+/f}\) (Δ), DI700: H\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2:H\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2:H\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2: H\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2:H\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2:A\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2:H\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2: H\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2:H\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2:A\(^{+/f}\) A\(^{+/f}\) (Δ), TUBA2:H\(^{+/f}\) A\(^{+/f}\) (Δ), and APPB2A3:A\(^{+/f}\) (X) cells to convert 1.8 μM [14C]hypoxanthine into nucleotides by >95% at high concentrations in DI700:H\(^{+/f}\) A\(^{+/f}\) and DI700:H\(^ {+/f}\) A\(^{+/f}\) cells, whereas wild type DI700:H\(^{+/f}\) A\(^{+/f}\) cells expressed a significant xanthine-refractory [14C]hypoxanthine uptake component.

**DISCUSSION**

The ability of *Leishmania* to undergo efficient homologous recombination (7, 10) and the relatively recent development of methodologies to transfect these parasites with exogenous DNA (13, 14) have enabled this genetic dissection of the purine salvage pathway by targeted gene replacement strategies. This genetic analysis has been greatly facilitated by the availability of mutationally derived cell lines deficient in either APRT (8) or AK (17) activity, thereby expediting the construction of cell lines with multiple mutations. Previous biochemical, genetic, and radiolabel incorporation studies have implied that HGPRT, APRT, and AK are three enzymes that play primary roles in purine salvage in parasitic hosts. *L. donovani* (8, 17, 28). XPRT may play an important role in accessing host guanylate pools (28, 29), but adenylate nucleotides are the predominant host purines. A nucleoside phosphotransferase activity has also been detected in *L. donovani*, albeit in low amounts, but is only known to recognize the purine analog, HPP riboside (25). As the *L. donovani* HGPRT (15) and APRT (16) and their respective flanking sequences have been isolated, a plethora of *Δhgp* and *Δapr* homologous null mutants have been generated by targeted gene replacement within the wild type, aprt−, and ak− backgrounds. Strains constructed for this genetic evaluation of the purine pathway include: *Δhgp*, *Δapr*, *Δhgp/Δapr* (as well as aprt−/Δhgp, *Δhgp/ak−*, *Δapr/ak−*, and *Δhgp/ Δapr/ak−*), and all of the obligate heterozygous progenitors. From a biochemical perspective, targeted gene replacement allowed the creation of mutant null phenotype parasites defective in the three purine salvage enzymes in every conceivable combination, including three single mutants, hgprt−, aprt−, and ak−; three double mutants, hgprt−/aprt−, hgprt−/ak−, and aprt−/ak−; and one triple mutant, hgprt−/aprt−/ak− (Fig. 1).
All of these strains are theoretically syngeneic except for the rearranged HGPRT and APRT loci encompassing the drug resistance cassettes and for the uncharacterized mutations that conferred deficiencies in either AK or APRT activity.

Critical to the creation of L. donovani strains with multiple mutations was the ability to generate homozygous null mutants at either the HGPRT or APRT locus with only a single targeting construct. This preserved the other drug resistance marker employed in these studies for subsequent genetic manipulations, thereby allowing the creation of cells with more than one homozygous mutation, and resulted in a substantial savings both in time required for the construction of all of the mutant cell lines and in financial resources. Eliminating both alleles with only a single targeting vector required first the creation of the heterozygotes by homologous recombination of drug resistance cassettes containing 5'- and 3'-flanking sequences of HGPRT or APRT followed by selection for LOH in subversive substrate. LOH has been demonstrated previously in Leishmania at the HGPRT, APRT, and dihydrofolate reductase-thymidylate synthase loci (21, 30), and these present studies embellish the previous findings by demonstrating that LOH at the HGPRT and APRT loci is neither marker- nor cell line-specific. The mechanism for LOH in Leishmania is not known but could include chromosome loss, chromosome nondisjunction, gene deletion, mitotic recombination, gene conversion, or possibly homologous recombination of stably maintained episomal DNAs originating either from the targeting construct or the rearranged chromosome locus. Simple chromosome loss (31), chromosome loss followed by duplication (31, 32), and mitotic recombination (32, 33) have all been demonstrated as mechanisms of LOH in mammalian cell systems. Overall, the ability to create null mutants with single targeting constructs greatly facilitates genetic investigations into complex and diverging metabolic pathways, such as that for purine salvage, and the availability of additional drug resistance markers for Leishmania parasites (34–36) should allow the construction of knockout parasites bearing even more than the 2 homozygous mutations.

The genetic studies with HGPRT and APRT targeting constructs in L. donovani have revealed some unique and unexpected features of the purine salvage pathway in this protozoan parasite. Foremost, mutants defective in HGPRT, APRT, and/or AK activity retain the capacity to proliferate in completely defined medium in which the sole exogenous purine

**FIG. 7.** Effect of purine nucleobases and nucleosides on [14C]hypoxanthine incorporation by DI700:H+/A+/+ (panel A), APPB2A3:H+/ (panel B), and TUBA2:H+/A+ cells (panel C) was determined in the absence (●) or presence of either adenine (○), guanine (■), hypoxanthine (□), xanthine (▲), adenosine (△), or inosine (+). [14C]hypoxanthine was present in the assay mixture at 1.8 μM, while nonradiolabeled purines were added to a final concentration of 100 μM. Each data point reflects the amount of radiolabel converted to the nucleotide level by 10^7 parasites.

**FIG. 8.** Effect of xanthine on [14C]hypoxanthine incorporation by wild type and Δhgprt L. donovani. [14C]hypoxanthine incorporation into DI700:H+/A+/+ (○), DI700:H+/A+/ (□), and DI700: H+/A+/ (●) cells was determined as a function of xanthine concentration. The data were normalized the amount of radiolabel incorporated by each strain in the absence of xanthine.

The genetic studies with HGPRT and APRT targeting constructs in L. donovani have revealed some unique and unexpected features of the purine salvage pathway in this protozoan parasite. Foremost, mutants defective in HGPRT, APRT, and/or AK activity retain the capacity to proliferate in completely defined medium in which the sole exogenous purine
source is any of the four naturally occurring purine nucleo-
bases, hypoxanthine, xanthine, guanine, or adenine, or the
nucleosides adenosine, inosine, or guanosine. In contrast,
mammalian cells rendered pharmacologically auxotrophic for
purines with inhibitors of purine biosynthesis cannot salvage
hypoxanthine through HGPRT (27), adenine through APRT
(37), or adenosine through AK (38) when the germaine enzyme
is missing. Indeed, this pharmacologically induced purine de-
privation has served as a valuable positive selectable marker for
expression of these purine salvage enzymes in animal cell
systems (27, 37–39). The viability of the mutant D. donovani
strains in any single purine can be attributed to their ability
to incorporate hypoxanthine efficiently into the purine pool.
HGPRT-deficient mammalian cells cannot incorporate hypox-
anthine (8, 27, 39). The uptake of other purine bases was not
evaluated in these mutant strains, since it has been previously
demonstrated that wild type D. donovani promastigotes funnel
adenine through hypoxanthine prior to incorporation into the
nucleotide pool and metabolize guanine and xanthine through
XPRT (28, 29). The channeling of other purines into hypoxan-
thine by L. donovani promastigotes is supported by our com-
petition studies that demonstrate that excess adenosine, in-
osine, and adenine are as effective as equimolar concentrations
of hypoxanthine in blocking [14C]hypoxanthine incorporation
into nucleotides (see Fig. 7). Guanine is a bit less effective at
impeding [14C]hypoxanthine incorporation, presumably be-
cause it is rapidly oxidatively deaminated to xanthine (28, 29).
The channeling of inosine, adenine, and adenosine into hypox-
anthine can be respectively ascribed to the high levels of of
nucleoside hydrolase (40), adenine deaminase (24), and adeno-
sine hydrolase/phosphoribase (17) activities present in L. do-
ovani promastigotes. None of these enzymes is expressed by
mammalian cells.

The mechanism by which hypoxanthine, as well as the pu-
rines that are transformed into hypoxanthine, are assimilated
into the nucleotide pool in Δhgprt/Δaprt L. donovani is cur-
rently under study. The fact that high concentrations of xan-
thine only partially inhibit [14C]hypoxanthine incorporation
into wild type parasites but virtually obliterate [14C]hypoxan-
thine uptake into Δhgprt strains (Fig. 8) implies that XPRT,
a enzyme that is expressed in L. donovani (41), plays a central,
but obviously not exclusive, role in hypoxanthine metabolism
in this protozoan parasite. Two mechanisms could account for
this inhibition of [14C]hypoxanthine uptake by xanthine; either
XPRT recognizes hypoxanthine as a substrate, or hypoxan-
thine is oxidized to xanthine prior to its phosphoribosylation.
The latter hypothesis is unlikely, however, since L. donovani
promastigotes lack detectable xanthine oxidase/dehydrogenase
activity (41). The different extents by which xanthine inhibits
[14C]hypoxanthine incorporation into wild type and Δhgprt
strains can be imputed to the fact that the former expresses
two enzymes that phosphoribosylate hypoxanthine, whereas
the Δhgprt mutants lacks the xanthine-refractory pathway, i.e.
HGPRT.

Little is known about XPRT from Leishmania, although mammalian cells lack the activity (41). However, it would ap-
pear that the enzyme might serve as a target for the selective
corporation of cytotoxic xanthine analogs, such as 6-thioxan-
thine, which is phosphoribosylated by the hypoxanthine-gua-
nine-xanthine PRT (HGXPRT) of Toxoplasma gondii (42, 43).
Tuttle and Krenitsky have chromatographically distinguished
the L. donovani XPRT and HGXPRT activities, but the substrate
specificity of XPRT could not be evaluated due to the inherent
instability of the native enzyme in solution (41). The L. do-
ovani HGXPRT, however, does not recognize xanthine (15, 41).
Attempts are currently under way to isolate and overexpress
the XPRT gene from L. donovani to provide sufficient recom-
binant protein for ultimate biochemical and structural charac-
terization.

Enzyme and radiolabel incorporation studies with other pro-
tozoan parasites have revealed considerable differences in the
purine salvage pathways among various genera (1). Thus, con-
clusions drawn from these studies on the purine pathway of L.
donovani promastigotes are unlikely to have general applica-
tion to other parasites, except perhaps to other parasites of the
Trypanosomatidae family, e.g. Trypanosoma brucei, the etiologic agent of African sleeping sickness, and Trypanosoma
cruzi, the parasite that causes Chagas disease. Moreover, there
are differences between the purine salvage pathways of pro-
mastigotes and amastigotes, the latter being the infective form
of the parasite. For instance, L. donovani amastigotes lack
adenine deaminase and cannot convert, therefore, other pu-
rines into hypoxanthine (44). A similar genetic dissection of
the purine pathway in amastigotes by targeted gene replacement
will be undertaken with L. mexicana, a species that can be
propagated as promastigotes, axenic amastigote-like forms
(45), and amastigotes in macrophage lines (46), and the genes
encoding L. mexicana HGPR and APRT have already been
isolated for these purposes. Extensive genetic studies on the
purine pathway of other protozoan parasites have not been
accomplished, although mutations in either AK (47) or HGX-
PR (42, 43) have no apparent effect on survival of T. gondii.

Preliminary experiments do suggest, however, that double
knock-outs of both AK and HGXPRT activities may be lethal.2

If these preliminary experiments are sustained, the HGXPRT
activity plays a sufficient but not obligatory role in purine
salvage in T. gondii. In contrast, neither HGPR, APRT, nor
AK, alone or in any combination, are essential to purine sal-
vage by or survival of L. donovani promastigotes.

The purine auxotrophy of Leishmania and its unique purine
salvage pathway offers a number of targets for therapeutic
consideration. One therapeutic paradigm has been inhibitor
design to block purine acquisition from the host. The channel-
ing of extracellular purines through hypoxanthine (28, 39) had
suggested that HGPR would be an appropriate target for
inhibitor design, but our genetic investigations demonstrate, at
least in promastigotes, that specific inhibitors of HGPR alone
would be therapeutically ineffective. Similar conclusions can
be drawn about the potential effectiveness of APRT and AK in-
hibitors as a consequence of these studies. Indeed, targeting all
three enzymes in combination may not be a valid therapeutic
paradigm. However, if XPRT in conjunction with HGPR
proves indispensable to purine salvage by this protozoan par-
aside, one might envision that a common mechanism-based
inhibitor might have therapeutic potential or that targeting
both enzymes with a combination of inhibitors might be a
rational therapeutic strategy. Targeting multiple steps in a
metabolic pathway has considerable precedence in antipara-
sitic therapies (48, 49). Testing XPRT function in both wild
type and Δhgprt backgrounds can be accomplished by similar
targeted gene replacement approaches, once the L. donovani
XPRT is isolated.

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