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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Leishmaniasis is a debilitating disease that afflicts more than 350 million people worldwide. It occurrence in many US soldiers deployed to the endemic area emphasizes its importance as a health problem for the military. Vaccine development is the ultimate solution for this problem. Our previous research indicates that Leishmania parasites secrete, excrete, or shed antigens into the medium during in vitro culture. These exo-antigens possess the features of eliciting strong protective immune response in animal models. This project aims at defining the molecular properties of these soluble exo-antigens. In this research period, we have thoroughly characterized a nucleoside hydrolase (NH) and its gene from *L. donovani*, the causative agent of severe visceral leishmaniasis. This enzyme is implicated as an important catalytic activity of purine salvage in parasites belong to the family Trypanosomatidae. We showed that NH is encoded by a single copy gene in the parasite, and its homologues are present in members representing other Leishmania species complexes, suggesting that chemotherapy targeting this molecule will have a broad spectrum for all Leishmania parasite species. We have also shown that this enzyme and its mRNA are constitutively expressed during the parasite growth in vitro. Enzyme kinetic study revealed that it is a nonspecific nucleoside hydrolase based on its substrate specificity. To this end, we have mass-produced this antigen for the evaluation of its antigenic potential. We have further screened an expression library and purified 10 clones for sequencing analysis. The result showed that only one clone encodes a hydrophilic protein, and the rest of the clones all encode the proteins of the surface glycoprotein GP46 complex. Our analysis of the GP46 protein complex allowed us to completely sequenced three genomic clones representing different GP46 genes, and partially characterized 6 additional clones. A cDNA of this gene was obtained by RT-PCR from total RNA of the parasite and the recombinant protein is being expressed in bacteria.

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1. Introduction

1.1. Leishmaniasis: diagnosis and treatments

Leishmaniasis, caused by a group of protozoan parasites of the genus *Leishmania*, is transmitted by sand flies in the genera *Phlebotomus* (in the Old World) and *Lutzomyia* (in the New World). The World Health Organization estimates that 350 million people worldwide are at risk of contracting leishmaniasis, and 1.5-2 million new cases occur annually (WHO, 2000). Moreover, leishmaniasis is a recurrent health problem that continues to disrupt military readiness and operational activities (Martin *et al.,* 1998). The epidemics of cutaneous leishmaniasis in Afghanistan where US soldiers are currently deployed further underline the importance of the leishmaniasis research for US military. In contrast to the rapid evolution of the disease, the tools to detect, diagnose, prevent and treat it are archaic or none-existent. The urgent need for a fast and reliable diagnostic tool has stimulated the development of a genus-specific immunodiagnostic method using native antigens secreted into a protein-free medium by *L. donovani* promastigotes (Martin *et al.*, 1998; Rajasekariah *et al.*, 2000). Control through vaccination, the most cost-effective means of disease eradication, has only been attempted for cutaneous leishmaniasis and research in this field is needed. Our major objective of this project is to characterize the major exo-antigens from *L. donovani* promastigotes, the causative agent of visceral leishmaniasis. This effort is in support of our other research on vaccine development against leishmaniasis and standardization of a genus-specific immunodiagnostic kit, which is being developed at Walter Reed Army Institute of Research (WRAIR).

2. Body of the Research

Our efforts for the control of leishmaniasis have focused on characterizing *Leishmania* promastigote exo-antigens for the development of novel immunodiagnostic measures and vaccines. Specifically, we proposed to 1) characterize a nucleoside hydrolase (NH), 2) identify 10 most abundant exo-antigens, and 3) obtain clones encoding the major surface glycoprotein GP46 complex and perform preliminary sequencing analysis. The following details our achievements under each objective.

2.1. Nucleoside hydrolase (NH) gene

Like many other trypanosomal parasites, *Leishmania* lacks the *de novo* purine biosynthetic pathway. They instead rely on preformed purine nucleosides or bases for the biosynthesis of purine ribonucleotides. As a result, the parasites have evolved the purine salvage pathway, which allows them to utilize preformed purine nucleosides or bases in the environment. Enzymes involved in the purine salvage pathway include purine nucleoside kinases, hypoxanthine-guanine-xanthine phosphoribosyl-1-pyrophosphate transferases, and the purine ribohydrolases and/or phosphorylases. One of these enzymes, purine N-ribohydrolase, has not been found in mammals. Therefore, it is a potential target for chemotherapeutic treatment of diseases caused by trypanosomatid

parasites. As its name shows, nucleoside hydrolase catalyzes the hydrolysis of the N-ribosidic bond between the base and the (deoxy-) ribose.

Leishmania parasites have different nucleoside hydrolase activities. Nucleoside hydrolase activity has been detected in crude lysates of *L. tropica* and *L. donovani* promastigotes. In *L. donovani*, three nucleoside hydrolases (particle weight of 33, 180, and 205 kDa) have been separated and purified from promastigotes, each with distinct catalytic and physical properties (Koszalka and Krenitsky, 1979). Recently, a nucleoside hydrolase gene homologue was found in the sequence database of the *L. major* genome (Shi *et al.*, 1999). The protein was expressed in *Escherichia coli* and purified, and the crystal structure was determined. Despite its similarity in structure to the *C. fasciculata* IU-nucleoside hydrolase apoenzyme, the *L. major* enzyme has substantially different catalytic specificities, which classify this enzyme as a nonspecific nucleoside hydrolase (Shi *et al.*, 1999). Two transition-state inhibitors, immucillin A and ACAP, compete favorably for the catalytic site of the enzyme, even in the presence of nanomolar inhibitor and micromolar substrate. The potential of these compounds as anti-*Leishmania* agents remains to be tested. Further investigation of the presence of this enzyme in other *Leishmania* species, and its molecular conservation, may provide additional information for molecular and immunological diagnosis of leishmaniasis.

We have isolated the NH gene by immuno-screening of *L. donovani* cDNA expression libraries using antibodies against promastigote exo-antigens. The 1.9-kbp cDNA clone of LdNH is terminated with a polyA tail, but is truncated at the 5' end, missing the translational initiation codon. The complete cDNA, 2.1-kbp in length, was obtained by RT-PCR. We performed BLASTP search of GenBank and showed that LdNH was significantly homologous to nucleoside hydrolase from various organisms. A close comparison was made for NH from different parasites. Southern blot analysis showed that LdNH and the homologous genes in other *Leishmania* parasites (*L. mexicana*, *L. tropica*, *L. major*, and *L. braziliensis*) are encoded by a single copy gene. The similarity in hybridizing patterns among these parasites suggests that these species have significant sequence conservation at the nucleoside hydrolase gene locus. Northern blot of total RNA detected the constitutive expression of the 2.1-kb LdNH mRNA in 1-6 day promastigote culture.

The complete reading frame of the nucleoside hydrolase was cloned into a bacterial expression vector pET22b(+) to produce recombinant protein fused with a six-histidine tag. It was purified by column affinity chromatography to almost homogeneity and was used to immunize a rabbit for antiserum production. I have provided the antiserum to the WRAIR Kenya Labs for the evaluation of sensitivity in disease diagnosis in an ELISA format.

Enzyme kinetic analysis showed that the recombinant LdNH possessed substrate specificity designated for a nonspecific nucleoside hydrolase because 1) it showed significant levels of activity for the naturally occurring purine and pyrimidine nucleosides tested (uridine, inosine, cytidine, adenosine, and guanosine), and 2) it efficiently utilized *p*-nitrophenyl-β-D-ribofuranoside as a substrate. Furthermore, the expression of LdNH in cultured promastigotes was estimated to be 3.6 μg/mg of promastigote protein extract. Western blot detected LdNH in *L. donovani* promastigote protein extracts with a molecular weight of ~34-kDa, in agreement with the predicted molecular size. In

addition, there was no significant difference in enzyme level during growth of *L. donovani* promastigotes, further corroborating the hypothesis of constitutive expression. Immunofluorescent assays (IFAs) with polyclonal antiserum to LdNH specifically detected immunoreactive foci in *L. donovani* promastigotes in contrast to the background labeling with the pre-immune serum. Such focalized labeling of LdNH may indicate that the enzyme is located in specific organelles or secretory vesicles.

We have achieved a detailed analysis of the genomic organization, gene expression, protein synthesis, enzymatic properties, and immunolocalization of LdNH. Detailed information of this work is published (Cui, Rajasekariah, and Martin, 2001, Gene, 280:153-162) and appended.

2.2. Other *L. donovani* exo-antigens.

During their growth *in vitro*, *L. donovani* parasites constitutively synthesize and secrete and/or shed over 40 different proteins (Bates *et al.*, 1988). Some of these antigens have been demonstrated to be cell surface antigens shed by the parasites during *in vitro* culture (Kaneshiro *et al.*, 1982). These promastigote-released factors have been designated as Leishmania exogenous, excretory factors, or extracellular antigens (El-On *et al.*, 1979; Kaneshiro *et al.*, 1982). Here, we refer to these antigens as soluble exo-antigens. To date, only a few of these proteins have been identified, including the secretary acid phosphatase (sAP) (Gottlieb and Dwyer, 1982; Shakarian *et al.*, 1997), chitinase (Schlein *et al.*, 1991; Shakarian and Dwyer, 1998, 2000), a thiol-specific antioxidant (Webb *et al.*, 1998), and a soluble glycoprotein GP63 released from the cell surface (Symons *et al.*, 1994). Therefore, characterization of individual proteins in the exo-antigen mixture will help 1) to elucidate the functions of these molecules in mediating interactions with the insect vector and the vertebrate host; 2) to standardize and optimize the immunodiagnostic tests; 3) to evaluate them as targets for the development of new chemotherapeutic measures and vaccines.

Our initial screening of an expression library with the antibody against the total exo-antigens from *L. donovani* promastigotes identified five clones, with one clone representing the LdNH gene and four clones GP46 genes. We have performed additional screening of the expression library and isolated 12 more clones. Sequencing of these clones and BLAST search of the GenBank demonstrated that nine clones were homologous to GP46 genes, one to the chitinase gene (Shakarian and Dwyer, 1998), and one to the hydrophilic protein from *L. major* (McKean et al., 1997; Alce et al., 1999). This result suggests that the antiserum against the *L. donovani* exo-antigens contains antibodies against only limited epitopes of a few abundant proteins. Further immunoscreening of the library would not be an efficient method for gene identification purpose. One of the proteins is the GP46 glycoprotein, which is encoded by multiple genes with significant homology and slight divergence of the sequences. This also suggests to us that the GP46 proteins may be the most important antigens in the exo-antigen mixture. Therefore, we have focused our efforts on the characterization of the GP46 protein complex.

2.3. Genes encoding the major surface glycoprotein GP46 complex.

Glycoprotein 46, also referred to as promastigote surface antigen 2, is one of the most abundant surface proteins of promastigotes and has been detected in all examined *Leishmania* species, except for the members of the *L. braziliensis* complex (McMahon-Pratt *et al.*, 1992; Symons et al., 1994). Genes encoding GP46 have been cloned and characterized in many *Leishmania* species, including *L. major, L. amazonensis, L. chagasi*, and *L. infantum* (Lohman *et al.*, 1990; Murray and Spithill, 1991; Beetham *et al.*, 1997; Jimenez-Ruiz *et al.*, 1998). The most salient features of the protein are the presence of leucine-rich internal repeats and a family of similar, but non-identical genes encoding GP46. Although the organization of this gene family has not been fully characterized, in those that have been examined, multiple GP46 genes are arranged in clusters (McMahon-Pratt *et al.*, 1992). While GP46 protein is anchored to the parasite cell membrane via a glycosylphosphatidylinositol moiety linked to a conserved GPI anchor signal sequence in the C-terminus of the protein, water soluble GP46 is also detected in culture medium (Jimenez-Ruiz *et al.*, 1998). The function of this gene family in the biology of the parasites is unkown. Owing to its similarity to *Trypanosoma cruzi* and human mucins and to the *Drosophila* glue protein, and the presence of Leu-rich repeats, GP46 may be involved in protein-protein interaction. Most importantly, data from McMahon-Pratt's lab indicate that vaccination with GP46 can produce protective immunity in mice against cutaneous leishmaniasis (Champsi and McMahon-Pratt, 1988; McMahon-Pratt *et al.*, 1993). Such protection is conveyed by recognition by host immune system of GP46 antigens expressed in amastigotes rather than in promastigotes (Handman *et al.*, 1995). It appears that expression of different GP46 genes in different developmental stages is regulated both transcriptionally and post-transcriptionally, which resembles the expression of the cell surface metalloprotease, GP63 (Beetham *et al.*, 1997).

Our emphasis on the GP46 complex is based on its potential as a vaccine candidate, and the multiple clone we have identified by our immunoscreening procedure. First, we selected 9 clones for sequencing analysis. Using universal primers we obtained sequences from both ends of the clones. The result showed that these 9 clones can be classified into 5 different groups. Among the groups, the differences single nucleotide polymorphisms (SNPs), short deletion of a codon triad, and long deletions of sequences. It is noteworthy that these deletions did not change the reading frame, suggesting the proteins encoded were not significantly altered. These mutations are illustrated in Fig. 1, where sequences between group 1 and 2 are aligned. Particularly, clone 1 shows two large deletions of 19 and 70 amino acids, respectively. So far, we have completely sequenced group 1 and 2 clones, and obtained partial sequences of other groups. The total amount of sequencing work is approximate to 10 kbp.

Fig. 1. Alignment of group 1 (upper sequence) and 2 (lower sequence) clones of the GP46 gene. Only the region with two deletions and multiple SNPs are shown here. Sequences were aligned using the GCG program. Identical nucleotides are indicated by vertical lines, and deletions by dots. Not clone 1 has two large deletions, 57 bp (19 amino acids), and second 210 bp (70 aa).

```
351 ACGTATTGCTGTGAACATCGAAGACGAACACAAGGGCCGCAAGTGCAAGC 399
    ||||||||||||| |||||| ||||| ||||| ||| ||||||||||||
201 ACGTATTGCTGTGACCATCGAGGACGAGCACAAGGGCAGCAAGTGCAAGC 250

400 TTGAGAATAAGTGCCGCCCGGCTGCACCCACCACGACGACCACGACCACA 449
    ||||||||||||||||||||||||||||
251 TTGAGAATAAGTGCCGCCCGGCTGCACC..................... 278

450 AGCACCACTACTAAGCGGCCAACTGCATCCACGAGCACCACGACgACCAC 499
                                    ||||||||||||||||
279 .................................CACCACGACGACCAC 293

500 CACTGTCCCGCCGACTGTACCCACCCCCACTGCCACCCCAACAGCTACGC 549
    |||||||||||||||||||||||||||||||||||||||||||| ||||
294 CACTGTCCCGCCGACTGTACCCACCCCCACTGCCACCCCAACAGCCACGC 343

550 CTACTCCTGCCCCCGAGACGGAGTGCGAGGTGGATGGGTGTGAGGTGTGC 599
    |||||||||||| |||||||||||| ||||||||||||||||||||||
344 CTACTCCTGCCCCAGAGACGGAGTGCGGGGTGGATGGGTGTGAGGTGTGC 393

600 GAGGGGGACTCCGCGGCGAGGTGCGCCAGGTGCCGTGAGGGCTACTTCCT 649
    ||||||||||||||||||||||||||||||||||||||||||||||||||
394 GAGGGGGACTCCGCGGCGAGGTGCGCCAGGTGCCGTGAGGGCTACTTCCT 443

650 GACGAGCGAGAAGACGTGCCGGGCCGACCGCGATGGCGGCGTTGCGGCCG 699
    ||||||||||||||||||||||| |||||||||||||||||||||||||
444 GACGAGCGAGAAGACGTGCCGGGCCAACCGCGATGGCGGCGTTGCGGCCG 493

700 CGTCGAGCGGAGCGGCTGCTGCCGCTGCTGTGTGCATGGCTGTGCTGCTG 749
     ||||||||||||||||||||||| |||||||||||||||||||||||
494 TGTCGAGCGGAGCGGCTGCTGCCGCTGTTGTGTGCATGGCTGTGCTGCTG 543

750 AGCGTGGGGCTGGCGGCGTGAGGATGCCGCTGCTGCCACGCGCAGGCGGC 799
    |||||||||||| |||||||||||||| ||||||| ||||||||||||||
544 AGCGTGGGGCTGGCGGCGTGAGGATGCTGCTGCTGTCACGCGCAGGCGGC 592

800 GGCACCCGCTGCGTGGCACACGGCT..................... 824
    |||||||||||||||||||||||||
593 GGCACCCGCTGCGTGGCACACGGCTGCGTTGCGTGCTTGCGTGCAGCACC 642
```

deletion of 210 bp in clone 1

```
825 ................................GCGTGCGTGCCTGTGG 840
                                    |||||| ||||||||||
793 TGTCGCTCCTCTGCCCTGACTCTCTCTCTCGCCCGCGTGTGTGCCTGTGG 842

841 GCTGATGGGGGCGGAGCGCGGGTGTGTGTGGAGCGTGGCAGCAGCCGCTG 890
    |||||||||||||||||||||||||||||||| |   ||||||||||
843 GCTGATGGGGGCGGAGCGCGGGTGTGTGTGGAGCGGG...GCAGCCGCTG 889

891 GAGAGAGGGGGAGGGCGATAGCCGAGGTGCAAGGGCCGCGGTGTTTGTGC 940
    |||||||||||||||||||| ||||||||||||||||||||||||||||
890 GAGAGAGGGGGAGGGCGATAGCGGAGGTGCAAGGGCCGCGGTGTTTGTGC 939
```

While sequencing of the GP46 clones, we have also obtained the complete cDNA clone of one GP46 gene. The open reading frame of this clone have been cloned in pET22b clone that will be expressed in bacteria for the evaluation in immunodiagnostic and vaccine studies.

3. Key Research Accomplishments

- **In this research period, we have thoroughly characterized a nucleoside hydrolase (NH) and its gene from *L. donovani*.** This enzyme is implicated as an important catalytic activity of purine salvage in parasites belonging to the family Trypanosomatidae. We showed that NH is encoded by a single copy gene in the parasite, and its homologues are present in other *Leishmania* species complexes. It is interesting that even the hybridizing patterns in Southern blot are conserved among these species, suggesting that the NH gene is conserved to a high degree. This further implicates that chemotherapy targeting this molecule will have a broad spectrum for all *Leishmania* parasite species. We have also shown that this enzyme and its mRNA are constitutively expressed during the parasite growth *in vitro*. Enzyme kinetic study revealed that it is a nonspecific nucleoside hydrolase based on its substrate specificity.
- **We have further screened an expression library and purified 12 more clones for sequencing analysis.** The result showed that one clone encodes a hydrophilic protein, another the chitinase, and the rest of the clones all encode the proteins of the surface glycoprotein GP46 complex.
- **We have completely sequenced two groups of GP46 clones and partially sequenced clones of additional three groups.** Our analysis of the GP46 protein complex allowed us to completely sequenced two genomic clones representing different GP46 genes, and partially characterized 6 additional clones. The total sequences obtained in this project period are more than 10 kb. A cDNA of a GP46 gene was obtained by RT-PCR from total RNA of the parasite and the recombinant protein is being expressed in bacteria.
- **Other supporting activities.** In addition, we have provided supporting works for other research activities under the same *Leishmania* project funded by the Department of Defense. These include evaluation of other antigens for the perfection of the immunodiagnostic tool for leishmaniasis, and for vaccine development. First, we have produced large amount of antiserum for the LdNH protein and provided to Dr. Samuel Martin at the WRAIR lab in Kenya for ELISA study. Second, we have mass-produced and purified 10 mg of LdNH protein to homogeneity. More than 5 milligrams of the protein have been sent to Dr. Richard Titus's group for the evaluation of antigenicity and potential protection against Leishmaniasis in an animal model.

4. Reportable Outcomes

1. **Publication:**
   Cui, L., Rajasekariah, G.R., and Martin, S.K. 2001. A nonspecific nucleoside hydrolase from *Leishmania donovani*: implications for purine salvage by the parasite. *Gene* 280, 153-162.

2. **Presentation:**
Cui L, Ryan,J. and Martin, S.K. Characterization of Leishmania exo-antigens for immunodiagnosis and vaccine development. *Research in Protozoan Pathogens. East African Regional Workshop.* 22-25 May 2002.

3. Materials and techniques:

We have produced a polyclonal antiserum for further evaluation of the LdNH protein in immunodiagnosis.

We have also procured the method for mass-production and purification of recombinant LdNH for evaluation as a vaccine candidate in animal models.

4. **Employment and training activities:**
This project has partially supported two undergraduate students, a technician and two postdocs. Under this project, two undergraduate students (Christina Pepple and Jennifer Sommer) have obtained excellent training in molecular parasitology. Both students are from the University WISER (Women In Science and Engineering Research) Program. Kimberley Rzomp has helped in maintaining the parasite culture and preparation of exo-antigens and parasite lysates for the immunoblotting work. In addition, Dr. Michael Kariuki, has performed most of the library and immunoblotting work. Dr. Kuijun Zhao performed much of the sequencing analysis and identification of a complete GP46 cDNA for bacterial expression.

5. Conclusions

We have so far achieved detailed characterization of a nucleoside hydrolase gene from *L. donovani*. This enzymatic activity is an essential step for the purine salvage pathway. The presence of this gene in all trypanosomatid parasites including all Leishmaia species examined in our work suggests that drugs targeting this enzyme may have broad spectrum effect on many parasites. This work is corroborated by the detection of high level of antibodies against the NH protein in dogs suffering from leishmaniasis. Therefore, future work will evaluate the effect of NH inhibitors on parasite growth, the detection of antibodies in human patients against the NH and the use of this enzyme for immunodiagnosis, and evaluation of NH as a vaccine candidate.

Our work has demonstrated the immunogenecity of the GP46 complex and its abundant presence in the exo-antigens. Future work should study the genomic organization and expression patterns of different GP46 genes, evaluate its potential in standardizing the immunodiagnostic kit, and further study to illustrate whether this multigene family undergoes antigenic switching and mediate protection of the parasite in its vertebrate and invertebrate hosts.

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7. Appendix

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A nonspecific nucleoside hydrolase from *Leishmania donovani*: implications for purine salvage by the parasite☆

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Abstract

In contrast to their mammalian hosts, protozoan parasites do not synthesize purines *de novo*, but depend on preformed nucleotides that they purportedly obtain by salvage pathways. Nucleoside hydrolases may play a crucial role in that salvage process. By screening *Leishmania donovani* libraries with polyclonal antibodies against promastigote soluble exo-antigens, we have identified a cDNA encoding a protein with significant homology to nonspecific and uridine–inosine-preferring nucleoside hydrolases. Sequence comparison demonstrated that all the residues involved in Ca^{2+}-binding and substrate recognition in the active site are conserved among the characterized protozoan nucleoside hydrolases. Genomic analysis suggests that it is a single copy gene in *L. donovani*, and its homologues are present in members representing other *Leishmania* species complexes. Both Northern blot and immunoblot analyses indicate that it is constitutively expressed in *L. donovani* promastigotes. The recombinant enzyme overexpressed in and purified from bacteria showed significant activity with all naturally occurring purine and pyrimidine nucleosides, and efficient utilization of *p*-nitrophenyl-β-D-ribofuranoside as a substrate. Altogether, the sequence comparison and substrate specificity data identify this *L. donovani* nucleoside hydrolase as a nonspecific nucleoside hydrolase. Further, the nucleoside hydrolase was localized to specific foci in *L. donovani* promastigotes by immunofluorescent assays. Although the conservation of the nucleoside hydrolases among protozoan parasites offers promise for the design of broad-spectrum anti-parasitic drugs, the existence of multiple and distinct nucleoside hydrolases in a single species demands special consideration.

Keywords: Nucleoside hydrolase; Purine salvage; Molecular conservation; Substrate preference; Intracellular localization

1. Introduction

Leishmania are a group of protozoan parasites that cause a wide variety of infectious diseases in human and other vertebrates. Clinical presentation ranges from self-healing cutaneous ulcers to potentially fatal visceral disease. The parasite routinely cycles between a vector sand fly and a reservoir host. They exist as extracellular flagellated promastigotes in the alimentary canal of the sand fly and as ovoid, intracellular, nonflagellate amastigotes within the phagolysosomes of mammalian macrophages.

Like many other pathogenic protozoa, *Leishmania* parasites are purine auxotrophs (Marr et al., 1978). Because they lack the pathways for *de novo* purine biosynthesis and rely on preformed purine nucleosides or bases to meet their growth demands (LaFon et al., 1982), the parasite may have evolved unique pathways to salvage purine nucleosides or bases from their environment. These unique pathways present targets against which safe and effective antileishmanial drugs can be designed. Unfortunately, present attempts to exploit this vulnerability have failed to meet clinical expectations. For example, *Leishmania donovani* incorporates hypoxanthine into both adenine and guanine via the hypoxanthine/guanine phosphoribosyl transferase (HGPRT) enzyme pathway. Allopurinol, a pyrazolo-pyrimidine, can be metabolized by HGPRT to intermediates that effectively inhibit parasite purine nucleotide biosynthesis (Looker et al., 1986) and protein synthesis (Marr, 1991). However, clinical trials with allopurinol show mixed results against cutaneous leishmaniasis. Our inability to abrogate nucleotide access by inhi-

Abbreviations: HGPRT, hypoxanthine/guanine phosphoribosyl transferase; IFA, indirect immunofluorescent assay; LdNH, *Leishmania donovani* nucleoside hydrolase; *Lds*/e, *Leishmania donovani* soluble exo-antigens; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reactions

☆ The nucleotide sequence reported in this paper has been deposited in GenBank with the Accession number AY033633.

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bition of the known enzymes of the salvage pathway may stem from redundancy. Alternatively, it may reflect the lack of a clear understanding of the dynamics of nucleoside availability to the parasite in various microenvironments where these organisms thrive. In the event that multiple mechanisms may have evolved in each organism to meet such a critical and obligatory requirement, parasite access to purines in defined compartments must be carefully delineated before effective interdiction strategies can be successfully imposed.

Leishmania shares the purine salvage pathway with most of the other human protozoan pathogens, including *Plasmodium* (Reyes et al., 1982), *Entamoeba histolytica* (Lo and Wang, 1985; Hassan and Coombs, 1986), *Giardia lamblia* (Wang and Aldritt, 1983), and *Toxoplasma gondii* (Krug et al., 1989). Enzymes involved in the purine salvage pathway include purine nucleoside kinases, HGPRTs, and the purine ribohydrolases and/or phosphorylases. Most of the enzymes in the pathway have been characterized at the molecular and biochemical levels. One of these enzymes, purine N-ribohydrolase, has not been found in mammals. The N-ribohydrolase isozymes differ by their substrate preference. In *Crithidia fasciculata*, two isozymes, the inosine–uridine-preferring and guanosine–inosine-preferring nucleoside hydrolases, account for more than 90% of the nucleoside salvage, and have been studied in great detail (Horenstein et al., 1991; Parkin et al., 1991; Estupinan and Schramm, 1994; Degano et al., 1996; Gopaul et al., 1996; Parkin, 1996). Nucleoside hydrolases have also been identified and characterized in other trypanosomatids including *Trypanosoma brucei gambiense* (Schmidt et al., 1975), *L. donovani* (Koszalka and Krenitsky, 1979; Looker et al., 1983), *Leishmania mexicana* (Davies et al., 1983), *Leishmania tropica* (Garin et al., 2001), *Trypanosoma cruzi* (Miller et al., 1984), and *Trypanosoma brucei brucei* (Parkin, 1996). Recently, the genes encoding ribohydrolases have been cloned from *C. fasciculata* (Gopaul et al., 1996), *T. brucei brucei* (Pelle et al., 1998), and *Leishmania major* (Shi et al., 1999). Here, we report the cloning and characterization of a nonspecific nucleoside hydrolase gene from *L. donovani*, the causative agent of human visceral leishmaniasis. We present evidence to show that *L. donovani* promastigotes constitutively express the nucleoside hydrolase, which could play a key role in *Leishmania* purine salvage and provide a readily accessible target for anti-parasitic drug design.

2. Materials and methods

2.1. Parasite and parasite antigens

Promastigotes of *L. donovani* clone WR0130 were cultured in RPMI-1640 medium supplemented with glutamine, 5.96 g/l of HEPES, 2.65 g/l sodium bicarbonate, and 10% heat inactivated fetal calf serum (pH 7.4) at 26°C. A standard protocol has been developed in our laboratory for the procurement of promastigote soluble exo-antigens comprised of products that were secreted, excreted and shed into the medium by the parasite (Martin et al., 1998). To prepare exo-antigens from the promastigotes, the parasites were washed and further incubated in a defined protein-free medium, XOM (Life Technologies) at 10^7 promastigotes per ml for an additional 3 days. Thereafter, the spent medium was harvested by centrifugation at $9000 \times g$ for 30 min and filtered through a 0.22 μm filter to obtain the soluble exo-antigens. *Leishmania donovani* soluble exo-antigens (*Lds*/e) have been utilized as immunodiagnostic antigens in an antibody-detection ELISA for kala-azar (Rajasekariah et al., 2001).

2.2. Production of polyclonal antibodies

Lds/e were used to immunize two New Zealand White rabbits using standard protocol (Harlow and Lane, 1988). The hyperimmune sera were affinity-purified by protein A chromatography. Affinity-purified antibodies, with a concentration of 13 mg/ml, were tested for specific immunoreactivity against *L. donovani* promastigotes. Antiserum against recombinant nucleoside hydrolase was similarly produced in rabbits. The serum against the nucleoside hydrolase was partially purified by ammonium sulfate precipitation and used in the immunoblots and indirect immunofluorescent assays (IFAs).

2.3. Cloning of L. donovani nucleoside hydrolase gene and sequence analysis

An expression genomic library of *L. donovani* was constructed in λZAP Express vector using parasite genomic DNA digested with *Sau* 3AI (Stratagene). A cDNA library was made in UniZAP vector from mRNA isolated from late log phase *L. donovani* promastigotes (Stratagene). Both libraries were screened with the affinity-purified antibodies against *Lds*/e using standard procedures (Sambrook et al., 1989). Purification of individual phage clones and excision of phagemids were performed according to the manufacturer's protocol (Stratagene). The phagemid inserts were sequenced on an automated sequencer ABI377 using the dye termination reaction. Sequence analysis was performed with the GCG program (version 9). The predicted protein sequence was used to search the GenBank for homology to other sequences with the BLASTP algorithm.

2.4. DNA and RNA extraction, Southern and Northern hybridization

Genomic DNA and total RNA were isolated from *L. donovani* promastigotes as described previously (Cui et al., 2000). For Southern blot, *L. donovani* (strain WR130E) genomic DNA was digested with *Hin*dIII, *Pst*I, *Sac*I, and *Sal*I. Genomic DNA from *L. mexicana* (ATCC50157), *L. major* (WR2167 strain), *L. tropica* (WR1063), and *Leishmania braziliensis panamensis* (WR676) was digested with *Hin*dIII and *Pst*I. Enzyme

digestion, electrophoresis, and transfer to nylon membrane were performed using standard protocols (Sambrook et al., 1989). To study the expression of the nucleoside hydrolase gene, Northern hybridization was performed on total RNA isolated daily from cultured *L. donovani* promastigotes (days 1, 3, 4, and 6). For Northern blot, total RNA (10 μg/lane) was separated in 1% agarose/formaldehyde gel and transferred to nylon membrane. Both Southern and Northern blots were probed with a randomly labeled open reading frame (ORF) of the *L. donovani* nucleoside hydrolase (LdNH) gene. High stringency hybridization was performed at 42°C in a hybridization buffer (50% formamide, 6× SSC, 5× Denhardt's, and 0.5% SDS).

2.5. Expression of LdNH in a bacterial expression system

The ORF of the LdNH gene was cloned in a bacterial expression vector pET 22b(+), and the recombinant enzyme was expressed in *Escherichia coli* strain BL21 (Cui et al., 1997). Six histidine residues were included at the carboxyl terminus to facilitate protein purification using TALON metal affinity resin (Clontech). Purification was done under native conditions using a buffer containing 50 mM NaH_2PO_4, 10 mM Tris–HCl (pH 8.0), and 100 mM NaCl. The expressed protein was eluted in the same buffer with 100 mM imidazole, and the enzyme was dialyzed extensively in 50 mM HEPES buffer (pH 8.0) at 4°C. The purity of the protein was estimated by SDS-polyacrylamide gel electrophoresis (PAGE). Glycerol was added to the purified protein to 5% and the protein was snap-frozen in liquid N_2 and stored at −70°C for later enzyme kinetic study. The concentration of the protein extract was determined by using the BioRad protein assay reagents with bovine serum albumin as the standard.

2.6. Substrate specificity

Nucleoside hydrolase activity was assayed using the purified recombinant enzyme at room temperature in 50 mM HEPES (pH 8.0). The hydrolysis of adenosine, guanosine, cytidine, and uridine was determined by the release of the reducing sugar, ribose, using a colorimetric assay (Parkin, 1996). The hydrolysis of inosine to hypoxanthine and ribose was determined by spectrometric assay through continuous reading of optical absorbance at 280 nm (Shi et al., 1999). The substrate *p*-nitrophenyl-β-D-ribofuranoside was a generous gift from Dr Vern L. Schramm. Its hydrolysis was determined by continuously measuring the optical absorbance at 400 nm (Shi et al., 1999). Initial rates of the reaction were measured with at least five substrate concentrations and the kinetic parameters were determined by fitting the data to the Michaelis–Menten equation. To determine the level of nucleoside hydrolase synthesis *in vivo*, *L. donovani* promastigotes were harvested from 1–6 day cultures. After parasites were lysed by three cycles of freeze-thaw in 50 mM HEPES buffer (pH 8.0), the lysate was centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was collected as the protein extracted for enzyme assays. A total of 5 μl of the protein extract was used to measure nucleoside hydrolase activity using 400 μM of *p*-nitrophenyl-β-D-ribofuranoside as the substrate. For each protein extract, the measurement was repeated three times. The purified recombinant LdNH was used to make a standard curve to estimate the amount of nucleoside hydrolase in the parasite protein extract.

2.7. Immunoblotting and IFA

Leishmania donovani promastigote exo-antigens were prepared as described (Martin et al., 1998) and further concentrated ~10× using a Centricon column with a 3 kDa molecular weight cutoff membrane. Promastigote protein extracts were prepared as described for enzyme activity study. Proteins (100 μg/lane) in the exo-antigens and the parasite extracts were separated by SDS-PAGE and transferred to nitrocellulose paper for immunoblotting. Promastigotes from a similar culture were used to prepare IFA slides. Western blots and IFAs were performed essentially as described previously (Cui et al., 1997) using rabbit polyclonal antiserum to recombinant LdNH as the primary antiserum diluted at 1:1000 and 1:100, respectively. For IFA, fluorescein-conjugated goat anti-rabbit IgG was used as the secondary antibody. As a control, parasites were incubated with pre-immune serum.

3. Results

3.1. Cloning and sequence analysis of the LdNH gene

To characterize individual exo-antigens from *L. donovani* promastigotes, we developed a polyclonal antiserum against the exo-antigen mixture and constructed two expression libraries in lambda phages. Initial screening of 4×10^5 phage plaques of each library with the polyclonal antibodies has isolated 11 genomic and four cDNA clones. The positive clones were purified and phagemids were excised. Sequencing of 15 inserts has identified a 1.9 kbp cDNA that is highly homologous to the *C. fasciculata* inosine–uridine-preferring nucleoside hydrolase gene and the *L. major* nonspecific nucleoside hydrolase gene (Gopaul et al., 1996; Shi et al., 1999). The 1.9 kbp cDNA clone is terminated with a polyA tail, but is truncated at the 5′ end, missing the translational initiation codon. To obtain the 5′ sequence, nested PCRs were performed using the cDNA library with two flanking primers from the vector and two internal primers within the LdNH cDNA. A ~420 bp amplicon was cloned in the TOPO cloning vector and sequenced. The PCR product had a ~150 bp extension of the 5′ sequence to include a 39 bp spliced leader at the 5′ end (Lamontagne and Papadopoulou, 1999). The composite cDNA is 2.1 kbp long, and has an ORF of 942 bp followed by a TGA stop codon (Fig. 1A). Amplification of the genomic sequence produced a PCR product of the same size,

further confirming the lack of introns in the ORF. The 3′ untranslated sequence between the stop codon and the polyA tail is ~1 kbp. The encoded LdNH has 314 amino acids with a predicted molecular weight of 34.24 kDa and a p*I* value of 6.5. Five potential N-glycosylation sites are present in the predicted amino acid sequence.

A BLASTP search of GenBank showed that LdNH was significantly homologous to *L. major* nonspecific nucleoside hydrolase (95% amino acid identity) and a number of inosine–uridine-preferring nucleoside hydrolases from *C. fasciculata* (80%) and several bacteria (e.g. *Pasteurella multocida* (69%), *Mesorhizobium loti* (42%), *E. coli* (41%), and *Caulobacter crescentus* (37%)). It showed only 27% identity to the inosine–adenosine–guanosine-preferring nucleoside hydrolase from *T. brucei brucei* (Pelle et al., 1998). No homology was found to the dUTP hydrolase from *L. major* (Camacho et al., 2000). Fig. 1B shows the alignment among nucleoside hydrolases from four protozoan parasites. The crystal structures of *C. fasciculata* and *L. major* nucleoside hydrolases revealed the binding of a Ca^{2+} ion at the active center (Degano et al., 1998; Shi et al., 1999); residues involved in chelating the Ca^{2+} ion are perfectly conserved in all four nucleoside hydrolases (Fig. 1B). Furthermore, all residues involved in hydrogen bonding with the hydroxyl groups of the substrates are conserved in LdNH.

3.2. Genomic organization and expression

To estimate the copy number of the LdNH gene and to detect this gene in other *Leishmania* species, the LdNH ORF was used to probe parasite genomic Southern blots (Fig. 2).

(a)

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AACTAACGCTATATAAGTATCAGTTTCTGTACTTTATTGCGTCATCGCTTACCCCTTGTT    60
CGGTTTCCCTTCAGCTGTCGCCGCTCCAACGCCCTCTTGCTTACGCCGCAGATACTGAAA   120
ACGGGTATTCGGCATTTGCGAGCCATGCCGCGCAAGATTATTCTCGATTGTGATCCCGGG   180
                          M  P  R  K  I  I  L  D  C  D  P  G
ATCGATGATGCCGTGGCCATCTTTCTCGCCCACGGCAACCCGGAGGTCGAGCTGCTGGCC   240
I  D  D  A  V  A  I  F  L  A  H  G  N  P  E  V  E  L  L  A
ATTACGACGGTGGTGGGCAACCAGACCCTGGAGAAGGTGACCCGGAACGCGCGGCTGGTA   300
I  T  T  V  V  G  N  Q  T  L  E  K  V  T  R  N  A  R  L  V
GCTGACGTAGCCGGCATCGTTGGTGTGCCCGTCGCGGCTGGTTGCACCAAGCCCCTCGTG   360
A  D  V  A  G  I  V  G  V  P  V  A  A  G  C  T  K  P  L  V
CGCGGTGTGCGGAATGCCTCTCAGATTCATGGCGAAACCGGCATGGGTAACGTCTCCTAC   420
R  G  V  R  N  A  S  Q  I  H  G  E  T  G  M  G  N  V  S  Y
CCACCAGAGTTCAAGACAAAGTTGGACGGCCGTCATGCAGTGCAGCTGATCATCGACCTT   480
P  P  E  F  K  T  K  L  D  G  R  H  A  V  Q  L  I  I  D  L
ATCATGTCGCACGAGCCGAAGACGATCACGCTTGTGCCTACGGGTGGCCTGACGAACATT   540
I  M  S  H  E  P  K  T  I  T  L  V  P  T  G  G  L  T  N  I
GCGATGGCTGTCCGTCTTGAGCCGCGCATCGTGGACCGTGTGAAGGAGGTGGTTCTGATG   600
A  M  A  V  R  L  E  P  R  I  V  D  R  V  K  E  V  V  L  M
GGTGGCGGCTACCATACTGGTAATGCGTCCCCTGTAGCGGAGTTCAACGTCTTCGTCGAC   660
G  G  G  Y  H  T  G  N  A  S  P  V  A  E  F  N  V  F  V  D
CCGGAGGCGGCGCACATTGTGTTCAACGAGAGCTGGAACGTAACGATGGTGGGGCTGGAC   720
P  E  A  A  H  I  V  F  N  E  S  W  N  V  T  M  V  G  L  D
CTAACGCACCAGGCACTCGCCACGCCGGCGGTCCAGAAGCGAGTGAAGGAGGTGGGCACG   780
L  T  H  Q  A  L  A  T  P  A  V  Q  K  R  V  K  E  V  G  T
AAGCCGGCTGCCTTCATGCTGCAGATTTTGGACTTTTACACGAAGGTGTACGAAAAGGAG   840
K  P  A  A  F  M  L  Q  I  L  D  F  Y  T  K  V  Y  E  K  E
CGCAACACGTACGCGACGGTGCACGATCCCTGCGCTGTGGCGTACGTGATTGACCCCACC   900
R  N  T  Y  A  T  V  H  D  P  C  A  V  A  Y  V  I  D  P  T
GTGATGACGACGGAGCAAGTGCCAGTGGACATCGAGCTCAATGGGGCACTGACGACTGGG   960
V  M  T  T  E  Q  V  P  V  D  I  E  L  N  G  A  L  T  T  G
ATGACGGTCGCGGACTTCCGCTACCCACGGCCAAAGCACTGCCACACGCAGGTGGCTGTG  1020
M  T  V  A  D  F  R  Y  P  R  P  K  H  C  H  T  Q  V  A  V
AAGCTGGACTTCGACAAGTTTTGGTGCCTCGTGATTGACGCACTCAAGCGCATCGGCGAT  1080
K  L  D  F  D  K  F  W  C  L  V  I  D  A  L  K  R  I  G  D
CCTCAATGACGTGTGTGTGTCGCGATTGGCCACCGACGCGGTGGCCAGCTCGCGGCCATA  1140
P  Q  *
AAGCTGTCAGAGCAACGGCACGCGCGTGCGGCGGCTTGTGTGAAGCGTCTTTTTTTTCCT  1200
CTCGCTACGAGGTTATTGCCACTGCACTGGTGCAGCTCCGTGTCGCATGAGGGAAAAGGG  1260
TGGGTGGGGAAGAGTGGAGACGAGGCAAGGTGGACAGGCCGTGGTGGAGCAGCTGGGAGG  1320
GTATTGATCGCAGTTATGAACCCGAGTAGGTGATTACACCACCTCCGCCGCCTGAATCGC  1380
CCTTTCTCGCGCCTTCTTACAGCATCCCCTGCATCCTTTTTTTTTCCGTCGGTTCAGCGA  1440
CCGCCTCATACCCTCCGTTTTCTCTACCGGCCGCGACAGGGACGAGACGCGTCGATGAGA  1500
CGCCGATCTATTGAGAGCGCGCATATGACGAAACCAAATGCGTATCTACATCGATATATG  1560
CATACATATATAGTATTTAGTGTTTTCCAACGTGTCCACCCCACCACGAACAAGTCAGCA  1620
CCAATACACGCATACGTAACCGCAGCCCACCCACCGCTGTGTCGCACGGCGGCAATGACA  1680
GGGAGCGGGGTCCACCGACGCCCTTCGGCGTCGCCCAGCCCACTGCCATTCTACTGCGCA  1740
TACACAGCAGCCGAGGCCCAGAACTACGCACGACATCACCGCAGCGCAGTAACAGCAGGA  1800
GAAGAGATGGTGGATGCGTGTACGTGCTGAATACGCGGGCAGGACTCGTAGTGGGCTGGT  1860
CCAGGCGTAGCATGGCTATACGTCTTTTGGCACAGCGCCAGCAAGCCCTGTCCACCGG  1920
CGCCAGAGAGCCCGCAGCCGCACCAGAGGGCAGGCTGCAGGCTGCTCGGCATTATCACAA  1980
CGAGTGAGTATTGGATCATGACGCCAGCGTGTGGTGACCGGCATTGTCAGGGCACGTGAG  2040
AGAGACGAAAAAAAAACGTTTTTGTTTTTTTGGTGGCCAATCGGAGCTCGACTGAAAAAA  2100
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(b)

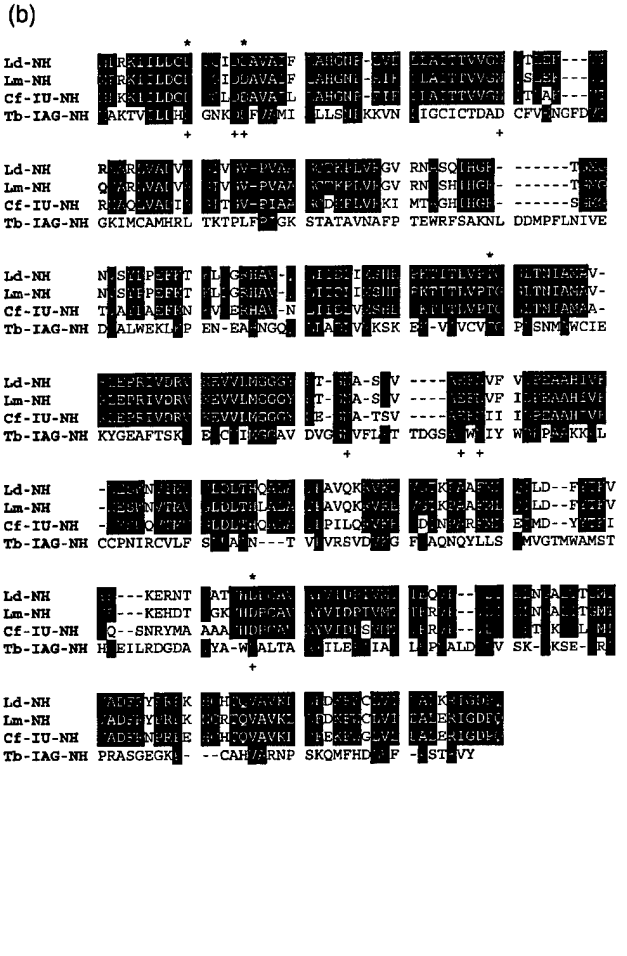

Fig. 1. Sequence analysis of nucleoside hydrolases. (A) The composite cDNA sequence and predicted amino acid sequence of LdNH. The conserved spliced leader sequence is underlined. Putative N-glycosylation sites are in bold. The stop codon TGA is indicated by an asterisk. (B) Alignment of predicted amino acid sequences of the nucleoside hydrolases from protozoan parasites. Ld-NH, nucleoside hydrolase from *L. donovani* (this paper); Lm-NH, the nonspecific nucleoside hydrolase from *L. major* (GenBank Accession number: 8569431); Cf-IU-NH, the inosine–uridine-preferring nucleoside hydrolase from *C. fasciculata* (U43371); Tb-IAG-NH, the purine-specific inosine–adenosine–guanosine-preferring nucleoside hydrolase from *T. brucei brucei* (AF017231). To optimize the alignments, gaps (-) are inserted. As determined for the Cf-IU-NH, amino acids in contact with Ca^{2+} are indicated with asterisks; those in contact with the ribosyl hydroxyl groups are indicated with a plus (+).

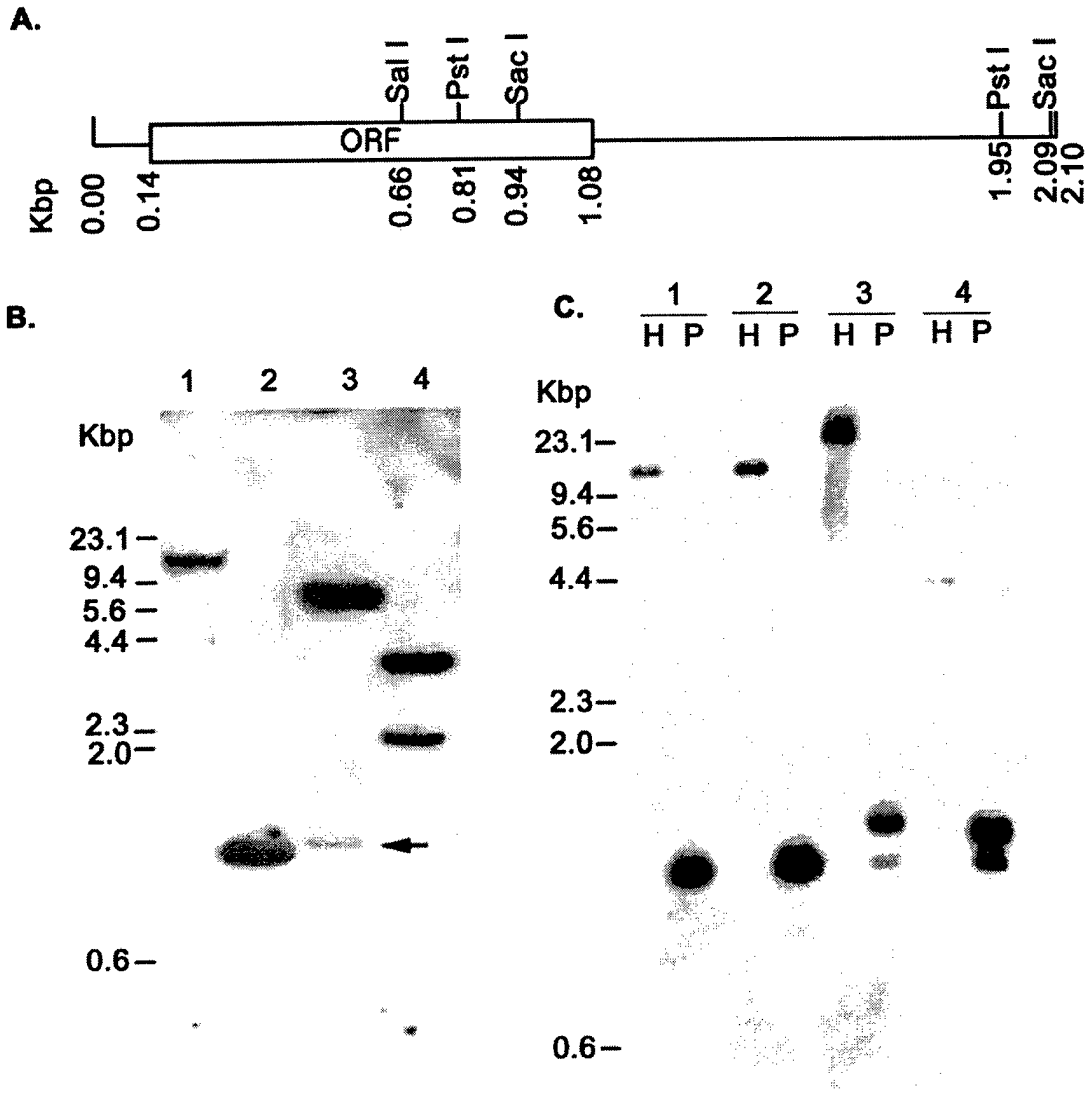

Fig. 2. Southern blots of the nucleoside hydrolase gene. (A) A physical map of the LdNH gene showing the sites for restriction enzymes *Pst*I, *Sac*I, and *Sal*I. The positions in the DNA in kbp are indicated. The box indicates the ORF of the gene, which was used for probe labeling. (B) Southern blot of *L. donovani* genomic DNA. *Leishmania donovani* DNA was digested with *Hin*dIII (lane 1), *Pst*I (lane 2), *Sac*I (lane 3), and *Sal*I (lane 4). The arrow indicates the 1.2 kbp fragments of *Pst*I and *Sac*I digestions predicted from the physical map. The sizes of the lambda HindIII marker are indicated in kbp. (C) Southern blot of genomic DNA from other *Leishmania* species. Two enzymes, *Hin*dIII (H) and *Pst*I (P), were used for each species, including *L. tropica* (1), *L. mexicana* (2), *L. major* (3), and *L. braziliensis* (4).

The physical map of the LdNH gene shows the presence of a *Pst*I, *Sac*I, and *Sal*I site in the ORF (Fig. 2A). Consistent with the expectation, *Hin*dIII produced one hybridizing band, whereas *Sal*I and *Sac*I digestions generated two hybridizing bands (Fig. 2B). The difference in the intensity of the two *Sac*I bands may reflect the fact that the 1.15 kbp *Sac*I fragment has only 140 bp overlap with the probe. However, *Pst*I yielded only one strong hybridizing band of 1.14 kbp, possibly resulting from two *Pst*I fragments of the same size. Altogether, the Southern blot suggests that the LdNH gene is probably a single copy gene (one copy per haploid genome). To determine if other *Leishmania* species have similar nucleoside hydrolase genes, Southern blots of *Hin*dIII and *Pst*I restricted genomic DNA from *L. mexicana*, *L. tropica*, *L. major*, and *L. braziliensis* were hybridized to labeled LdNH ORF under high stringency. DNA from all tested parasite species produced strong hybridizing bands, which is an indication of the existence of similar nucleoside hydrolase genes in these species (Fig. 1C). The appearance of a 1.14 kbp *Pst*I fragment in all parasite species tested indicates that two *Pst*I sites within the nucleoside hydrolase gene are conserved among these species. Interestingly, similar hybridization patterns were observed among *L. donovani*, *L. tropica*, and *L. mexicana* for both enzymes, and

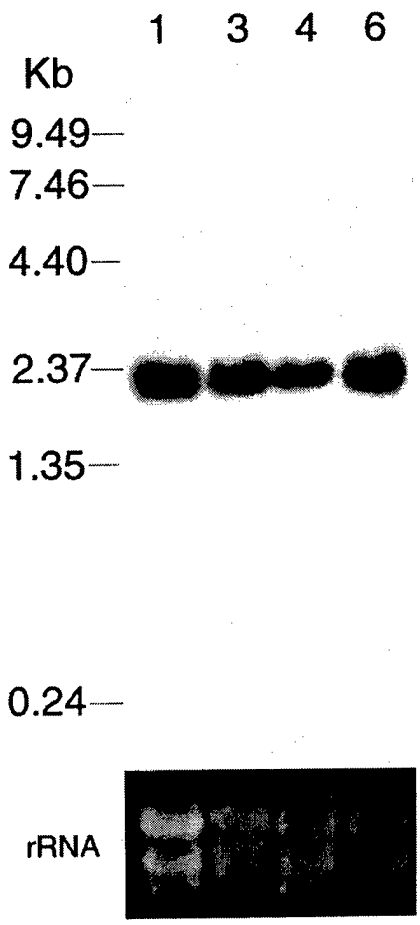

Fig. 3. Northern blot of nucleoside hydrolase gene expression. Total RNA (10 μg) isolated from day 1, 3, 4, and 6 (lanes 1, 3, 4, and 6, respectively) *in vitro* cultures of *L. donovani* promastigotes, representing early to late log phases of parasite growth, was loaded in each lane. The RNA ladder is indicated in kb. The lower panel indicates ethidium bromide-stained rRNAs as loading controls.

between *L. major* and *L. braziliensis* with *Pst*I digestion, suggesting that these species have significant sequence conservation at the nucleoside hydrolase gene locus.

The expression of the nucleoside hydrolase gene was studied in *L. donovani* promastigotes. Northern blot of total RNA detected the 2.1 kb nucleoside hydrolase mRNA in 1–6 day promastigote culture (Fig. 3). The size of the nucleoside hydrolase mRNA is consistent with that predicted from the cloned cDNA. The hybridization intensity also suggested that the nucleoside hydrolase gene might be constitutively expressed.

3.3. Expression, purification, and kinetic analysis of the recombinant LdNH

The complete reading frame of the nucleoside hydrolase was cloned into a bacterial expression vector pET22b(+) to produce recombinant protein fused with a six-histidine tag. The recombinant protein, with a molecular weight of 35.27 kDa, was detected by immunoblotting with anti-His tag antibody (data not shown). It was purified by column affinity chromatography to almost homogeneity and was used to immunize a rabbit for antiserum production (Fig. 4). A total of ~1 mg of the recombinant protein was purified from a liter of bacterial culture. The purified protein used for enzyme activity assay has a concentration of 80 μg/ml.

Kinetic analysis showed that the recombinant LdNH possessed similar substrate specificity to the *L. major* nonspecific nucleoside hydrolase, which is consistent with the expectation from their highly homologous amino acid sequences. The recombinant LdNH showed significant levels of activity for the naturally occurring purine and pyrimidine nucleosides tested (uridine, inosine, cytidine, adenosine, and guanosine), while it also efficiently utilized *p*-nitrophenyl-β-D-ribofuranoside as a substrate (Table 1). Based on Shi et al. (1999), LdNH is defined as a nonspecific nucleoside hydrolase.

3.4. Expression of native enzyme by promastigotes

To study the expression of LdNH in cultured promastigotes, parasite protein extracts were used to measure the nucleoside hydrolase activity using *p*-nitrophenyl-β-D-ribofuranoside as the substrate. The result was compared to the purified recombinant LdNH as the standard. The amount of LdNH produced in the promastigotes was estimated to be 3.6 μg/mg of protein extract, if we assume that the recombinant LdNH had no significant alteration in the enzyme

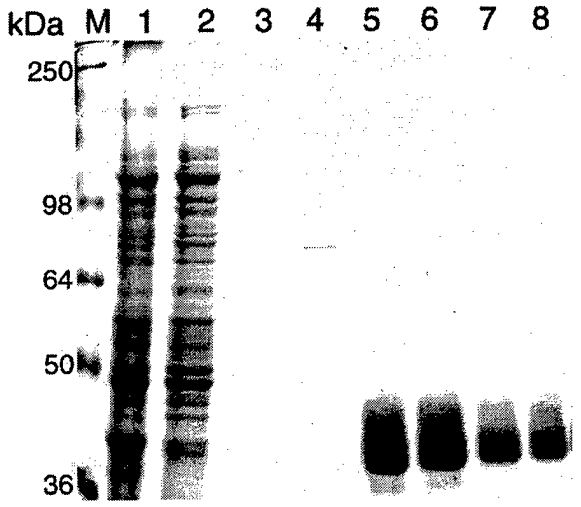

Fig. 4. Expression and purification of LdNH protein in *E. coli*. Coomassie Blue stained 10% SDS-PAGE shows the expression and purification of the recombinant protein. Lane M, molecular weight marker in kDa (SeeBlue marker, Invitrogen); lane 1, lysate of BL21 cells after IPTG induction; lane 2, lysate after binding to Ni-NTA column; lane 3, first wash using the buffer (see Section 2) with 10 mM imidazole; lane 4, second wash using the buffer with 20 mM imidazole; lanes 5 and 6, elutes with the buffer containing 50 mM imidazole; lanes 7 and 8, elutes with the buffer containing 200 mM imidazole.

Table 1
Catalytic properties of recombinant LdNH[a]

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1}/s^{-1})
Inosine	329 ± 143 (445 ± 209)	7.6 ± 4.2 (119 ± 3.4)	2.3×10^4 (2.7×10^5)
Uridine	241 ± 34 (234 ± 112)	9.5 ± 2.0 (32 ± 6)	3.9×10^4 (1.3×10^5)
Cytidine	362 ± 74 (422 ± 175)	16.1 ± 2.2 (0.36 ± 0.05)	4.4×10^4 (8.5×10^2)
Adenosine	182 ± 88 (145 ± 46)	1.1 ± 0.2 (0.57 ± 0.04)	6.0×10^3 (3.1×10^3)
Guanosine	144 ± 23 (140 ± 23)	2.3 ± 0.2 (0.59 ± 0.03)	1.6×10^4 (4.2×10^3)
p-NPR[b]	178 ± 39 (185 ± 31)	26.7 ± 3.4 (220 ± 17)	1.5×10^5 (1.2×10^6)

[a] The substrate specificity of the recombinant *L. major* nucleoside hydrolase is included in parentheses for comparison (Shi et al., 1999).
[b] *p*-Nitrophenyl-β-D-ribofuranoside.

activity, and the nonspecific nucleoside hydrolase was the only enzyme in the protein extract that efficiently utilized *p*-nitrophenyl-β-D-ribofuranoside as a substrate. The molecular weight of LdNH was estimated by immunoblotting using parasite protein extracts and concentrated exo-antigens separated by SDS-PAGE. The anti-LdNH antiserum was specific and reacted strongly with the nucleoside hydrolase recombinant protein even at a dilution of 1:10,000 (data not shown). Western blot detected LdNH in *L. donovani* promastigote protein extracts with a molecular weight of ~34 kDa, in agreement with the predicted molecular size (Fig. 5). The slightly diffused appearance of the bands might be due to glycosylation, as several N-glycosylation sites are found in the enzyme. However, there was no apparent deviation in molecular weight from the predicted size, which implies that post-translational modifications, if present, should be minor. In addition, there was no significant difference in enzyme level during growth of *L. donovani* promastigotes, further corroborating the hypothesis of constitutive expression. The antiserum also recognized a protein of ~60 kDa, probably resulting from cross-reaction or common shared epitopes with LdNH. Interestingly, the Western blot revealed only a very faint band in the lane for exo-antigens (data not shown). Even though the LdNH gene was isolated by screening the expression libraries with antibodies to whole *Lds*/e, this finding implies that LdNH is probably not secreted by the parasite. The antibodies to *L. donovani* exo-antigens probably reacted to LdNH that leaked out from the parasites or was released from lysed parasites. IFAs with polyclonal antiserum to LdNH specifically detected immunoreactive foci in *L. donovani* promastigotes in contrast to the background labeling with the preimmune serum (Fig. 6). Such focalized labeling of LdNH may indicate that the enzyme is located in specific organelles or secretory vesicles.

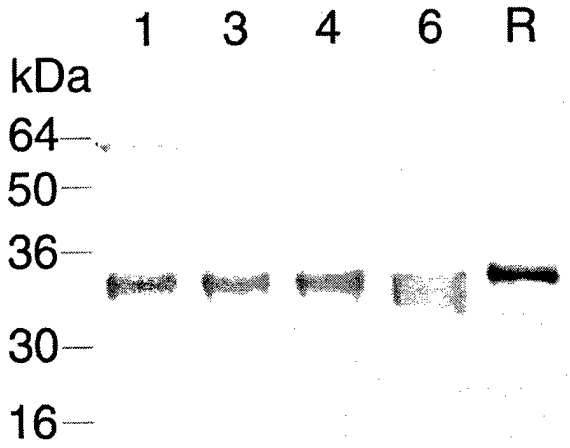

Fig. 5. Western blot of *L. donovani* promastigote protein extracts. Protein extracts were made from *L. donovani* promastigotes at days 1, 3, 4, and 6 of an *in vitro* culture (lanes 1, 3, 4, and 6, respectively). The protein extracts (100 μg/lane) were separated in 12% SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting with rabbit polyclonal antiserum against the recombinant LdNH. Note the slightly larger size of the recombinant LdNH (lane R, 35.27 kDa) due to the presence of six histidines at the carboxyl terminus, compared to the native LdNH from the parasite (34.24 kDa).

4. Discussion

Numerous reports demonstrate the essential roles of nucleoside salvage for parasitic protozoa, and biochemical properties of the enzymes involved in the pathways have largely been characterized (Hassan and Coombs, 1988). Nucleoside N-hydrolase is implicated as an important catalytic activity of purine salvage in several trypanosomatid and other parasites. The absence of this enzyme in the mammalian hosts makes it an ideal target for chemotherapy. Although many nucleoside hydrolases have been biochemically characterized from protozoan parasites, only a few genes have been identified, including the nonspecific or inosine–uridine-preferring nucleoside hydrolases from *L. major* and *C. fasciculata*, the purine-specific N-ribohydrolase from *T. brucei brucei*, and the dUTP hydrolase from *L. major* (Gopaul et al., 1996; Camacho et al., 1997; Pelle et al., 1998; Shi et al., 1999). In this paper, we have presented a detailed study of a nonspecific nucleoside hydrolase from *L. donovani*. This enzyme shows 95% sequence identity to the *L. major* enzyme, despite the fact that they cause human leishmaniasis with totally different clinical manifestations. Among the trypanosomatids, the three sequenced inosine–

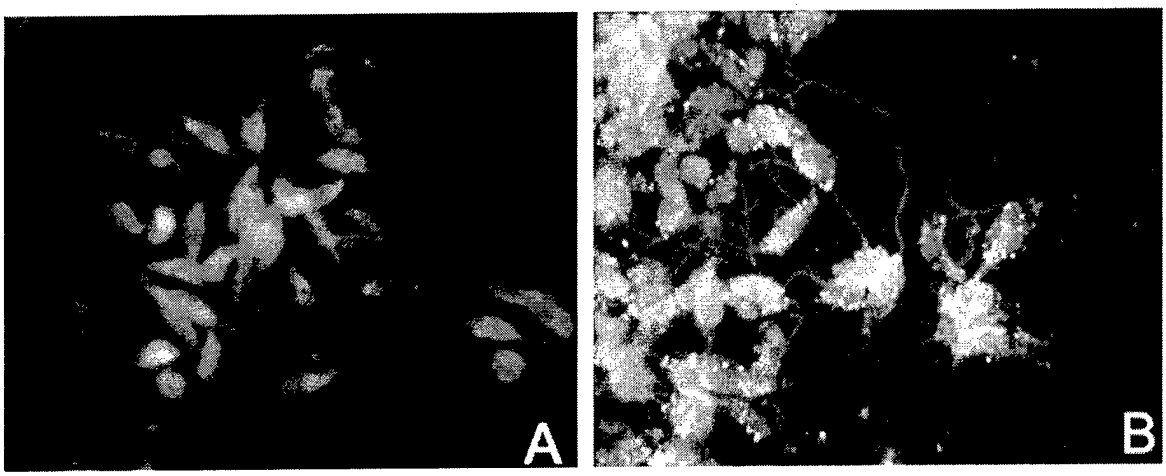

Fig. 6. Immunofluorescent staining of *L. donovani* promastigotes. Parasites incubated with pre-immune serum showed background staining (A), whereas parasites incubated with anti-LdNH antiserum showed specifically stained fluorescent microbodies (B). 400×.

uridine-preferring and nonspecific ribohydrolases are highly homologous to each other, but quite diverged from the purine-specific ribohydrolase from *T. brucei brucei*. Nevertheless, they are perfectly conserved at the catalytic center, and therefore likely employ similar mechanisms of catalysis. Conservation of the nucleoside hydrolases among these trypanosomatid species indicates that this enzyme may play an essential role in the purine salvage pathway. We have further shown by Southern analysis that there is a high degree of conservation of the nonspecific nucleoside hydrolase gene among several Old and New World *Leishmania* species, which strongly suggests the ubiquitous existence of similar enzymes in *Leishmania*. Accordingly, drugs targeting nucleoside hydrolase in one parasite species may be effective against a wide range of parasite species. Such a ubiquitous presence and sequence conservation in *Leishmania* may provide us with a useful tool for molecular phylogenetic study of these species and disease diagnosis. In fact, nucleoside hydrolase activity has been exploited for quantitative determination of *Leishmania infantum* promastigote growth *in vitro* (Garin et al., 2001).

Most protozoan parasites studied to date have multiple nucleoside hydrolases with distinct catalytic properties (Estupinan and Schramm, 1994; Parkin et al., 1991). In *L. major*, in addition to the nonspecific nucleoside hydrolase, a dUTP nucleotidohydrolase has been identified and cloned (Camacho et al., 1997, 2000). In *L. donovani*, three distinct nucleosidases have been purified (Koszalka and Krenitsky, 1979). Two are ribonucleosidases with different substrate preferences: one is a purine nucleosidase; the other cleaves both purines and pyrimidines. The LdNH identified here resembles the latter based on the similarity in (1) p*I* value (predicted 6.5 vs. estimated 6.3), (2) substrate preference, and (3) molecular weight (predicted tetramer of 137 kDa vs. estimated particle weight of 180 kDa). The presence of different nucleoside hydrolases in a parasite species may reflect the parasite's response to the nucleoside compositions in their living environments. It may also represent certain degrees of redundancy, where deficiency of one enzyme may be well compensated by the presence of others to ensure nucleoside salvage. Therefore, further functional analyses of individual nucleoside hydrolases in purine and pyrimidine metabolisms are necessary for more rational drug designs targeting these vulnerable metabolic steps.

Both *L. major* and *L. donovani* nonspecific nucleoside hydrolases have relatively high K_m values for the preferred substrates – inosine, uridine, and cytidine (Shi et al., 1999). Yet, it is not known how the parasites achieve higher intracellular concentrations of these pyrimidine nucleosides above the low levels found in their environment. The sources of the nucleosides during the parasite growth are not known. Even though internalization of degradation of host nucleic acid products is a tempting hypothesis, data to implicate a plausible mechanism are presently lacking. In *L. donovani* promastigote, for example, a membrane associated 3′ nucleotidase has been described which is capable of converting RNA into nucleotides (Sacci et al., 1990; Debrabant et al., 2000). This and earlier *in vitro* uptake studies have documented the ability of *L. donovani* promastigotes to obtain purines from nucleic acids in the medium. However, a clear definition of a sustainable external source of nucleosides is lacking. It is in this regard that data indicating parasite nucleoside hydrolase activity in spent medium are appealing. Yet, we want to emphasize that it is more likely that nucleosides are transported into the cell and then cleaved into bases and ribose, as the majority of nucleoside hydrolase activity is located intracellularly. Hydrolytic enzymes released by the parasite may create a purine-rich environment external to the parasite, which may also be imported to the cell via compatible nucleoside transporters. Evidence of purine transport into the parasite cytoplasm is provided by the cloning and characterization of nucleoside transporters from many protozoan parasites (reviewed in Carter et al., 2001). It is noteworthy that

some nucleoside transporters can also transport purine bases efficiently.

Rabbit polyclonal antiserum detected a major protein in the parasite cell lysates that migrated with a molecular mass approximate to that predicted from the LdNH ORF. It is intriguing that both enzyme activity assays and immunoblotting did not reveal a significant amount of LdNH in the exo-antigens in spite of the fact that the LdNH cDNA clone was identified using antibodies to whole *Lds*/e. More importantly, the characterized nucleoside hydrolases do not have a signal peptide for secretion. Immunofluorescence analysis with FITC-conjugated secondary antibodies revealed a punctate pattern for LdNH localization, suggesting that the enzyme may be compartmentalized. A recent study demonstrates that the *L. donovani* HGPRT, another enzyme of the purine salvage pathway, is localized exclusively in the glycosome, and the tripeptide SKV was determined to be the targeting signal (Shih et al., 1998). However, it is not clear why HGPRT is targeted to the glycosomes, which usually harbor fuel-metabolizing enzymes. The compartmentalized locations of purine salvage enzymes in parasites require additional attention when designing drugs, as drugs targeting the nucleoside salvage enzymes need to traverse intracellular membranes in order to reach their targets. Whether nucleoside hydrolase is localized to special cellular compartments in the parasite remains to be determined.

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