TWO PHYLOGENETICALLY DIVERGENT IMP DEHYDROGENASES IN THE LIFE CYCLE OF *TRYPANOSOMA BRUCEI*

by

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(Under the Direction of Boris Striepen)

ABSTRACT

Millions world-wide are afflicted by diseases caused by the trypanosomatids, *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania*. These parasites are unable to synthesize purines *de novo*; instead, they acquire pre-assembled nucleobases and nucleosides from the host through the salvage pathway. Enzymes involved in this pathway are essential for parasite viability and differ from those of the mammalian host. Therefore, they are potential drug targets. One central enzyme is IMP dehydrogenase which catalyzes the rate-limiting, penultimate step in the production of guanine nucleotides and has been described as an excellent drug target in other systems. Interestingly, two highly-divergent IMPDHs are present in the genomes of *T. brucei*, *T. cruzi* and *L. donovani*. One of these has been characterized in the insect stages of *T. b. gambiense* and *L. donovani*. However, the role of the second gene is unknown. In this study the role of the two IMPDHs in trypanosomatids was determined using *T. brucei* as a model.

INDEX WORDS: Inosine Monophosphate Dehydrogenase, Purine Salvage pathway, *Trypanosoma brucei*, *Trypanosoma cruzi, Leishmania*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Trypanosomatids are vector-borne protozoan parasites that are the causative agents of a variety of tropical diseases. These parasites include *Trypanosoma brucei*, *Trypanosoma cruzi* and several subspecies of *Leishmania*.

Table 1.1. Diseases caused by Trypanosomatids

Disease	Causative Agent	Insect Vector	Disease Burden	Distribution	Treatment
African Trypanosomiasis (Sleeping Sickness)	*Trypanosoma brucei*	Tsetse fly	300-500 Thousand	Sub-Saharan Africa	*Early stage*: Suramin, Pentamidine *Late stage:* Melarsoprol, Eflornithine, Nifurtimox
American Trypanosomiasis (Chagas' Disease)	*Trypanosoma cruzi*	Triatomine bug	18-20 Million	Central and South America	Benznidazole, Nifurtimox
Leishmaniasis (Visceral, Mucocutaneous and Cutaneous)	*Leishmania* spp	Sand fly	12 million	Africa, Asia, Europe, North & South America	Pentostam, Amphotericin

African Trypanosomiasis

Trypanosoma brucei is the causative agent of African Trypanosomiasis or Sleeping Sickness that is a reemerging disease and currently afflicts at least 300,000 to 500,000 individuals in 36 countries of Sub-Saharan Africa [1, 2]. Two clinically distinct forms of Sleeping Sickness are prevalent in different geographic areas. Disease caused by *Trypanosoma*

brucei rhodesiense is prevalent in East Africa and progresses very rapidly with more than 80% of mortality occurring within the first six months. Prevalent in West Africa, the progression of disease caused by *Trypanosoma brucei gambiense* is rather slow, with mortality occurring several years after the initial onset of disease [3]. *Trypanosoma brucei brucei* is a subspecies of *T. brucei* that infects mammals, but not humans and causes nagana, a severe, often fatal disease in cattle [1]. Transmitted by the tsetse fly, the parasites initially replicate and produce a small sore or chancre at the bite site. Parasites then enter the blood and lymph where they reproduce massively. During this time, the host undergoes many cycles of high and low parasitemia due to antigenic variation, a parasitic mechanism that allows the parasites to evade the host immune response. The genome of *T. brucei* contains hundreds of variant surface glycoprotein (VSG) genes, only one of which is expressed at any given time. Millions of these VSG molecules form a dense coat on the surface of the parasite and the host immune system produces antibodies against and eliminates the parasite population that is expressing that particular VSG. However, a portion of the parasite population in a host randomly switches expression to an antigenically distinct VSG. Continuous antigenic switching allows the parasites to evade the host immune response and establish a long-lasting and chronic infection [4]. Symptoms characteristic of this phase are sporadic periods of fever, headaches, joint pain and itching. The second or the neurological phase occurs when the parasites cross the blood-brain barrier. This stage is characterized by confusion, sensory disturbances and poor coordination. Perhaps the most characteristic feature of the disease is sleep cycle disturbances, which gives the disease its name [5, 6].

Treatment for African Trypanosomiasis involves chemotherapy and is stage-specific. Pentamidine and Suramin are the drugs usually used to treat the first phase of the disease while Melarsoprol and Eflornithine are used to treat the second phase of the disease. However, several

limitations are associated with these drugs including high toxicity and the emergence of drug resistance [7, 8].

American Trypanosomiasis

American Trypanosomiasis or Chagas Disease is caused by *Trypanosoma cruzi* and afflicts 18-20 million people in Central and South America [9]. Disease distribution includes the southern United States where it is currently limited to animals: Chagas disease was recently found in San Benito, TX where three dogs died due to Chagas mediated cardiomyopathy [10]. Transmission occurs when a triatomine bug takes a blood meal and simultaneously defecates on the skin of the host. The parasite-infested feces are accidentally introduced into the bite site or near by mucosal membranes of the eye and mouth. Although, the parasites are able to invade any kind of host tissue, they more readily invade cells of the central nervous system and muscle cells of the heart and digestive system. *T. cruzi* invades and multiplies inside these cells, generating large numbers of parasites that eventually burst out and proceed to invade other cells. The disease is characterized as two stages, the acute and the chronic stage. The former is usually asymptomatic while the latter is associated with loss of muscle tone resulting in enlargement of the organs of the digestive system. Furthermore, cardiomegaly can also develop leading to cardiac failure and sudden death. Approximately 40% of individuals infected with *T. cruzi* develop Chagas mediated cardiac disease and only 10% develop Chagas mediated digestive system abnormalities [9, 11].

Treatment for American Trypanosomiasis consists entirely of chemotherapy. However, treatment is only effective during the acute stage of the disease. The drugs used are benznidazole or nifurtimox. No drugs are in clinical use for the treatment of the chronic stage. Instead, treatment is usually focused on managing the symptoms associated with the disease [12].

Leishmaniasis

Leishmania is transmitted by the bite of an infected sand fly and is the causative agent of Leishmaniasis. At least 12 million people world-wide in five continents are believed to be afflicted with some form of the disease [13]. Three distinct clinical manifestations exist which display different degrees of disease severity, morbidity and mortality. The least severe of these is Cutaneous Leishmaniasis (CL) caused by *L. major* and *L. tropica*. When these parasites are introduced into the skin by the sand fly, they invade and replicate inside reticulo-endothelial and lymphoid cells in and around the bite site causing lesions that are usually self-curing. Mucocutaneous Leishmaniasis (MCL), caused by *L. mexicana* and *L. braziliensis*, occurs when parasites are able to move from the bite site to mucocutaneous regions where they produce lesions that can cause severe damage to the nose, mouth and throat. The disease manifestation that is the most severe and is responsible for the most morbidity and mortality is Visceral Leishmaniasis or Kala Azar caused by *L. donovani*. *L. donovani* is able to move from the bite site to the visceral organs of the spleen and liver, causing fever, weight loss, anemia and swelling of these organs. In all manifestations of the disease, the parasites continually invade and replicate inside reticulo-endothelial and lymphoid cells, the very cells that are important in host defense [13, 14].

Pentavalent antimonials are the first line of treatment against Leishmaniasis. Recent emergence of drug resistance has been associated with these drugs, however. Amphotericin B and pentamidine are the second line of treatment when the first line is ineffective. However, the use of these drugs is limited due to their high toxicity [15].

As aforementioned, the fight against diseases caused by the trypanosomatids, *T. brucei*, *T. cruzi* and *Leishmania* relies primarily on chemotherapeutic strategies. Most of these drugs were developed over 50 years ago and are associated with several shortcomings including

limited efficacy in the treatment of all three diseases, as well as high toxicity and the emergence of drug resistance in the treatment of African Trypanosomiasis and Leishmaniasis [7, 12, 15]. This has prompted the search and development of novel drug targets.

The Salvage Pathway of Purine Biosynthesis as a drug target

Protozoan parasites including the trypanosomatids are entirely dependent on their host to provide them with nutrients. For many essential nutrients the parasites have abandoned the cost and effort of *de novo* synthesis and instead have evolved highly efficient salvage pathways. This phenomenon has been well-studied for purine metabolism.

The purines, guanine and adenine, are essential for the viability of all organisms. Firstly, they are the basic building blocks of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Additionally, purines are also involved in the transport of chemical energy as phosphate groups or electrons and as coenzyme components.

Purine biosynthesis is typically carried out via two distinct pathways, the *de novo* and the salvage pathway. In the *de novo* pathway, step-wise assembly of the purine ring system occurs from ribose-phosphate, amino acids, carbon dioxide and ammonia. In the salvage pathway, however, the nucleosides and nucleobases are obtained extrinsically from the environment and are subsequently recycled. Unlike their mammalian hosts, which utilize the *de novo* as well as the salvage pathway, most protozoan parasites including trypanosomatids are completely dependent on the salvage pathway for purine biosynthesis [16, 17]. These parasites have a high replication rate and thus are dependent on a constant and ready supply of purine precursors from their hosts. Additionally, the enzymes involved in the salvage pathway in the trypanosomatids are distinct from those of the host. Since the pathway is crucial for the viability of the parasites, and diverges from that used by the mammalian host, the enzymes involved could be potential drug targets. In fact, allopurinol has been shown to be toxic to all insect and mammalian forms

of *T. cruzi* [18] as well as mammalian forms of *L. donovani* [19, 20] and has been tested to be therapeutically effective against chronic Chagas' disease in many cases [21]. Allopurinol is an analog of hypoxanthine and inhibits xanthine oxidase thus preventing the production of xanthine.

The Salvage Pathway in the glycosomes of Trypanosomatids

The trypanosomatid cell contain numerous (up to 65), identical organelles that resemble peroxisomes of higher eukaryotes. Similar to peroxisomes, these glycosomes are surrounded by a single phospholipid bilayer membrane, have a matrix that is electron and protein-dense and do not contain any detectable organellar DNA [22]. Glycosome localized proteins are synthesized by free ribosomes in the cytoplasm and contain a peroxisomal targeting sequence or PTS. There are three types of PTSs and the best characterized of these is PTS1 which is found on the C-terminus of many glycosomal proteins and usually consists of the tri-peptide, Serine-Lysine-Leusine (SKL). Variations include alanine or cysteine at the first position, and lysine, histidine or arginine at the second position [23, 24]. Import of proteins into the glycosome first involves recognition and interaction of PTS with a cytosolic receptor. After interaction of the bound receptor with a docking complex located at the glycosomal membrane, import across the membrane occurs [25]. Glycosomes are essential for the viability of the parasite. The organelle is home to enzymes of the following pathways: Beta-oxidation of fatty acids, ether lipid synthesis, glycolysis, isoprenoid synthesis, as well as purine and pyrimidine salvage [26]. It is interesting that enzymes involved in the purine salvage pathway, which are cytosolic in all other eukaryotic organisms, are localized entirely in the glycosome in trypanosomatids [27-29]. Currently it is not known why this is the case, however it may be speculated that compartmentalization might increase efficiency of the pathway given that it is crucial for parasite viability and survival.

As in most organisms, trypanosomatids harbor redundant salvage pathways. Therefore, the parasites are able to obtain any purine nucleobase (adenine, guanine, hypoxanthine or xanthine) or nucleoside (adenosine, guanosine, or inosine) as they have all the enzymes necessary to interconvert between adenine monophosphate (AMP), inosine monophosphate (IMP) and guanine monophosphate (GMP) [30-33]. However, the trypanosomatids have preference for the salvage of nucleobases over nucleosides [30].

Translocation of purines, in the form of nucleobases or nucleosides across the trypanosomal membrane is the initial step in the salvage of purines from the host. Nucleoside and nucleobase transporters have been extensively studied in *Trypanosoma brucei*.

The insect stage of *T. brucei* harbors a single nucleoside transporter, P1, which transports adenosine and inosine and a single nucleobase carrier, H1, which transports hypoxanthine and other purine nucleobases [35]. The mammalian stage harbors additional nucleoside and nucleobase transporters. In this stage P1 transports adenosine and inosine as in the insect form [36], P2 is involved in the transport of adenosine and adenine [36] and H2 and H3 are involved in the transport of purine nucleobases [37].

Salvageable purines in the human host include hypoxanthine and inosine [38]. Therefore, the enzymes associated with these purines are very important for the viability of the parasite. After translocation of hypoxanthine, Hypoxanthine-phosphoribosyltransferase (HGPRT) converts it to inosine monophosphate (IMP). When inosine is translocated, it is first converted to hypoxanthine and then to IMP. IMP is then converted to XMP by IMP dehydrogenase (IMPDH). The final step in the purine salvage pathway is the conversion of XMP to guanine monophosphate [Figure 1.2].

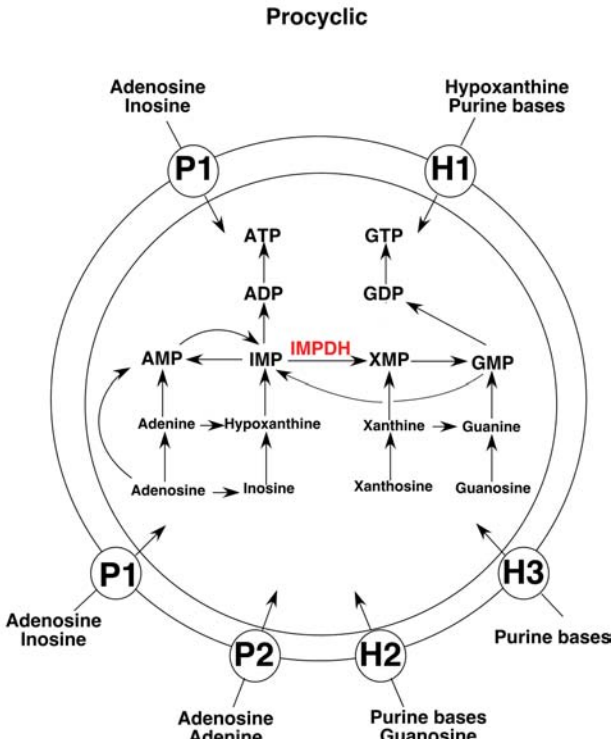

Figure 1.2: Schematic of the *T. brucei* purine salvage pathway (modified from 34)

Inosine 5'-Monophosphate Dehydrogenase

IMP dehydrogenase catalyzes the oxidation reaction of IMP to XMP with the concomitant reduction of NAD to NADH [Figure 1.2]. The crystal structures of several IMPDHs have been characterized including the *Streptococcus pyogenes* [39], *Tritrichomonas foetus* [40], and the human Type II [41] IMPDH. The enzyme exists as a tetramer of alpha and beta barrels and the active site is present in the mobile loop at the C-terminal end of the beta barrels. The IMP and NAD interact in a long cleft at the active site and are covered by a flap. First, cysteine residues on IMP undergo a nucleophilic attack forming a tetrahedral structure with the enzyme. This is followed by a hydride transfer from the enzyme-IMP intermediate to NAD+. After the release of NADH, the flap repositions itself onto the empty NAD site, which causes transformation of the enzyme into a hydrolase. Subsequent hydrolysis of the enzyme-XMP intermediate to XMP and recovery of the free enzyme occurs [42, 43].

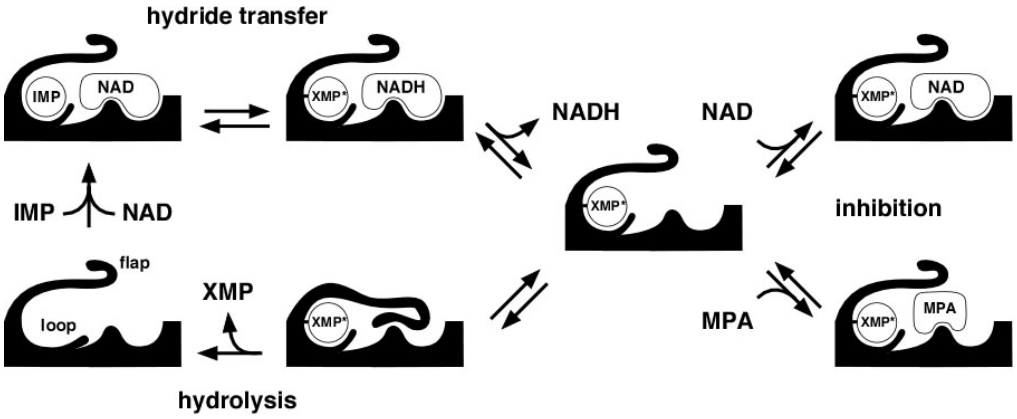

Figure 1.2: Oxidation of IMP to XMP catalyzed by Inosine Monophosphate Dehydrogenase [reproduced from 39]

This reaction is the rate-limiting, penultimate step in both the *de novo* and the salvage pathway of guanine nucleotide biosynthesis. Therefore, IMPDH is an attractive drug target since it plays such a central role in nucleotide biosynthesis. Many IMPDH inhibitors have already been identified as having anticancer [44], antiviral [45] or immunosuppressive [46] properties. One of these, Mycophenolic acid (MPA) is a non-competitive inhibitor of IMPDH that binds to the empty NADH site, thereby prohibiting the repositioning of the flap, and in turn preventing hydrolysis to produce XMP. MPA is species-specific and strongly inhibits eukaryotic but poorly inhibits prokaryotic IMPDHs. In the NAD binding sites, two residues are not conserved between eukaryotic and prokaryotic IMPDHs. Eukaryotic enzymes contain Arginine and Glutamine, which are conducive to MPA binding, whereas prokaryotic enzymes contain Lysine and Glutamic acid at these positions. Furthermore, after the release of NADH, eukaryotic enzymes remain in the open flap conformation while prokaryotic enzymes remain in the closed flap conformation. This allows MPA to bind to eukaryotic enzymes more readily then to prokaryotic enzymes. [47-51]. Differences in drug selectivity suggest that the eukaryotic and prokaryotic IMPDHs while providing the same catalytic function have unique structural and kinetic

characteristics. These characteristic differences could be exploited to design and develop species-specific drugs.

Wilson et al. have identified an IMP dehydrogenase gene in the insect stages of *Trypanosoma brucei gambiense* [52] and *Leishmania donovani* [53]. They found that the enzyme was susceptible to a low mycophenolic acid concentration with the EC50 value of 0.45 uM for *T. brucei* and 1 uM for *L. donovani*. Furthermore, they isolated parasites that were resistant to mycophenolic acid-associated cell death and found that these parasites had amplified the IMPDH gene locus ten-fold or over. These resistant parasites had an EC50 value of 7.5 uM and 100 uM for *T. brucei* and *L. donovani* respectively. Interestingly, we found the presence of a second IMPDH gene in the genomes of *T. brucei*, *T. cruzi* and *L. donovani* that appears to be highly-divergent from the one previously described by Wilson et al.

Previously in our lab, we found an IMP dehydrogenase of prokaryotic origin in the apicomplexan parasite, *Cryptosporidium parvum* [54]. By analyzing the phylogeny, we concluded that the *C. parvum* IMP dehydrogenase gene was obtained via horizontal gene transfer (HGT) from a proteobacterium. This finding was further supported by analysis of the kinetic characteristics of the *C. parvum* enzyme. Major differences were observed in the amino acid sequences between the NAD binding sites of the human and the *C. parvum* enzymes which paralleled differences measured in the affinity of the two enzymes for NAD and mycophenolic acid. Similar to other prokaryotic IMPDHs, *C. parvum* IMPDH has a higher affinity for NAD and is resistant to MPA-mediated inhibition. These marked differences in the NAD binding sites between the human and *C. parvum* enzymes can be exploited to develop drugs that are parasite-specific [55]. Furthermore, additional transfers were identified in *C. parvum* of genes some of which are involved in pyrimidine metabolism, energy production, amino acid biosynthesis and carbohydrate metabolism [56, 57, 58]. Genes acquired by HGT have also been identified in

other protozoan parasites [59] namely, *Giardia lamblia* [60] and *Entamoeba histolytica* [60, 61]. These include genes involved in iron-sulfur cluster assembly in *E. histolytica*. Iron-sulfer proteins are integral in energy metabolism, DNA repair, regulation of transcription as well as nucleotide and amino acid biosynthesis [61]. Additionally, in both *E. histolytica* and *G. lamblia*, genes acquired by lateral transfer encode enzymes involved in the fermentation pathway which is essential for survival in the anaerobic conditions that these parasites are exposed to in the intestines of their hosts [60]. In summary, recent work has shown that horizontally transferred genes play key roles in the metabolism of a number of parasitic protozoa. Why have these organisms seemingly abandoned their eukaryotic machinery for a prokaryotic gene? This might be due to sampling bias but could also have some biological explanation. One attractive hypothesis is that evolutionary processes such as HGT might have served as mechanisms to generate metabolic diversity and pre-adapted parasites for the colonization of new hosts, which exposed them to a divergent nutritional environment. On a more applied note, enzymes acquired from prokaryotes through HGT might provide novel parasite specific drug targets due to the large phylogenetic distance between parasite and host enzyme. In this study I test these hypotheses using the two phylogenetically divergent IMPDH genes in the trypanosomatids as a model.

Trypanosoma brucei as a model to study IMP Dehydrogenase in Trypanosomatids

In these studies I used *Trypanosoma brucei* as a model to study the role of the highly-divergent IMPDHs in trypanosomatids. *T. brucei* serves as an excellent model due to the ease with which different life cycle stages can be cultivated and manipulated through highly effective RNA interference approaches. *T. brucei* develops through a series of defined life cycle stages in the insect and mammalian host [62, 63]. These stages show differential morphologies and gene expression patterns. Stumpy forms are taken up by the tsetse fly during the course of a blood

meal. A drop in temperature along with chemical cues results in transformation into procyclic trypomastigotes that grow and replicate in the midgut of the insect [64]. These migrate to the salivary glands of the tsetse fly where they proliferate as epimastigote forms. Subsequent differentiation into the infectious and non-dividing metacyclic trypomastigote stage occurs. During a blood meal, thousands of these forms are injected into the vertebrate host where they differentiate into slender trypomastigotes which proliferate in the blood stream. These bloodstream trypomastigotes enter the blood through the lymphatic system, are carried to different sites of the body and can differentiate into non-dividing stumpy forms that are taken up by another tsetse fly during.

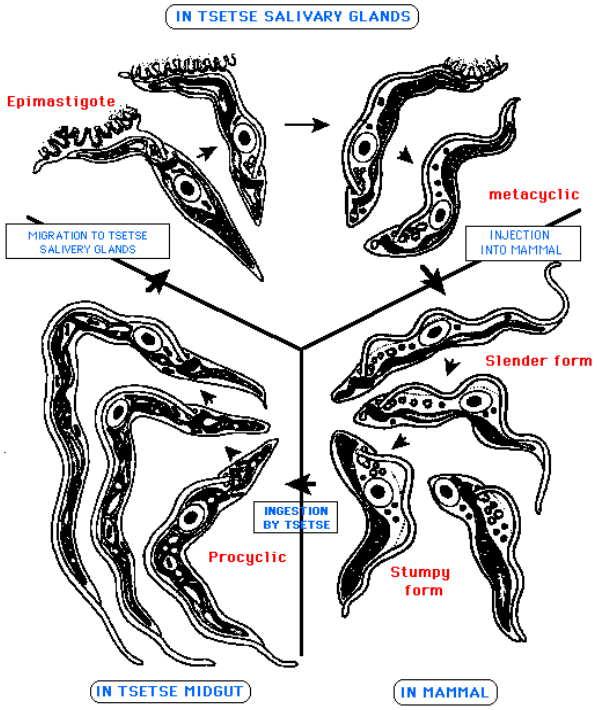

Figure 1.3: Life cycle of *Trypanosoma brucei* [reproduced from 63]

Both the procyclic trypomastigotes and the bloodstream trypomastigotes are entirely extracellular and can be grown in culture, whereas in *T. cruzi* and *L. donovani* the mammalian stage is primarily intracellular and therefore more difficult to cultivate and manipulate experimentally.

The recent development of RNA interference in *T. brucei* has made it easier to analyze the role of a particular gene of interest. One potential cellular function of RNAi is hypothesized to be the protection of the genome against dsRNA of viruses and transposons. In the presence of dsRNA, an endonuclease (DICER) binds to and cleaves the dsRNA, forming 21-25 nucleotide sequences called small interfering RNAs (or siRNAs). These siRNAs then bind to and activate the RNAi-induced silencing complex (RISC). Subsequent binding of the siRNA-RISC to homologous mRNA causes its degradation by RISC [65, 66, 67]. Therefore, induction of dsRNA, can lead to the degradation of target mRNA which has a complementary sequence. RNAi can cause target gene repression in both the insect [68, 69] as well as in the mammalian [70] stages of *T. brucei*.

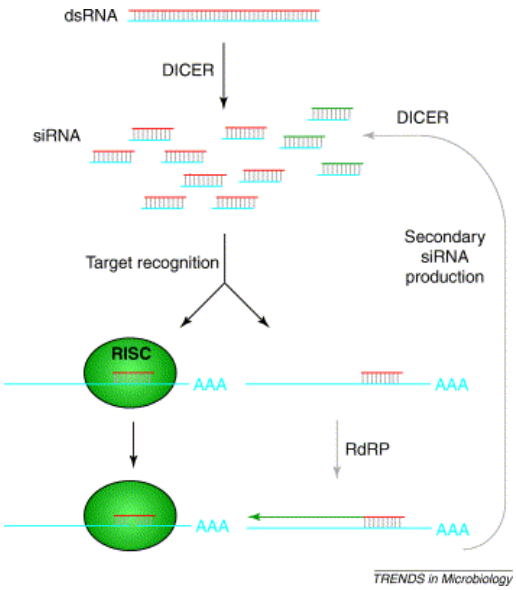

Figure 1.4: RNA interference [reproduced from 65]

Some limitations are associated with the technique, however. For example, RNAi does not cause repression of all targeted genes. Furthermore, an "RNAi escape" mechanism has emerged in which, after a certain period of time, the RNAi ceases to be effective. These parasites might display resistance due to loss of the dsRNA cassette by recombination of the flanking repeats. Lastly, the RNAi is known to result in target gene repression in *T. brucei* but

not in *T. cruzi* [71] or in *Leishmania* [72]. Nevertheless, RNAi has truly provided a powerful tool in the study of gene function in the insect and mammalian life cycle stages of *Trypanosoma brucei*.

References

[1] World Health Organization (2001). African Trypanosomiasis, Fact sheet number 259 (WHO Publications, Geneva).

[2] Moore, A., Richer, M. (2001). Re-emergence of epidemic sleeping sickness in southern Sudan. Tropical Medicine and International Health. 6 (5): 342-347.

[3] Welburn, S.C., Fevre, E.M., Coleman, P.G., Odiit, M., Maudin, I. (2001). Sleeping Sickness: a tale of two diseases. Trends in Parasitology. 17 (1): 19-24.

[4] Pays, Etienne, Vanhamme, L., Perez-Morga, D. (2004). Antigenic Variation in *Trypanosoma brucei*: facts, challenges and mysteries. Current opinion in Microbiology. 7: 369-374.

[5] Lundkvist, G.B., Kristensson, K., Bentivoglio, M. (2004). Why Trypanosomes Cause Sleeping Sickness. Journal of Physiology. 19: 198-206.

[6] Mhalanga, J.D., Bentivoglio, M., Kristensson, K. (1997) Neurobiology of cerebral malaria and African sleeping sickness. Brain Research Bulletin. 44(5): 579-589.

[7] Fairlamb, A.H. (2003). Chemotherapy of human African trypanosomiasis: current and future prospects. Trends in Parasitology. 19 (11): 488-494.

[8] Matovu, E., Seebeck, T., Enyaru, J.C.K., Kaminsky, R. (2001). Drug resistance in *Trypanosoma brucei* spp., the causative agents of sleeping sickness in man and nagana in cattle. Microbes and Infection. 3: 763-770.

[9] World Health Organization. 1998. Chagas' disease: tropical diseases progress in research, 1997-1998. WHO technical report series. World Health Organization, Geneva, Switzerland.

[10] Beard, C.B., Pye, G., Steurer, F.J., Rodriguez, R., Campman, R., Peterson, A.T., Ramsey, J., Wirtz, R.A., Robinson, L.E. (2003). Chagas Disease in a Domestic Transmission Cycle in Southern Texas, USA. Emerging Infectious Diseases. 9 (1): 103-105.

[11] Tanowitz, H.B., Kirchhoff, L.V., Simon, D., Morris, S.A., Weiss, L.M., Wittner, M. (1992). Chagas' disease. Clinical Microbiology Review. 5 (4): 400-419.

[12] Urbina, J.A., Docampo, R. (2003). Specific chemotherapy of Chagas disease: controversies and advances. Trends in Parasitology. 19 (11): 495-501.

[13] World Health Organization (2000). The leishmaniasis and Leishmania/HIV co-infections, Fact sheet number 116 (WHO Publications, Geneva).

[14] Herealdt, B.L. (1999). Leishmaniasis. Lancet. 354(9185): 1191-1199.

[15] Croft, S.L., Coombs, G.H. (2003). Leishmaniasis—current chemotherapy and recent advances in the search for novel drugs. Trends in Parasitology. 19 (11): 502-508.

[16] Berens, R.L., Krug, E.C, Marr, J.J. (1995) Purine and pyrimidine metabolism. In Biochemistry and Molecular Biology of Parasites. J.J. Marr, Muller, M., editor. Academic Press, London. 89-117.

[17] Berens, R.L., Marr, J.J., LaFon, S.W., Nelson, D., J. (1981). Purine Metabolism in *Trypanosoma cruzi*. Molecular and Biochemical Parasitology. 3: 187-196.

[18] Berens, R. L., Marr, J. J., Da Cruz, F. S., Nelson, D. J. (1982). Effect of Allopurinol on *Typranosoma cruzi*: Metabolism and biological activity in intracellular and bloodstream forms. Antimicrobial Agents and Chemotherapy. 22(4): 657-661.

[19] Berman, J.D., Webster, H.K. (1982). In vitro effects of Mycophenolic Acid and Allopurinol against *Leishmania tropica* in Human Macrophages. Antimicrobial Agents and Chemotherapy. 21(6): 887-891.

[20] Momeni, A.Z., Reiszadae, M.R., Aminjavaher, M. (2002). Treatment of cutaneous leishmaniasis with a combination of allopurinol and low-dose meglumine antimoniate. International Journal of Dermatology. 41: 441-443.

[21] Gallerano, R., Marr, J., Soza, R. (1990). Therapeutic efficacy of allopurinol in patients with chronic Chagas disease. American Journal of Tropical Medicine and Hygiene. 43: 159-166.

[22] Tetley, L., Vickerman, K. (1991). The glycosomes of trypanosomes: number and distribution as revealed by electron spectroscopic imaging and 3-D reconstruction. Journal of Microscopy. 162 (1): 83-90.

[23] Keller, G., Krisans, S., Gould, S.J., Sommer, J.M., Wang, C.C., Schliebs, W., Kunau, W., Brody, S., Subramani, S. (1991). Evolutionary Conservation of a Microbody Targeting signal that targets proteins to peroxisomes, glyoxysomes, and glycosomes. The Journal of Cell Biology. 114(5): 893-904.

[24] Blattner, J., Swinkels, B., Dorsam, H., Prospero, T., Subramani, S., Clayton, C. (1992). Glycosome assembly in Trypanosomes: Variations in the acceptable degeneracy of a COOH-terminal Microbody Targeting Signal. The Journal of Cell Biology. 119 (5): 1129-1136.

[25] Parsons, M., Furuya, T., Pal, S., Kessler, P. (2001). Biogenesis and function of peroxisomes and glycosomes. Molecular & Biochemical Parasitology. 115:19-28.

[26] Michels, P.A.M., Hannaert, V., Bringaud, F. (2000). Metabolic aspects of glycosomes in trypanosomatidae—New data and views. Parasitology Today. 16 (11): 482-489.

[27] Shih, S., Stenberg, P., Ullman, B. (1998). Immunolocalization of *Trypanosoma brucei* hypoxanthine-guanine phosphoribosyltransferase to the glycosome. Molecular and Biochemical Parasitology. 92: 367-371.

[28] Shih, S., Hwang, H.Y., Carter, D., Stenberg, P., Ullman, B. (1998). Localization and targeting of the *Leishmania donovani* hypoxanthine-guanine phophoriolsyltransferase to the glycosome. Journal of Biochemical Chemistry. 273 (3): 1534-1541.

[29] Zarella-Boitz, J.M., Rager, N., Jardim, A., Ullman, B. (2004). Subcellular localization of adenine and xanthing phophoriosyltransferases in *Leishmania donovani*. Molecular and Biochemical Parasitology. 134(1): 43-51.

[30] Davies, M.J., Ross, A.M., Gutteridge, W.E. (1983). The enzymes of purine salvage in *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania mexicana*. Parasitology. 87: 211-217.

[31] Fish, W.R., Looker, D.L., Marr, J.J., Berens, R.L. (1982). Purine metabolism in the bloodstream forms of *Trypanosoma gambiense* and *Trypanosoma rhodesiense*. Biochimica et Biophysica Acta. 719: 223-231.

[32] Ceron, C.R., Caldes, R.A., Felix, C.R., Mundim, M.H., Roitman, I. (1979). Purine Metabolism in Trypanosomatids. J. Protozoology. 26 (3): 479-483.

[33] Ogbunude, P.O., Ikediobi, C.O. (1983). Comparative aspects of Purine Metabolism in some African Trypanosomes. Molecular and Biochemical Parasitology. 9: 279-287.

[34] Ullman, B., Carter, D. (1997). Molecular and Biochemical Studies on the Hypoxanthine-guanine Phosphoribosyltransferases of the pathogenic Haemoflagellates. International Journal of Parasitology. 27 (2): 203-213.

[35] De Koning, H.P., Watson, C.J., Jarvis, S.M. (1998) Characterization of a Nucleoside/Proton Symporter in procyclic *Trypanosoma brucei brucei*. The Journal of Biological Chemistry. 273 (16): 9486-9494.

[36] Landfear, S.M., Ullman, B., Carter, N.S., Sanchez, M.A. (2004). Nucleoside and Nucleobase Transporters in Parasitic Protozoa. Eukaryotic Cell. 3(2): 245-254.

[37] de Koning, H.P., Jarvis, S.M. (1997). Purine nucleobase transport in bloodstream forms of *Trypanosoma burcei brucei* is mediated by two novel transporters. Molecular and Biochemical Parasitology. 89: 245-258.

[38] Hartwick, R.A., Krustulovic, A. M. and Brown, P.R. (1979). Identification and quantification of nucleosides, bases and other uv-absorbing compounds in the serum using reversed-phase high-performance liquid chromatography. II. Evaluation of human sera. Journal of Chromatography. 186: 659-676.

[39] Zhang, R., Evans, G., Rotella, F.J., Westbrook, E.M., Beno, D., Huberman, E., Joachimiak, A., Collart, F.R. (1999). Characteristics and Crystal Structure of Bacterial Inosine-5'-monophosphate Dehydrogenase. Biochemistry. 38: 4691-4700.

[40] Whitby, F.G., Luecke, H., Kuhn, P., Somoza, J.R., Huete-Perez, J.A., Philips, J.D., Hill, C.P., Fletterick, R.J., Wang, C.C. (1997). Crystal structure of *Tritrichomonas foetus* Inosine-5'-monophosphate Dehydrogenase and the Enzyme-Product Complex. Biochemistry. 36: 10666-10674.

[41] Colby, T.D., Vanderveen, K., Strickler, M.D., Marham, G.D., Goldstein, B.M. (1999). Crystal structure of human type II inosine monophosphate dehydrogenase: Implications for ligand binding and drug design. PNAS. 96: 3531-3536.

[42] Sintchak, M.D., Nimmesgern, E. (2000). The structure of inosine 5'-monophosphate dehydrogenase and the design of novel inhibitors. Immunopharmacology. 47: 163-184.

[43] Hedstrom, L. (1999). IMP Dehydrogenase: Mechanism of Action and Inhibition. Current Medicinal Chemistry. 6: 545-560.

[44] Robins, R. (1982). Nucleoside and nucleotide inhibitors of inosine monophosphate (IMP) dehydrogenase as potential antitumor inhibitors. Nucleosides & Nucleotides. 1: 35-44.

[45] Malinoski, F., Stollar, V. (1981). Inhibitors of IMP dehydrogenase prevent Sindbis virus replication and reduce GTP levels in *Aedes albopictus* cells. Virology. 110: 281-291.

[46] Allison AC, Eugui EM. (1993). Inhibitors of *de novo* purine and pyrimidine synthesis as immunosuppressive drugs. Transplantation Proc 25: 8-18.

[47] Franklin, T.J., Cook, J.M. (1969) The inhibition of nucleic acid synthesis by mycophenolic acid. Biochem. J. 113: 515-524.

[48] Allison, A.C., Engui, E.M. (2000) Mycophenolate mofetil and its mechanisms of action. Immunopharmacology. 47: 85-118.

[49] Digits, J.A. and L. Hedstrom. (1999) Species-specific inhibition of inosine 5'-monophosphate dehydrogenase by mycophenolic acid. Biochemistry. 38(46): 15388-97.

[50] Digits, J.A., Hedstrom, L. (2000). Drug selectivity is determined by coupling across the NAD+ site of IMP dehydrogenase. Biochemistry. 39: 1771-1777.

[51] Gan, L., Petsko, G. A., Hedstrom, L.A. (2002). Crystal structure of a ternary complex of Trichomonas foetus inosine 5' monophosphate dehydrogenase: NAD+ orients the active site loop for catalysis. Biochemistry. 41: 13309-13317.

[52] Wilson, K., Berens, R.L., Sifri, C.D., Ullman, B. (1994). Amplification of the Inosinate Dehydrogenase Gene in *Trypanosoma brucei gambiense* due to an increase in chromosome copy number. The Journal of Biological Chemistry. 269(46): 28979-87.

[53] Wilson, K., Collart, F.R., Huberman, E., Stringer, J.R., Ullman, U. (1991). Amplification and molecular cloning of the IMP dehydrogenase gene of *Leishmania donovani*. The Journal of Biological Chemistry. 266 (3): 1665-1671.

[54] Striepen, B., White, M.W., Li, C., Guerini, M.N., Malik, S.B., Logsdon, J.M., Liu, C., Abrahamsen, M.S. (2002). Genetic complementation in apicomplexan parasites. PNAS. 99(9):6304-6309.

[55] Umejiego, N.N., Li, C., Riera, T., Hedstrom, L., Striepen, B (2004). *Cryptosporidium parvum* IMP Dehydrogenase. The Journal of Biological Chemistry. 279 (39): 40320-40327.

[56] Striepen, B., Pruijssers, A.J.P., Huang, J., Li, C., Gubbels, M., Umejiego, N.N., Hedstrom, L., Kissinger, J. (2004). Gene transfer in the evolution of parasite nucleotide biosynthesis. PNAS. 101 (9): 3154-3159.

[57] Huang, J., Mullapudi, N., Lancto, C.A., Scott, M., Abrahamsen, M.S., Kissinger, J.C. (2004). Phylogenomic evidence supports past endosymbiosis, intracellular and horizontal gene transfer in *Cryptosporidium parvum*. Genome Biology. 5(11).

[58] Huang, H., Mullapudi, N., Sicheritz-Ponten, T., Kissinger, J.C. (2004). A first glimpse into the pattern and scale of gene transfer in the Apicomplexa. International Journal for Parasitology. 34: 265-274.

[59] Richards, T.A., Hirt, R.P., Williams, B.A.P., Embley, T.M (2003). Horizontal Gene Transfer and the Evolution of Parasitic Protozoa. Protist. 154: 17-32.

[60] Nixon, J.E.J., Wang, A., Field, J., Morrison, H.G., McArthur, A.G., Sogin, M.L., Loftus, B.J., Samuelson, J. (2002). Evidence for Lateral Transfer of Genes Encoding Ferredoxins, Nitroreductases, NADH Oxidase, and Alcohol Dehydrogenase 3 from Anaerobic Prokaryotes to *Giardia lamblia* and *Entamoeba histolytica*. Eukaryotic Cell. 1(2): 181-190.

[61] Van der Giezen, M., Cox, Sian, Tovar J. (2004). The iron-sulfur cluster assembly genes iscS and iscU of *Entamoeba histolytica* were acquired by horizontal gene transfer. Evolutionary Biology. 4.

[62] Matthews, K.R., Ellis, J.R., Paterou, A. (2004). Molecular regulation of the life cycle of African trypanosomes. Trends in Parasitology. 20 (1): 40-47

[63] Vickerman, K. (1985). Developmental cycles and biology of pathogenic trypanosomes. British Medical Bulletin. 41 (2): 105-114.

[64] Engstler, M., Boshart, M. (2004). Cold shock and regulation of surface protein trafficking convey sensitization to inducers of stage differentiation in *Trypanosoma brucei*. Genes and Development. 18(22): 2798-2811.

[65] Cottrell, T., Doering, T.L., Silence of the strands, RNA interference in eukaryotic pathogens. Trends in Microbiology. 11 (1): 37-43.

[66] Agrawal, N., Dasaradhi, P.V.N., Mohmmed, A., Malhotra, P. (2003). RNA interference: Biology, Mechanism, and Applications. Microbiology and Molecular Biology Reviews. 67(40): 657-685.

[67] Ullu, E., Tschudi, C., Chakraborty, T. (2004). RNA interference in protozoan parasites. Cellular Microbiology. 6(6): 509-519.

[68] Ngo, H., Tschudi, C., Gull, K., Ullu, E., Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. PNAS. 95: 14687-14692.

[69] Wang, Z., Morris, J.C., Drew, M.E., Englund, P.T. (2000). Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. The Journal of Biological Chemistry. 275 (51): 40174-40179.

[70] Morris, J.C., Wang, Z., Drew, M.E., Paul, K.S., Englund, P.T. (2001). Inhibition of bloodstream form *Trypanosoma brucei* gene expression by RNA interference using the pZJM dual T7 vector. Molecular & Biochemical Parasitology. 11: 111-113.

[71] DaRocha, W.D., Otsu, K., Teixeira, S.M.R., Donelson, J.E. (2004). Tests of cytoplasmic RNA interference (RNAi) and construction of a tetracycline-inducible T7 promoter system in *Tryapanosoma cruzi*. Molecular and Biochemical Parasitology. 133: 175-186.

[72] Robinson, K.A., Beverley, S.M. (2003). Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite *Leishmania*. Molecular & Biochemical Parasitology. 128: 217-228

CHAPTER 2

TWO PHYLOGENETICALLY DIVERGENT IMP DEHYDROGENASES IN THE LIFE CYCLE OF *TRYPANOSOMA BRUCEI*[1]

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[1]Kanchagar, C., Malik, B., Subramanya, S., Logsdon, J., Hedstrom, L., Mensa-Wilmot, K, Tarleton, R.L., and Striepen, B. To be submitted to *Molecular Microbiology.*

2.1 Abstract

The ability to efficiently salvage nucleotide precursors from the host is critical for the survival and viability of all protozoan parasites. Multiple gene transfers from bacteria have previously been documented in this pathway in *Cryptosporidium parvum* which led us to hypothesize that metabolic diversity generated by evolutionary processes such as gene transfer, could pre-adapt parasites for the colonization of new hosts which exposed them to divergent nutritional environments. To test this hypothesis we mined the genomes of several protozoan parasites for genes involved in the salvage pathway.

Interestingly, we observed that the genomes of *T. brucei*, *T. cruzi* and *L. donovani* harbor two genes encoding inosine monophosphate dehydrogenase (IMPDH). IMPDH is a central enzyme in the salvage pathway and catalyzes the penultimate step in the production of guanine nucleotides. One of the trypanosomatid IMPDHs has been previously described in the insect stages of *T. b. gambiense* and *L. donovani* and is of clear eukaryotic origin (IMPDH 1). However, phylogenetic analysis of the second, previously unknown gene revealed that it is highly divergent from IMPDH 1 and consistently groups with prokaryotic IMPDHs (IMPDH 2). In this study we demonstrate IMPDH 1 and 2 are differentially expressed in the life cycle of *T. brucei*. RNA and protein for IMPDH 1 is only detected in the insect stage whereas IMPDH 2 is detected in the mammalian stage. Furthermore, down regulation of IMPDH 1, by RNAi, results in reduced growth in procyclics while bloodstream trypomastigotes show no adverse effects. The opposite is found for IMPDH 2. Finally, bloodstream trypomastigotes show reduced sensitivity to the IMPDH inhibitor, mycophenolic acid, when compared with procyclics. This result is consistent with bloodstream form dependence on IMPDH 2. IMPDH 2 shows a high degree of similarity to prokaryotic enzymes in its NAD binding site which are indicative of mycophenolic acid resistance.

2.2 Introduction

Parasites are entirely dependent on their host to provide them with nutrients. For many essential nutrients the parasites have abandoned the cost and effort of *de novo* synthesis and instead have evolved highly efficient salvage pathways. This phenomenon has been well studied for purine metabolism. Protozoan parasites are entirely dependent on salvage for the acquisition of purine precursors (nucleobases and nucleosides) from the host [1]. Purines are essential for the viability of all organisms: they are the basic building blocks of DNA and RNA, are involved in the transport of chemical energy as phosphate groups or electrons and are essential components of several enzymatic reactions. An important enzyme in this pathway is inosine monophosphate dehydrogenase (IMPDH), which catalyzes the oxidation of inosine monophosphate (IMP) to xanthine monophosphate (XMP) with the concurrent reduction of NAD to NADH. IMP and NAD interact in a long cleft at the active site, which is enclosed by a mobile flap. IMP undergoes a nucleophilic attack and a hydride transfer occurs from the enzyme-IMP intermediate to NAD. After the release of NADH, the flap repositions itself onto the empty NAD site, transforming the enzyme into a hydrolase. The enzyme-XMP intermediate is then hydrolyzed which causes the subsequent release of XMP [2, 3]. This reaction is the rate-limiting, penultimate step in the production of guanine nucleotides. IMPDH is an essential enzyme in most cells and has been established as a drug target in anti-viral, cancer and immunosuppressive therapy [4, 5, 6].

Previously we have shown that the protozoan parasite, *Cryptosporidium parvum*, harbors an IMPDH gene that groups with eubacteria in phylogenetic analysis, which suggested that the gene was acquired by horizontal gene transfer [7]. Major differences in the amino acid sequences between the NAD binding sites of the human and the *C. parvum* enzymes which paralleled differences measured in the affinity of the two enzymes for NAD and mycophenolic

acid (MPA). Unlike the human enzyme and similar to other bacterial IMPDHs, *C. parvum* IMPDH has a higher affinity for NAD. MPA is a potent inhibitor of eukaryotic IMPDHs and a poor inhibitor of prokaryotic IMPDHs. MPA binds to the NAD binding site and traps the enzyme-XMP intermediate. Interestingly, *C. parvum* IMPDH was found to be resistant to MPA-mediated inhibition. Differences in the NAD binding sites between the human and *C. parvum* enzymes can be exploited to develop drugs that are parasite-specific [8]. Comparative genomic studies have identified a number of additional gene transfers in *C. parvum*, most of these genes encode metabolic enzymes [9, 10, 11]. Genes acquired by HGT are not restricted to *C. parvum* but have also been identified in other protozoan parasites [12] namely, *Giardia lamblia* and *Entamoeba histolytica* [13]. These include genes involved in iron-sulfur cluster assembly in *E. histolytica*. Iron-sulfer proteins are integral in energy metabolism, DNA repair, regulation of transcription as well as nucleotide and amino acid biosynthesis. Additionally, in both *E. histolytica* and *G. lamblia*, genes acquired by HGT encode enzymes involved in the fermentation pathway which is essential for survival in the anaerobic conditions that these parasites are exposed to in the intestines of their hosts [14].

In summary, recent work has shown that horizontally transferred genes play key roles in the metabolism of a number of parasitic protozoa. Why have these organisms seemingly abandoned their eukaryotic machinery for a prokaryotic gene? One attractive hypothesis is that evolutionary processes such as HGT might have served as a mechanism to generate metabolic diversity and pre-adapted parasites for the colonization of new hosts, which exposed them to a divergent nutritional environment. On a more applied note, enzymes acquired from prokaryotes through HGT might provide novel parasite specific drug targets due to the large phylogenetic distance between parasite and host enzyme.

Therefore, we searched the genomes of several protozoan parasites for an IMPDH gene that seemed to be of divergent origin. Interestingly, we found that the genomes of the trypanosomatids, *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* harbor two highly divergent IMPDH genes. One of these has been previously described in the insect stages of *T. brucei gambiense* [15] and *L. donovani* [16] and displays clear eukaryotic origin (IMPDH 1). The significance of the highly-divergent IMPDH 2 is not known.

Trypanosomatids are vector-borne protozoan parasites that cause a variety of tropical diseases afflicting millions of individuals world-wide [17, 18, 19]. These diseases cause significant morbidity and mortality and the available options for drug treatment are associated with high toxicity, low potency and threatened by emerging resistance [20, 21, 22]. The identification of highly divergent enzymes might provide avenues for the development of parasite specific inhibitors.

We have studied the expression and biological relevance of the two trypanosomatid IMPDH genes using *T. brucei* as model system. In this system the insect and mammalian life cycle stages are easily cultivated [23, 24] and are very amenable to genetic experimentation. Excellent tools to down-regulate and study essential gene functions are available [25] and can be applied to the insect [26, 27] as well as the mammalian [28] life cycle stages.

2.3 Materials and Methods

Parasites

T. b. brucei procyclic forms of the strain 427 were cultured and maintained by serial passage at 28ºC in SM medium [23] supplemented with 15% heat inactivated fetal bovine serum (HyClone), and 1% antibiotic-antimytotic solution (Cellgro; 10,000 I.U. penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B). *T. b. brucei* bloodstream forms, strain 427, were

cultured and maintained by serial passage at 37ºC in HMI-9 medium [24] supplemented with 10% fetal bovine serum and 1% antibiotic-antimytotic solution.

Procyclic *T. b. brucei* strain 29-13 and bloodstream *T. b. brucei* strain 90-13 [29] were used for the induction of RNA interference. Procyclic forms were grown in SM media additionally supplemented with 15 μg/ml G418 and 50 μg/ml hygromycin (Calbiochem) to maintain the T7 RNA polymerase and the tetracycline repressor transgenes respectively. Bloodstream forms were grown in HMI-9 media supplemented with 2.5 μg/ml G418 and 5 μg/ml hygromycin.

Reagents

All restriction enzymes were obtained from New England Biolabs (Beverly, MA). All chemicals except for those noted were obtained from J.T. Baker (Phillipsburg, NJ), Fisher Scientific (Suwanee, GA) or Sigma-Aldrich (St.Louis, MO).

Database mining and sequence analysis

Protein sequences homologous to IMPDH in eukaryotes, bacteria and archaea were found by using *Cryptosporidium parvum*, *Plasmodium falciparum*, *E. coli* and Human Type 1 IMPDH amino acid sequences as queries for BLASTP searches of the NCBI non-redundant database and BLASTP and TBLASTN searches of the NCBI eukaryotic genomes database [30]. Similarly, homologs of IMPDH were retrieved from the protist genome sequence databases of *Trypanosoma brucei, Trypanosoma cruzi, Toxoplasma gondii, Theileria parva, Perkinsus marinus* and *Tetrahymena thermophila* at The Institute for Genomic Research (TIGR)*, Theileria annulata*, *Cyanidioschyzon merolae* [31], *Dictyostelium discoideum*, *Phytophthora sojae* and *Thalassiosira pseudonana* [32] and of *Paramecium tetraaurelia* [33] by BLASTP and TBLASTN with both *L. major* IMPDH homologs as queries. The genome databases of *Trichomonas vaginalis*, *Entamoeba histolytica* (TIGR) and *Giardia intestinalis* were also searched by TBLASTN.

Putative open reading frames for *Theileria, Paramecium* and *Perkinsus* IMPDH homologs were inferred and annotated from genomic nucleotide sequences using the Sequencher software (Genecodes). The inferred translated peptide sequences for all of the IMPDH homologs were aligned using ClustalX [34] and the multiple sequence alignment inspected and adjusted manually using MacClade [35]. Gaps in the alignment representing insertion/deletion events, and ambiguous portions of the alignment flanking such gaps, were deleted from the alignments that were used for phylogenetic analyses.

Phylogenetic trees were inferred from these alignments using Tree-Puzzle5.0 [36] and MrBayes 3.0b4 [37] software, in both cases using the WAG model for amino acid substitutions and accounting for among-site substitution rate heterogeneity with an invarying and eight gamma-distributed substitution rate categories (abbreviated as WAG+I+8G). Trees were drawn using the Neighbor program in the PHYLIP package [38] based on the distance matrices inferred using Tree-Puzzle. A 185 sequence alignment of 430 amino acids (including the CBS domain) was analyzed with Tree-Puzzle, and those sequences that failed the chi-squared test for amino acid composition were removed from the alignment. The alignment was then subjected to analysis with MrBayes for 1000000 generations, with four Markov chains, the temperature parameter set to 0.5, and every 1000th tree sampled. Trees were drawn using Treeview [39].

Expression of *T.brucei* IMPDH 1 and IMPDH 2 in *E.coli*

The coding region of *T. brucei* IMPDH 1 and IMPDH 2 was amplified by polymerase chain reaction using primers that contained synthetic *BamHI* and *HindIII* sites (IMPDH 1: ATCGGATCCATGGAAAACACCAACCTACGCACC, GATAAGCTTTTAGAGCTTCGAGGCAAAGAGTTT; IMPDH 2: CGGATCCATGTCCTTCAATGAATCGGCATCC, GATAAGCTTTTAAAGTTTGGCAACACCGTGAC). The PCR products were purified,

restricted with *BamHI* and *HindIII* and ligated into the expression vector, pTacTac [40]. The cloned vectors were transformed into TX685 or STL8224 *E. coli* cells that lack endogenous IMPDH expression due to a mutation in the GuaB locus [41, 42]. To test for complementation, the transformed TX685 cells were plated on LB and minimal media (5x M9, 1M $MgSO_4$, 1M $CaCl_2$, 0.0005% $FeSO_4$, 2% glucose, 9.6 µg/ml tryptophan, 48 µg/ml histidine, 5 µg/ml tyrosine, 0.1 ng/ml thymine)

Western Blot assay

$2.5x10^6$ parasites were harvested, resuspended in 1X sample loading buffer and 1X reducing agent (Invitrogen), and heated to 75ºC for 10 minutes. The sample was loaded on a pre-cast 10% SDS-PAGE gel (Invitrogen), and electrophoreses was carried out at 100V for 2 hours. Proteins were transferred to a nitrocellulose membrane (Invitrogen) for two hours at 100V or overnight at 20V in blotting buffer (25 mM Tris, 190 mM glycine (Biorad) in 20 % Methanol). Membranes were blocked (5% milk, 1% BSA, 0.1% tween (Omnipur) in PBS) for 30 minutes and then incubated with either antisera against *L. donovani* IMPDH 1 or IMPDH 2 a kind gift of Dr. Armando Jardin (McGill University) or anti-HGPRT antiserum (obtained from Dr. Buddy Ullman, Oregon Health and Science University) for one hour at a dilution of 1:500. The membrane was then incubated with goat anti-guinea pig or anti-rabbit antibody conjugated to alkaline phosphatase (Biorad) at a dilution of 1:3000 for one hour. IMPDH or HGPRT bound antibody on the membrane were visualized by incubation with developing buffer (100 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$) containing Nitroblue tetrazolium (NBT, Biorad—AP Color Kit) and 5-bromo,4-chloro,3-indolylphosphate (BCIP, Biorad—AP Color Kit)

RNA interference experiments

RNAi Vector

Vector pZJM was used to induce RNA interference in *T. b .brucei*, a kind gift of Dr. James Morris at Clemson University. A 500 bp fragment of the coding region of the IMPDH 1 and 2 gene sequence was amplified by PCR using primers that introduced flanking *XhoI* and *HindIII* sites (ACTAAGCTTTCTAGATGTCCTTCAATGAATCGGCATCC, AGTCTCGAGGGATGTATTCGTCGAAACCACC, ACTAAGCTTTGGAAAACACCAACCTACGCACC, and GATCTCGAGTCTAGATATTGCGACACCGGAGCACTGG). The PCR products were purified, restricted with *XhoI* and *HindIII* and ligated in between the two opposing T7 promoters in the pZJM plasmid replacing the tubulin sequence [Figure 2.7A, 43].

Trypanosome transfection and selection

$2.5x10^7$ Procyclic trypomastigotes and 25 µg of *Not I* linearized plasmid DNA were used for each transfection. Parasites were harvested, washed and resuspended in cytomix (120 mM KCL, 0.15 mM $CaCl_2$, 10 mM K2HPO_4, 25 mM Hepes, 2 mM EDTA, 5 mM $MgCl_2$). Electroporation was carried out with a single pulse at 1.45 kV in a 4mm cuvette. Transfection of bloodstream trypomastigotes followed the same protocol with slight modifications. For each transfection, $1x10^8$ bloodstream trypomastigotes and 100 µg of *Not I* linearized plasmid DNA were used. Furthermore, electroporation was carried out at 1.7 kV. Subsequent to transfection stable transformants were selected in media supplemented with G418 and hygromycin using culture conditions suitable for procyclic and bloodstream trypomastigotes. Sixteen hours after transfection, the parasites were placed under selection by addition of 2.5 µg/ml phleomycin (Calbiochem). In order to induce the formation of dsRNA, parasites were cultured in the presence of 1.0 µg/ml tetracycline (Calbiochem). Parasite density was determined every twenty-

four hours for procyclic trypomastigotes and every 12 hours for bloodstream trypomastigotes using a Becton Dickinson FACS Calibur cytometer. For long-term experiments, parasites were diluted 10-fold when the density reached $1x10^6$.

RNA isolation, generation of cDNA and RT-PCR

Total RNA from $1x10^7$ tetracycline-induced and induced parasites was extracted with TriZol (Invitrogen) three days after induction. Parasites were incubated for 5 minutes in Trizol reagent, followed by addition of 0.2 ml chloroform. After 3 min incubation, phases were separated by centrifugation and 0.5 ml of isoproponol was added to the upper aqueous layer containing the RNA. The RNA was then pelleted by centrifugation, washed with 75% ethanol and air-dried briefly. The RNA was then dissolved in DEPC treated H_20 (Omnipur) and quantified spectrometrically.

1 µg RNA was incubated with 10 mM deoxynucleotide (dNTP) mix and 500 µg/ml oligo $(dT)_{12-18}$ (Invitrogen) for ten minutes at 65ºC. The RNA was then mixed with 0.1M dithiothreitol (Invitrogen), 40 U/ul Recombinant Ribonuclease inhibitor (Promega), and incubated at 42ºC for two minutes and after the addition of Superscript II Reverse Transcriptase (Invitrogen) incubated at 42ºC further for 50 minutes. The reaction was terminated by a 15 minute incubation at 70ºC after which the RNA was digested by addition of RNase H (New England Biolabs) for 20 minutes at 37ºC.

The subsequent PCR step was conducted using the cDNA from above as a template, gene-specific primers used for creating the RNAi construct, and DNA Polymerase. Primers against alpha-tubulin were used as a control (CACCTCGAGATGCGTGAGGCTATTGCATC and CACAAGCTTAGGTTGCGGCGAGTCAAATC). The PCR was conducted in a Peltier Thermal Cycler 200 (MJ Research) as follows: 1 min, 94ºC; 30 sec, 94ºC; 30 sec, 55ºC; 2 min,

68ºC for 40 cycles; and finally 10 min, 72ºC. PCR products were analyzed by agarose gel electrophoreses, and viewed by an electronic ultraviolet transilluminator.

Mycophenolic Acid Treatment

Cultures initiated with $5x10^4$ procyclic or bloodstream trypomastigotes were treated with 0, 0.5, 1, 5, or 10 μM mycophenolic acid (6-(4-Hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid). Parasites were counted every 24 hours for procyclic forms and every 12 hours for bloodstream forms using a Becton Dickinson FACS Calibur cytometer.

2.4 Results

Trypanosomatids harbor two phylogenetically divergent genes for IMP dehydrogenase

To test the hypothesis that evolutionary processes might serve as a mechanism to generate metabolic diversity and pre-adapt parasites for the colonization of new hosts, genomes of protozoan parasites were first mined for divergent IMPDHs using the BLAST algorithm and IMPDHs of various organisms as query sequences. Interestingly, we observed that the genomes of the trypanosomatids, *Tryanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* encode two distinct IMPDH genes. One of these (referred to subsequently as IMPDH 1) has previously been cloned from the insect stages of *T. brucei* and *L. donovani*. The second, previously unknown enzyme (IMPDH 2) is quite divergent and only shows about 30% amino acid identity with IMPDH 1. Furthermore, its nearest neighbors as identified by BLAST searches against GenBank were prokaryotic IMPDH genes. To account for sequence differences between the two IMPDHs both the *E. coli* as well as the human IMPDH genes were used as query sequences in blast searches (Figure 2.1). At the protein level the IMPDH 1 and 2 enzymes are highly conserved among the trypanosomatids showing 71% amino acid identity and 20% similarity for IMPDH 1 (Figure 2.2A) and 65% identity and 28% similarity for IMPDH 2 (Figure 2.2B).

To determine the evolutionary origin of IMPDH 2, amino acid sequences were aligned and sites that make contact with substrates (IMP and NAD) and/or an inhibitor (MPA) were analyzed in detail. IMPDH enzymes of organisms representative of the three domains of life, archea (*Halobacterium salinarum* and *Pyrococcus furiosus*), eubacteria (*Escherichia coli*) and eukarya (Human) were used in the analysis. The crystal structure of the human type 2 IMPDH has been elucidated [43] which assisted in the identification of sites of interest. Analysis of the multiple sequence alignments by Clustal W [34] demonstrated that the IMPDH 2 of trypanosomatids are quite divergent and appear to be more similar to the prokaryotic IMPDHs than the trypanosomatid IMPDH 1 or the human type 2 IMPDH (Figure 2.2). The active site loop and part of the IMP binding site seems to be conserved in all the IMPDHs analyzed. However, as shown in Figure 2.2, analysis of several residues in the NAD and IMP binding site revealed that IMPDH 2 of trypanosomatids are more similar to the prokaryotic IMPDH (identical residues in red) while IMPDH 1 are more similar to the eukaryotic IMPDHs (identical residues in black). Consistent with this dissimilarity is the analysis of the MPA binding which suggests that the IMPDH 2 enzyme might be more resistant to MPA-mediated inhibition than IMPDH 1.

To further investigate the evolutionary origin of these enzymes we conducted extensive phylogenetic analyses using 185 IMPDH amino acid sequences from a representative sampling of organisms across the tree of life. These analyses revealed that IMPDH 1 of trypanosomatids group within the core group of eukaryotic IMPDHs. On the other hand, IMPDH 2 groups with prokaryotic IMPDH sequences with *Rhodospirillum rubrum*, *Bdellovibrio bacteriovorus* and *Legionaells pneumophila* being its closest neighbors. Data mining of several unassembled protozoan genomes produce two additional eukaryotic homologs from the ciliates *Tetrahymena thermophila* and *Paramecium tetraaurelia*, which belong to the alveolate group. Inspection of the multiple sequence alignment lends support that the trypanosomatid, ciliate and the bacterial

IMPDHs are indeed more related to one another than to the other IMPDHs in the alignment. Thus one could argue that this group is not an artefact of "long branch attraction" (LBA) which may cause divergent sequences to group together randomly rather than due to a shared ancestry [44]. Interestingly, the trypanosomatid IMPDH 2 groups very distinctly separately from either the IMPDHs of Kinetoplastid endosymbionts [45] related to *Bordetella pertussis* or *Pseudomonas putida*, or the IMPDH of the endosymbiont of the tsetse fly, *Wigglesworthia glossinidia* (NCBI GI# 32491354), or, for that matter, mammals.

IMPDH 1 and IMPDH 2 are differentially expressed in the insect and mammalian stages of *T.brucei*

Trypanosomatids harbor two phylogenetically divergent IMPDH genes. To test if both of these genes encode active IMPDH enzymes we cloned the respective coding sequences of *Trypanosoma brucei* IMPDH 1 and 2 into the bacterial expression plasmid, ptactac [40]. The resulting plasmids were introduced into *E. coli* strain TX685 [41] or STL8224 [42] both of which do not express an endogenous IMPDH. To determine whether transformation with *T. brucei* IMPDH 1 or 2 complemented these mutants, these cells were grown on minimal medium lacking guanosine. Only cells that are expressing an active IMPDH will be able to grow under these conditions. While initial assays in TX685 cells failed to demonstrate complementation, robust growth was observed of strain STL8224 expressing *T. brucei* IMPDH 1 or 2 (data not shown). Furthermore, no growth was observed of the parental STL8224 strain. This might suggest that expression in TX685 was not sufficiently robust to allow for complementation. Using a sensitive Western Blot assay, however, expression of recombinant protein was observed in all of the strains. As seen in Figure 2.5 A, anti-sera developed against the *Leishmania donovani* IMPDH 1 and 2 (a kind gift from Dr. Armando Jardim, McGill University) detected recombinant protein in lysates of transformed bacteria that were absent in the parental TX685 strain (Figure 2.5A). The

apparent molecular masses of these protein bands matched the predicted masses of IMPDH 1 (55 kDa) and IMPDH 2 (52 kDa) precisely. The anti-sera were cross-reactive and recognized both the recombinant IMPDH 1 and 2 proteins; however, the difference in size nevertheless permits us to distinguish between the two enzymes. For subsequent analysis we used anti-sera against the *L. donovani* IMPDH 1. This anti-serum equally detects the two proteins in western blot analysis of cell lysates prepared from *T. brucei* procyclic and bloodstream trypomastigotes. However, only a single band was detected in the respective lysates. A band consistent with IMPDH1 is soley detected in lysates of procyclic trypomastigotes while IMPDH 2 seems to be restricted to bloodstream trypomastigotes (Figure 2.5B). Furthermore, a higher molecular weight band was also detected that probably represents a dimer form of IMPDH. An antibody against *T. brucei* HGPRT (a kind gift of Dr. Ullman, 46) was used as a control to eliminate gel-loading discrepancies. As expected, this antibody detected a band of ~28 kDa in western analyses of both bloodstream and procyclic trypomastigotes. We noted a slight (~2 kDa), but reproducible higher gel mobility of IMPDH derived from *T. brucei* lysates when compared to bacterial lysates. This could be due to posttranslational modification, perhaps a processing step that occurs during import of the protein to the glycosomes of the parasite.

Therefore, our western blot analyses demonstrated the differential expression of IMPDH 1 and IMPDH 2 in the insect and mammalian life cycle stages of *Trypanosoma brucei*.

Down-regulation of IMPDH mRNA results in stage-specific *T. brucei* growth inhibition

To study the significance of the highly-divergent IMPDHs for parasite survival and development we used RNA interference to disrupt IMPDH 1 and 2 in *Trypanosoma brucei*. 500 bp fragments of the coding region of IMPDH 1 and 2 (nucleotides 1-500) were amplified by PCR and introduced into plasmid pZJM (a kind gift from Dr. James Morris, 27). In the resulting construct, the IMPDH fragments were flanked by two opposable T7 promoters, which are under

the control of a tetracycline operator (Figure 2.6 A, modified from 27). These plasmids were linearized, and transfected into the *T.brucei* 29-13 procyclic forms and 13-90 bloodstream forms that express the T7 polymerase and the tetracycline repressor [29]. Stable parasite lines were obtained by selection with phleomycin. In order to induce the formation of double stranded RNA, 1 μg/ml tetracycline was added to the culture medium. To study the fate of IMPDH 1 and 2 mRNAs under these conditions, we isolated RNA from tetracycline-uninduced and induced parasites, purified the mRNAs and conducted a reverse-transcription reaction to produce cDNA. RT-PCR experiments detected transcript by amplifying the 500 bp fragment of the IMPDH 1 or 2 coding region from the cDNA. As shown in figure 2.6B, in RNA isolated from bloodstream forms, we detected an IMPDH 2 transcript but no IMPDH 1 transcript. On the other hand, in the procyclic trypomastigote RNA we detected only an IMPDH 1 transcript and no IMPDH 2 transcript. Furthermore, no IMPDH transcript was detected in RNA isolated from tetracycline-induced bloodstream or procyclic trypomastigotes, which demonstrates that induction of dsRNA did cause RNAi mediated down regulation of IMPDH 1 and IMPDH 2 gene expression (Figure 2.6B).

Parasite growth was measured by counting bloodstream forms every 12 hours and procyclic forms every 24 hours for a total of six generations. These experiments demonstrated that in procyclic trypomastigotes, RNAi-mediated down regulation of IMPDH 1, but not IMPDH 2 had an effect on parasite growth. The growth defect due to RNAi-mediated down regulation of IMPDH 1 was observed starting at four days after induction. Additionally, six days after RNAi induction, 62% reduction in parasite number was observed. In bloodstream trypomastigotes, the opposite was true: RNAi of IMPDH 2 but not IMPDH 1 had affected parasite growth. The growth defect due to RNAi-mediated knockdown of IMPDH 2 was observed starting at 60 hours after induction. At 72 hours post-induction, a 51% reduction in parasite number was observed

(Figure 2.6C). Additional experiments were carried out to determine the effect of RNAi-mediated down-regulation of IMPDH 1 or 2 on parasite growth over a longer period of time. In procyclic trypomastigotes, we observed that RNAi of IMPDH 1 resulted in an 88% reduction in parasite number twelve days after induction. In bloodstream trypomastigotes, RNAi of IMPDH 2 led to a 99% reduction in parasite number 168 hours after induction.

To ensure that the growth defect observed was specifically due to RNAi-mediated knock down of IMPDH 1 or 2, a metabolic complementation experiment was performed by adding excess guanine to the medium. *T. brucei* harbors redundant salvage pathways; including an HGPRT [47], which is able to salvage guanine directly and hence, circumvent a block of IMPDH. Figure 2.6 D shows that addition of guanine to the medium complemented the growth defect to some degree in procyclic trypomastigotes and entirely in bloodstream trypomastigotes. Collectively, these results suggest that the highly-divergent IMPDH 1 and 2 are differentially expressed in the insect and mammalian life cycle stages of *T. brucei*. Furthermore, given that RNAi of IMPDH 2 causes a growth defect in bloodstream forms demonstrates its significance for parasite survival and viability during this stage.

Effect of Mycophenolic acid on the growth of *Trypanosoma brucei* procyclic and bloodstream trypomastigotes.

Due to differences in the structure and dynamic properties, eukaryotic and prokaryotic IMPDHs show differential sensitivity to certain inhibitors. Mycophenolic acid (MPA), for example, causes strong inhibition of eukaryotic enzymes but prokaryotic IMPDHs are considerably more resistant. The species-specificity of MPA is partly due to the differences in the amino-acid residues at the NAD binding site of the eukaryotic and prokaryotic enzymes. Arginine 322 and Glutamine 441 (human IMPDH 2 numbering, 43) which is conducive to MPA binding in eukaryotic enzymes is replaced by Lysine and Glutamic acid in prokaryotic enzymes

[48-51]. We inspected residues known to affect MPA sensitivity in both trypanosomatid IMPDHs and found that IMPDH 2 contains Lysine and Glutamine at the NAD site similar to that observed in prokaryotic enzymes (Figure 2.3). This observation predicts that IMPDH 2 should be less susceptible to MPA-mediated inhibition than IMPDH 1. To test this hypothesis, bloodstream and procyclic trypomastigotes were treated with varying concentrations of mycophenolic acid (0, 0.5, 1, 5 and 10 uM) and parasite numbers were measured every 24 hours (procyclic forms) or every 12 hours (bloodstream forms) using a cytometer. As shown in Figure 2.7 A and reported previously by Wilson et al., procyclic trypomastigotes are sensitive to growth inhibition by mycophenolic acid. The effective concentration$_{50}$ (EC50) value in our experiments was 1 μM, which is in the range of what was previously found for *T. b. gambiense* (0.45μM, 15) and *L. donovani* (1 μM, 16). On the other hand, growth inhibition is observed in the bloodstream forms only with treatment of over 10 μM MPA. Thus, a ten-fold higher MPA concentration is needed to inhibit growth of bloodstream when compared with the procyclic trypomastigotes. However, when parasites were treated with more than 20 uM MPA, parasite growth was completely inhibited (data not shown) even after five days of treatment.

2.2 Discussion

Salvage of purine precursors from the host is essential for parasite survival. We have previously shown that gene transfers from bacteria have shaped this pathway in the protozoan parasite, *C. parvum* [7, 8, 9]. We have exploited the abundant genome sequence information that is available for protozoan parasites to determine the extent of the role that HGT plays in purine metabolism. Interestingly, we found the presence of two distinct IMP dehydrogenases (IMPDH 1 and 2) in the genomes of the trypanosomatids, *T. brucei*, *T. cruzi*, and *L. donovani*. IMPDH 1 has been previously cloned and described in the insect stages of *T. brucei* and *L. donovani*,

however, the discovery of a second IMPDH gene in the trypanosomatid genomes is novel and the significance of this is not known.

Through extensive comparative sequence and phylogenetic analyses, we found that trypanosomatid IMPDHs are highly divergent (Figures 2.3 and 2.4). The evolutionary history of IMPDH in the eukaryotes is very complex. Several hypotheses consistent with the phylogentic data set can be built to explain the taxonomic representation of these genes across the eukaryotic tree. Firstly, if Kinetoplastids, Ciliates and Eukaryotes represent a true group, this may support an ancient gene duplication event that occurred early during eukaryotic evolution, prior to the divergence of extant lineages. In this scenerio, one of the paralogs was lost in eukaryotes, ciliates lost the other paralog, while kinetoplastids retained both. Second, the group of Kinetoplastid, Ciliate and *Rhodospirillum* IMPDHs may be due to bacteria to eukaryote or eukaryote to eukaryote horizontal gene transfers (HGT). HGT from bacteria may be explained by Kinetoplastids and Ciliates both independently harboring endosymbionts related closely enough to *Rhodospirillum* to share its divergent IMPDH. Elucidation of the identity of bacterial endosymbionts of the vectors of parasitic kinetoplastids, or of the kinetoplastids themselves, may also help to shed light on putative donors for HGT of IMPDH. However, the Kinetoplastid IMPDH 2 is not apparently nested with strong support within a clade of related bacteria, as is, for example, the *C. parvum* IMPDH, which falls within the group of epsilon-proteobacteria [7]. Therefore, the prokaryotic affiliation of IMPDH 2 is not entirely clear. In terms of eukaryote to eukaryote HGT, ciliates, being heterotrophs, may have engulfed either an autotrophic or colorless euglenozoon that was ancestral to the kinetoplastids included in this study [52, 53], and may have acquired their IMPDH *via* this route, either ancestrally or independently. Lastly, the group of Kinetoplastids, Ciliates and *Rhodospirillum* IMPDHs may be an artifact, due to similar selective pressures or Long Branch Attraction. The availability of IMPDH sequence data from

the genomes of additional prokaryotic and eukaryotic microorganisms in the future may help to distinguish between all of these possibilities with the use of phylogenetic tools.

An obvious question is why do the trypanosomatid genomes harbor two phylogenetically divergent IMPDHs? *T. brucei* develops through a series of defined and morphologically distinct life cycle stages in the insect and mammalian host, which suggests that they are exposed to varying host environments. Therefore, one attractive hypothesis is that evolutionary processes such as gene duplication or horizontal gene transfer generated metabolic diversity and allowed the parasites to survive in the divergent nutritional environments of different hosts. In agreement with this hypothesis, we found that IMPDH 1 and 2 are differentially expressed in the insect and mammalian life stages of *T. brucei*. IMPDH 1 is expressed in the insect or procyclic forms as previously described [15] and IMPDH 2, we found, is expressed in the mammalian or bloodstream forms. This finding was supported by western blot analyses using antibodies developed against the *L. donovani* IMPDH 1 or 2 (Figure 2.5B) as well as RT-PCR analyses (Figure 2.6B), which additionally suggested that differential regulation of IMPDH is occurring at the RNA level. Furthermore, RNAi mediated downregulation of only IMPDH 1 mRNA had an affect on insect stage growth while downregulation IMPDH2 mRNA had an affect on the growth of the mammalian stage (Figure 2.6 C, D). This is consistent with the stage-specific regulation of IMPDH 1 and 2.

Several studies have reported that the mammalian and insect forms are morphologically quite distinct [54]. Several metabolic adaptations to the environment have been identified in the insect stage. An important example that is life cycle stage specific is energy metabolism, which differs dramatically between the mammalian and insect stages [55]. In the mammalian bloodstream the parasite is avail to high concentrations of glucose. Therefore, the parasite is able to acquire glucose from the blood and convert it to pyruvate through the glycolytic pathway.

The insect midgut is depleted of glucose, but is rich in amino acids especially proline (since it is principally used by the tsetse fly during flight) [56]. Therefore, proline may be metabolized by the parasites to produce energy. Interestingly, procyclic trypomastigotes that are grown in a medium that is glucose rich are susceptible to glycolytic inhibitors and those grown in medium that is depleted of glucose are resistant [57]. Furthermore, it has been shown that procyclic trypomastigotes regulate glucose metabolism depending on the availability of proline [58] . Additionally, glucose depletion or RNAi mediated inhibition of glycolysis results in an adaptation in the expression of certain surface antigens [59]. Collectively, these studies indicate that trypanosomes have the ability to regulate their metabolism and use several different pathways during their life cycle depending on the nutrients available in the host environment. Similarly, there might be differences in the availability of nucleobases and nucleosides in the insect and mammalian host, driving the parasites to express one IMPDH or another.

Since IMPDH 2 is phylogenetically distant than the human IMPDH enzyme, it might be selectively targeted. Here, we have shown that disruption of IMPDH gene expression by RNAi led to a reduction in parasite growth (Figures 2.6 C, D). We had predicted that IMPDH 2 would be less sensitive to mycophenolic acid (MPA) based on the multiple sequence alignment of the MPA binding sites (Figure 2.3) and found that bloodstream trypomastigotes (that express IMPDH 2) are in fact less sensitive to MPA-mediated growth inhibition than IMPDH 1 (Figure 2.7). In procyclic trypomastigotes, Wilson et al. observed amplification of the IMPDH gene locus as a result of MPA treatment [15]. These parasites were more resistant to MPA-mediated growth inhibition than wild-type parasites. Although, growth inhibition is observed in procyclic trypomastigotes, these parasites begin to grow similar to untreated parasites after five days of MPA treatment. This might be associated with amplification of the IMPDH locus as described by Wilson et al. However, in bloodstream trypomastigotes, treatment with more than 20uM

MPA resulted in complete growth arrest even after five days of treatment. Hence, it appears that the IMPDH gene amplification that eventually conferred resistance to MPA in procyclic trypomastigotes might not occur as readily in bloodstream forms. Furthermore, bloodstream trypomastigotes, which replicate twice as fast as procyclic trypomastigotes, might be more dependent on a constant pool of guanine nucleotides, and a block in this pathway might be lethal to the parasite. However, additional factors could also contribute to MPA resistance, including the level of IMPDH expression, or potential differences in the purine salvage pathway between the insect and mammalian stages. Nevertheless, IMPDH 2 might be a good choice for a drug target, not only because it is different than its mammalian counterpart, but because it is also very important for parasite survival and viability.

2.3 References

[1] Berens, R.L., Krug, E.C, Marr, J.J. (1995). Purine and pyrimidine metabolism. In Biochemistry and Molecular Biology of Parasites. J.J. Marr, Muller, M., editor. Academic Press, London. 89-117.

[2] Sintchak, M.D., Nimmesgern, E. (2000). The structure of inosine 5'-monophosphate dehydrogenase and the design of novel inhibitors. Immunopharmacology. 47: 163-184.

[3] Hedstrom, L. (1999). IMP Dehydrogenase: Mechanism of Action and Inhibition. Current Medicinal Chemistry. 6: 545-560.

[4] Robins, R. (1982). Nucleoside and nucleotide inhibitors of inosine monophosphate (IMP) dehydrogenase as potential antitumor inhibitors. Nucleosides & Nucleotides. 1: 35-44.

[5] Malinoski, F., Stollar, V. (1981). Inhibitors of IMP dehydrogenase prevent Sindbis virus replication and reduce GTP levels in Aedes albopictus cells. Virology. 110: 281-291.

[6] Allison AC, Eugui EM. (1993). Inhibitors of de novo purine and pyrimidine synthesis as immunosuppressive drugs. Transplantation Proc. 25: 8-18.

[7] Striepen, B., White, M.W., Li, C., Guerini, M.N., Malik, S.B., Logsdon, J.M., Liu, C., Abrahamsen, M.S. (2002). Genetic complementation in apicomplexan parasites. PNAS. 99(9): 6304-6309.

[8] Umejiego, N.N., Li, C., Riera, T., Hedstrom, L., Striepen, B. (2004). *Cryptosporidium parvum* IMP dehydrogenase. The Journal of Biological Chemistry. 27 (39): 40320-40327.

[9] Striepen, B., Pruijssers, A.J.P., Huang, J., Li, C., Gubbels, M., Umejiego, N.N., Hedstrom, L., Kissinger, J. (2004). Gene transfer in the evolution of parasite nucleotide biosynthesis. PNAS. 101 (9): 3154-3159.

[10] Huang, J., Mullapudi, N., Lancto, C.A., Scott, M., Abrahamsen, M.S., Kissinger, J.C. (2004). Phylogenomic evidence supports past endosymbiosis, intracellular and horizontal gene transfer in *Cryptosporidium parvum*. Genome Biology. 5(11).

[11] Huang, H., Mullapudi, N., Sicheritz-Ponten, T., Kissinger, J.C. (2004). A first glimpse into the pattern and scale of gene transfer in the Apicomplexa. International Journal for Parasitology. 34: 265-274.

[12] Richards, T.A., Hirt, R.P., Williams, B.A.P., Embley, T.M (2003). Horizontal Gene Transfer and the Evolution of Parasitic Protozoa. Protist. 154: 17-32.

[13] Van der Giezen, M., Cox, Sian, Tovar J. (2004). The iron-sulfur cluster assembly genes iscS and iscU of *Entamoeba histolytica* were acquired by horizontal gene transfer. Evolutionary Biology. 4.

[14] Nixon, J.E.J., Wang, A., Field, J., Morrison, H.G., McArthur, A.G., Sogin, M.L., Loftus, B.J., Samuelson, J. (2002). Evidence for Lateral Transfer of Genes Encoding Ferredoxins, Nitroreductases, NADH Oxidase, and Alcohol Dehydrogenase 3 from Anaerobic Prokaryotes to *Giardia lamblia* and *Entamoeba histolytica*. Eukaryotic Cell. 1(2): 181-190.

[15] Wilson, K., Berens, R.L., Sifri, C.D., Ullman, B. (1994). Amplification of the Inosine Dehydrogenase Gene in *Trypanosoma brucei gambiense* due to an increase in chromosome copy number. The Journal of Biological Chemistry. 269 (46): 28979-87.

[16] Wilson, K., Collart, F.R., Huberman, E., Stringer, J.R., Ullman, U. (1991). Amplification and molecular cloning of the IMP dehydrogenase gene of *Leishmania donovani*. The Journal of Biological Chemistry. 266 (3): 1665-1671.

[17] World Health Organization (2001). African Trypanosomiasis, Fact sheet number 259 (WHO Publications, Geneva).

[18] World Health Organization. 1998. Chagas' disease: tropical diseases progress in research, 1997-1998. WHO technical report series. World Health Organization, Geneva, Switzerland.

[19] World Health Organization (2000). The leishmaniasis and Leishmania/HIV co-infections, Fact sheet number 116 (WHO Publications, Geneva).

[20] Fairlamb, A.H. (2003). Chemotherapy of human African trypanosomiasis: current and future prospects. Trends in Parasitology. 19 (11): 488-494.

[21] Urbina, J.A., Docampo, R. (2003). Specific chemotherapy of Chagas disease: controversies and advances. Trends in Parasitology. 19 (11): 495-501.

[22] Croft, S.L., Coombs, G.H. (2003). Leishmaniasis—current chemotherapy and recent advances in the search for novel drugs. Trends in Parasitology. 19 (11): 502-508.

[23] Brun, R., Schonenberger, M. (1979). Cultivation and in vitro cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Acta Tropica. 36(3): 289-292.

[24] Hirumi, H., Hirumi, K. (1989). Continuous cultivation of *Trypanosoma brucei* forms in a medium containing a low concentration of serum protein without feeder cell layers. Journal of Parasitology. 75(6): 985-989.

[25] Ullu, E., Tschudi, C., Chakraborty, T. (2004). RNA interference in protozoan parasites. Cellular Microbiology. 6(6): 509-519.

[26] Ngo, H., Tschudi, C., Gull, K., Ullu, E., Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. PNAS. 95: 14687-14692.

[27] Wang, Z., Morris, J.C., Drew, M.E., Englund, P.T. (2000). Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. The Journal of Biological Chemistry. 275 (51): 40174-40179.

[28] Morris, J.C., Wang, Z., Drew, M.E., Paul, K.S., Englund, P.T. (2001). Inhibition of bloodstream form *Trypanosoma brucei* gene expression by RNA interference using the pZJM dual T7 vector. Molecular & Biochemical Parasitology. 11: 111-113.

[29] Wirtz, E., Leal, S., Ochatt, C., Cross, G.A. (1999). A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Molecular & Biochemical Parasitology. 99(1): 89-101.

[30] Altschul, S. F., Madden, T.L, Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research. 25: 3389-3402.

[31] Matsuzaki, M., Misumi, O., Shin-I, T., Maruyama, S., Takahara, M., Miyagishima, S.Y., Mori, T., Nishida, K., Yagisawa, F., Nishida, K., Yoshida, Y., Nishimura, Y., Nakao, S., Kobayashi, T., Momoyama, Y., Higashiyama, T., Minoda, A., Sano, M., Nomoto, H., Oishi, K., Hayashi, H., Ohta, F., Nishizaka, S., Haga, S., Miura, S., Morishita, T., Kabeya, Y., Terasawa, K., Suzuki, Y., Ishii, Y., Asakawa, S., Takano, H., Ohta, N., Kuroiwa, H., Tanaka, K., Shimizu, N., Sugano, S., Sato, N., Nozaki, H., Ogasawara, N., Kohara, Y., Kuroiwa, T. (2004). Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. Nature. 428: 653-657.

[32] Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., Zhou, S., Allen, A.E., Apt, K.E., Bechner, M., Brzezinski, M.A., Chaal, B.K., Chiovitti, A., Davis, A.K., Demarest, M.S., Detter, J.C., Glavina, T., Goodstein, D., Hadi, M.Z., Hellsten, U., Hildebrand, M., Jenkins, B.D., Jurka, J., Kapitonov, V.V., Kroger, N., Lau, W.W., Lane, T.W., Larimer, F.W., Lippmeier, J.C., Lucas, S., Medina, M., Montsant, A., Obornik, M., Parker, M.S., Palenik, B., Pazour, G.J., Richardson, P.M., Rynearson, T.A., Saito, M.A., Schwartz, D.C., Thamatrakoln, K., Valentin, K., Vardi, A., Wilkerson, F.P., Rokhsar, D.S. (2004). The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. Science. 306: 79-86.

[33] Sperling, L., Dessen, P., Zagulski, M., Pearlman, R.E., Migdalski, A., Gromadka, R., Froissard, M., Keller, A.M., Cohen, J. (2002). Random sequencing of Paramecium somatic DNA. Eukaryotic Cell. 1: 341-352.

[34] Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G., Thompson, J.D. (2003). Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Research. 31: 3497-3500.

[35] Maddison, W.P., and Maddison, D.R. (2003). MacClade. Sinauer Associates, Sunderland MA.

[36] Schmidt, H. A., Strimmer, K., Vingron, M., and von Haeseler, A. (2002). TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics.18: 502-504.

[37] Huelsenbeck, J. P., Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics.17: 754-755.

[38] Felsenstein, J. (2003). PHYLIP, Phylogeny Inference Package, University of Washington, Seattle.

[39] Page, R.D (1996). TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci.12: 357-358.

[40] Muchmore, D.C., McIntosh, L.P., Russell, C.B., Anderson, D.E., Dahlquist, F.W. (1989). Expression and nitrogen-15 labeling of proteins for proton and nitrogen-15 nuclear magnetic resonance. Methods Enzymology. 177: 44-73.

[41] Tiedeman, A.A., Smith, J.M. (1985). Nucleotide sequence of the guaB locus encoding IMP dehydrogenase of *Escherichia coli* K12. Nucleic Acids Research. 13: 1303-1316.

[42] Datsenko, K.A., Wanner, B. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. PNAS. 97(12): 6640-6645.

[43] Colby, T.D., Vanderveen, K., Strickler, M.D., Marham, G.D., Goldstein, B.M. (1999). Crystal structure of human type II inosine monophosphate dehydrogenase: Implications for ligand binding and drug design. PNAS. 96: 3531-3536.

[44] Felsenstein, J. (1978). Cases in which parsimony or compatibility methods will be positively misleading. Syst. Zool. 27:401-410.

[45] Motta, M.C., Picchi, G.F., Palmie-Peixoto, I.V., Rocha, M.R., de Carvalho, T.M., Morgado-Diaz, J., de Souza, W., Goldenberg. S., Fragoso, S.P. (2004) The microtubule analog protein, FtsZ, in the endosymbiont of trypanosomatid protozoa. J Eukaryot Microbiol. 51:394-401.

[46] Shih, S., Stenberg, P., Ullman, B. (1998). Immunolocalization of *Trypanosoma brucei* hypoxanthine-guanine phosphoribosyltransferase to the glycosome. Molecular and Biochemical Parasitology. 92: 367-371.

[47] Ullman, B., Carter, D. (1997). Molecular and Biochemical Studies on the Hypoxanthine-guanine Phosphoribosyltransferases of the pathogenic Haemoflagellates. International Journal of Parasitology. 27 (2): 203-213.

[48] Allison, A.C., Engui, E.M. (2000) Mycophenolate mofetil and its mechanisms of action. Immunopharmacology. 47: 85-118.

[49] Digits, J.A. and L. Hedstrom. (1999) Species-specific inhibition of inosine 5'-monophosphate dehydrogenase by mycophenolic acid. Biochemistry. 38(46): 15388-97.

[50] Digits, J.A., Hedstrom, L. (2000). Drug selectivity is determined by coupling across the NAD+ site of IMP dehydrogenase. Biochemistry. 39: 1771-1777.

[51] Gan, L., Petsko, G. A., Hedstrom, L.A. (2002). Crystal structure of a ternary complex of Trichomonas foetus inosine 5' monophosphate dehydrogenase: NAD+ orients the active site loop for catalysis. Biochemistry. 41: 13309-13317.

[52] Leander, B. S. (2004). Did trypanosomatid parasites have photosynthetic ancestors? Trends Microbiol 12: 251-258.

[53] Simpson, A. G., Roger, A.J., (2004). Protein phylogenies robustly resolve the deep-level relationships within *Euglenozoa*. Mol Phylogenet Evol 30: 201-212.

[54] Mathews, K.R., Ellis, J.R., Paterou,A. (2004). Molecular regulation of the life cycle of African trypanosomes. Trends in Parasitology. 20(1): 40-47.

[55] Besteiro, S., Barrett, M.P., Riviere, L., Bringaud, F. (2005). Energy generation in insect stages of *Trypanosoma brucei*: metabolism in flux. Trends in Parasitology. 21(4): 186-191.

[56] Bursell, E. (1963). Aspects of the metabolism of amino acids in the tsetse fly, *Glossina* (Diptera). Journal of Insect Physiology. 9: 439-452.

[57] Drew, M.E. et al. (2003). The adenosine analog tubericidin inhibits glycolysis in *Trypanosoma brucei* as revealed by an RNA interference library. Journal of Biological Chemistry. 278: 46596-46600.

[58] ter Kuile, B.H. (1997). Adaptation of metabolic enzyme activities of *Trypanosoma brucei* promastiogtes to growth rate and carbon regiment. Journal of Bacteriology. 179: 4699-4705.

[59] Morris, J.C. et al. (2002). Glycolysis modulates trypanosome glycoprotein expression as revealed by an RNAi library. EMBO J. 21: 4429-4438.

Figure 2.1

Presence of two distinct IMPDHs in the genomes of *T. brucei*, *T. cruzi* and *L. infantum*.

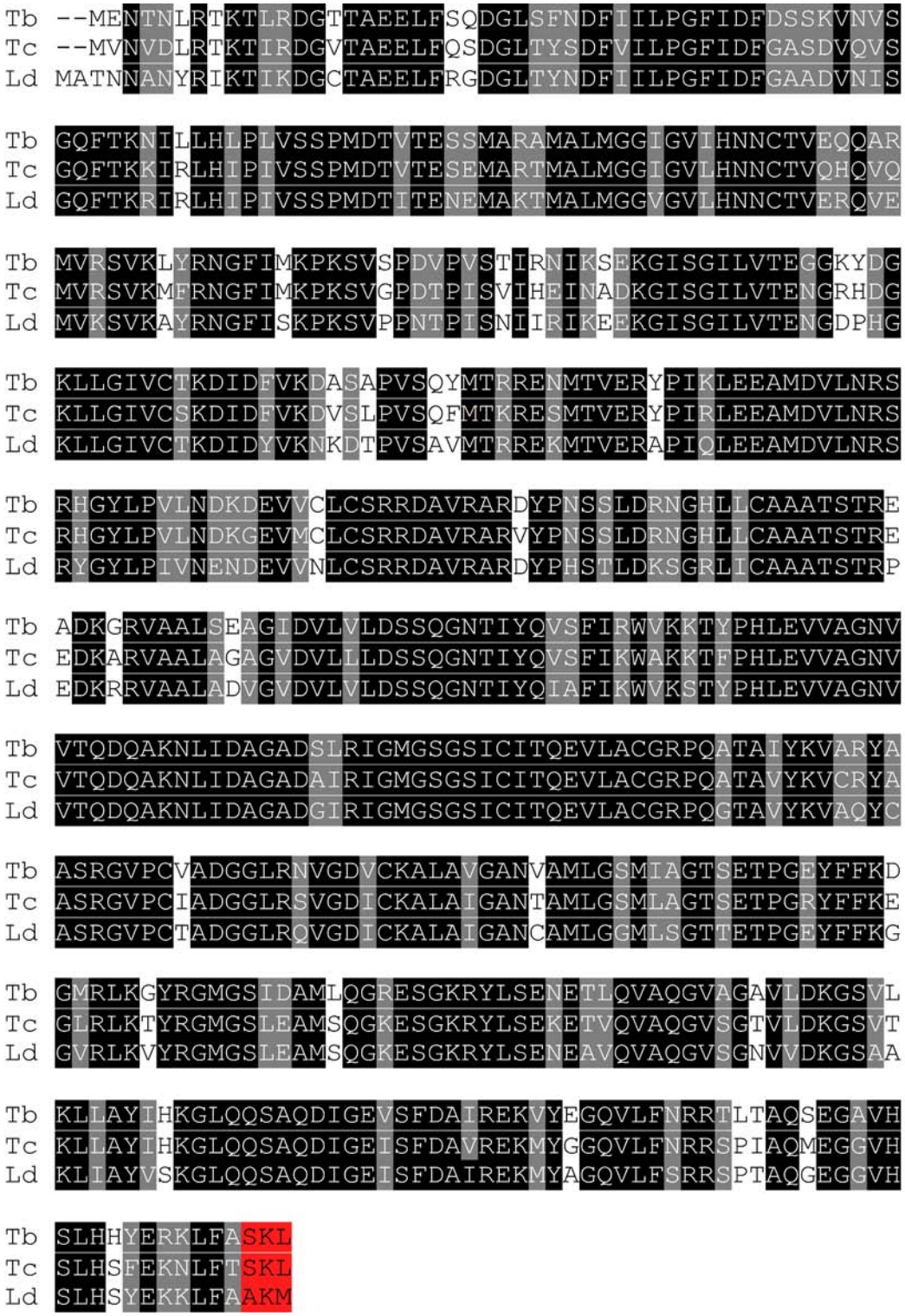
Query	***T. brucei***	***T. cruzi***	***L. infantum***
E.coli **IMPDH**	P-value: 1.20E-91 Putative IMPDH Tb05.1P6.110 NN: *Aquifex aeolicus*	P-value: 2.20E-89 Putative IMPDH Tc00.1047053508909.20 NN: *Thermatoga maritima*	P-value: 7.90E-91 IMPDH LinJ17.0630 NN: *Thermus thermophilus*
Human Type 2	P-value: 1.60E-127 IMPDH Tb10.61.0150 NN: *Xenopus laevis*	P-value: 1.50E-141 Putative IMPDH Tc00.1047053507211.40 NN: *Xenopus laevis*	P-value: 1.20E-144 IMPDH LinJ19.1230 NN: Human IMPDH

Figure 2.1: Presence of two distinct IMPDHs in the genomes of *T. brucei*, *T. cruzi*, and *L. infantum*.

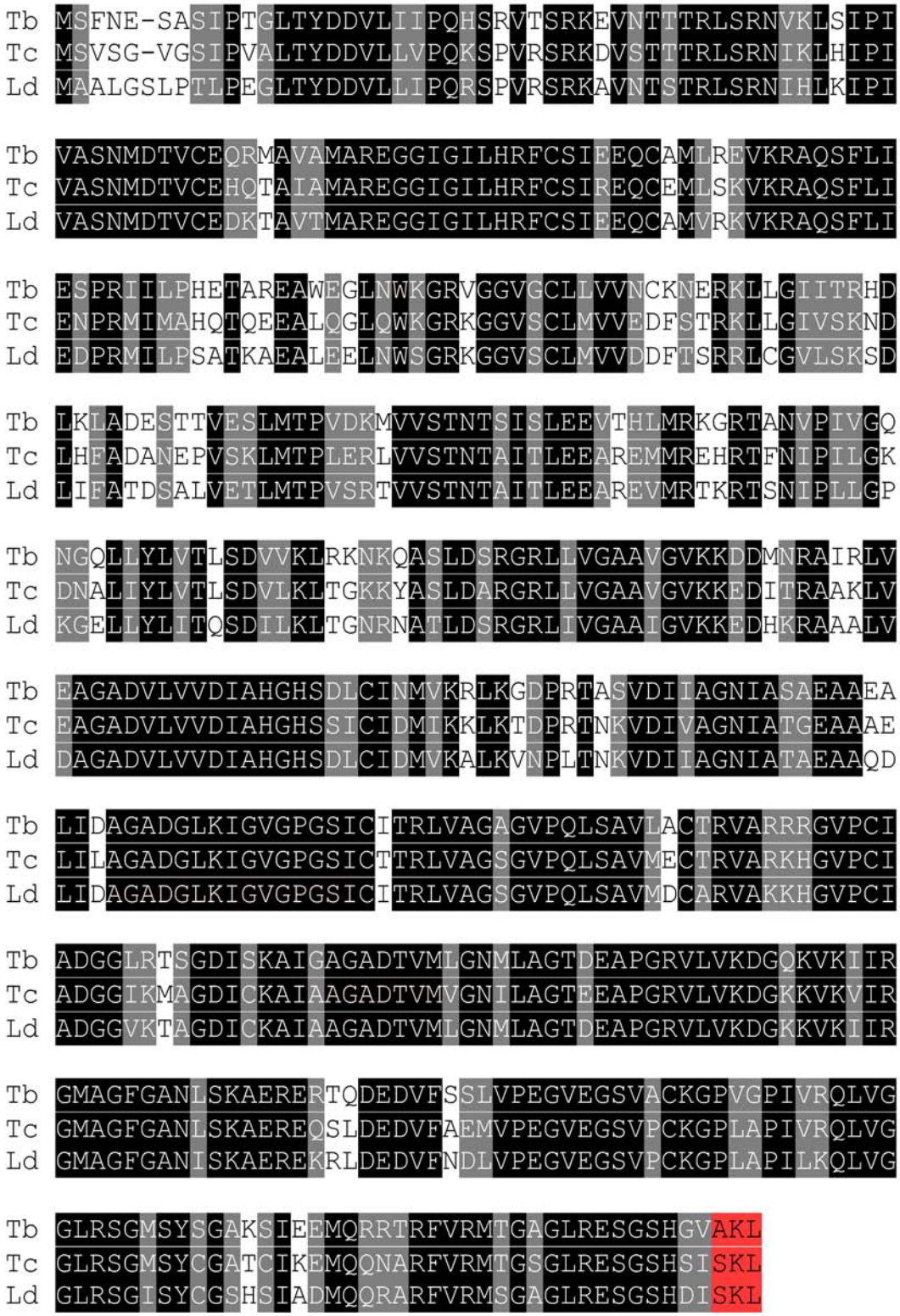
GeneDB was used to search for an IMPDH gene of divergent origin in the Trypanosomatids, *Trypanosoma brucei*, *Trypansoma cruzi* and *Leishmania donovani*. *E.coli* IMPDH and Human IMPDH Type 2 were used as query sequences in blast analysis of predicted proteins databases of the trypanosomatids. The analysis revealed the presence of two distinct IMPDHs in the trypanosomatid genomes. Statistical significance is represented by P values. The accession number and the protein annotation are also indicated. Also displayed are nearest neighbours outside of the kinetoplastid taxon as identified by blast analysis against the non-redundant protein database at Genbank using the trypanosomatid IMPDH amino acid sequences as query sequences. The nearest neighbors identified for IMPDH 1 belong to the eukaryotic domain while those for identified for IMPDH 2 belong to the prokaryotic domain. NN: Nearest Neighbor.

Figure 2.2: Amino acid sequence alignments of IMPDH 1 and IMPDH 2

A.



B.


Tb MSFNE-SASIPTGLTYDDVLIIPQHSRVTSRKEVNTTTRLSRNVKLSIPI
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Ld MAALGSLPTLPEGLTYDDVLLIPQRSPVRSRKAVNTSTRLSRNIHLKIPI
Tb VASNMDTVCEQRMAVAMAREGGIGILHRFCSIEEQCAMLREVKRAQSFLI
Tc VASNMDTVCEHQTAIAMAREGGIGILHRFCSIREQCEMLSKVKRAQSFLI
Ld VASNMDTVCEDKTAVTMAREGGIGILHRFCSIEEQCAMVRKVKRAQSFLI
Tb ESPRIILPHETAREAWEGLNWKGRVGGVGCLLVVNCKNERKLLGIITRHD
Tc ENPRMIMAHQTQEEALQGLQWKGRKGGVSCLMVVEDFSTRKLLGIVSKND
Ld EDPRMILPSATKAEALEELNWSGRKGGVSCLMVVDDFTSRRLCGVLSKSD
Tb LKLADESTTVESLMTPVDKMVVSTNTSISLEEVTHLMRKGRTANVPIVGQ
Tc LHFADANEPVSKLMTPLERLVVSTNTAITLEEAREMMREHRTFNIPILGK
Ld LIFATDSALVETLMTPVSRTVVSTNTAITLEEAREVMRTKRTSNIPLLGP
Tb NGQLLYLVTLSDVVKLRKNKQASLDSRGRLLVGAAVGVKKDDMNRAIRLV
Tc DNALIYLVTLSDVLKLTGKKYASLDARGRLLVGAAVGVKKEDITRAAKLV
Ld KGELLYLITQSDILKLTGNRNATLDSRGRLIVGAAIGVKKEDHKRAAALV
Tb EAGADVLVVDIAHGHSDLCINMVKRLKGDPRTASVDIIAGNIASAEAAEA
Tc EAGADVLVVDIAHGHSSICIDMIKKLKTDPRTNKVDIVAGNIATGEAAAE
Ld DAGADVLVVDIAHGHSDLCIDMVKALKVNPLTNKVDIIAGNIATAEAAQD
Tb LIDAGADGLKIGVGPGSICITRLVAGAGVPQLSAVLACTRVARRRGVPCI
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Ld LIDAGADGLKIGVGPGSICITRLVAGSGVPQLSAVMDCARVAKKHGVPCI
Tb ADGGLRTSGDISKAIGAGADTVMLGNMLAGTDEAPGRVLVKDGQKVKIIR
Tc ADGGIKMAGDICKAIAAGADTVMVGNILAGTEEAPGRVLVKDGKKVKVIR
Ld ADGGVKTAGDICKAIAAGADTVMLGNMLAGTDEAPGRVLVKDGKKVKIIR
Tb GMAGFGANLSKAERERTQDEDVFSSLVPEGVEGSVACKGPVGPIVRQLVG
Tc GMAGFGANLSKAEREQSLDEDVFAEMVPEGVEGSVPCKGPLAPIVRQLVG
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Tc GLRSGMSYCGATCIKEMQQNARFVRMTGSGLRESGSHSISKL
Ld GLRSGISYCGSHSIADMQQRARFVRMSGAGLRESGSHDISKL

Figure 2.2: IMPDH 1 and IMPDH 2 amino acid sequence alignments**.** Tb, *T. brucei*; Tc, *T. cruzi*; Ld, *L. donovani*. Alignments of Trypanosomatid IMPDH 1 (A) and IMPDH 2 (B) amino acid sequences were prepared and analyzed with CLUSTALW. Residues in black are identical within the trypansomatids and those represented in grey display some similarity. At the protein level, IMPDH 1 is 71% identical and 20% similar and IMPDH 2 is 65% identical and 28% similar within *T. brucei*, *T. cruzi*, and *L. donovani*. Residues in red constitute the glycosome-targeting signal that suggests the localization of both IMPDH 1 and 2 in the glycosome of the parasite.

Figure 2.3

Multiple sequence alignments of trypanosomatid IMPDH 1 and 2 with prokaryotic and eukaryotic IMPDHs.

A.

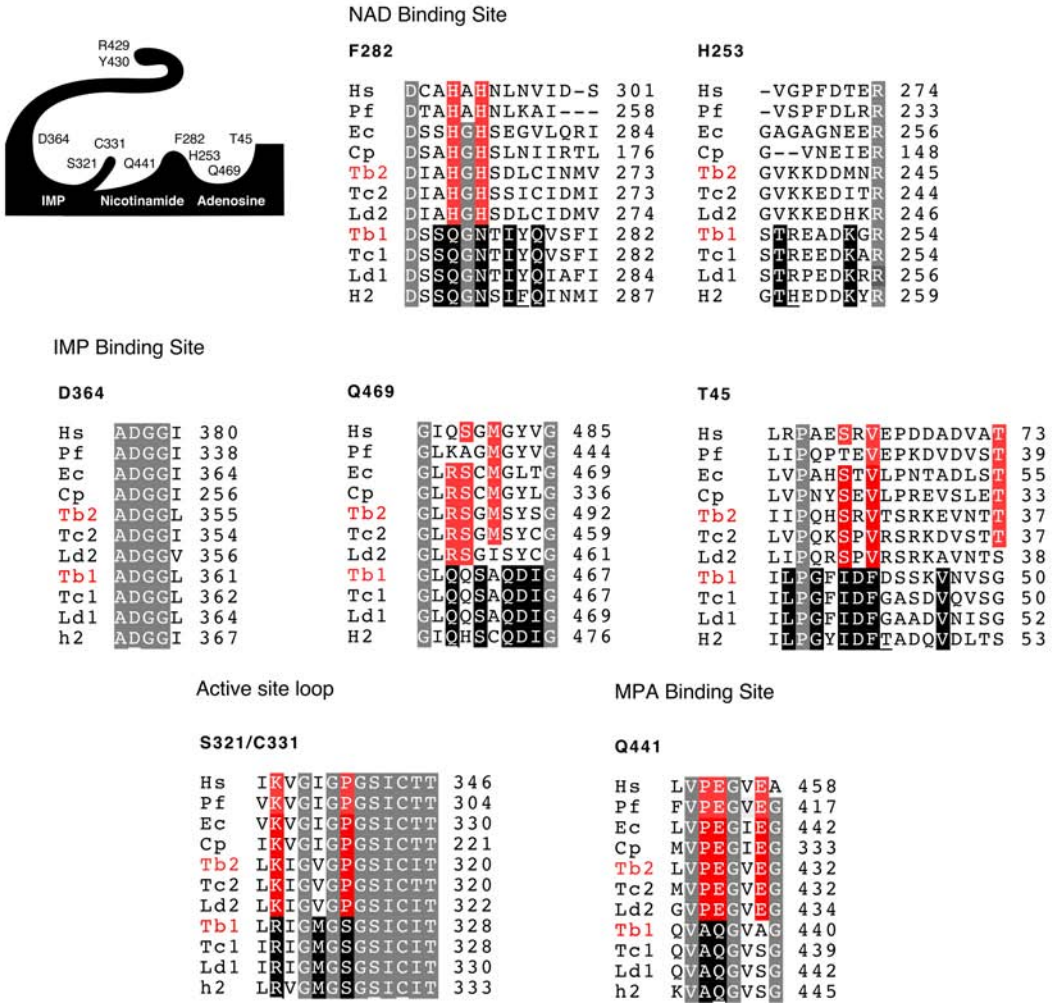


Figure 2.3:

Multiple sequence alignments of trypanosomatid IMPDH 1 and 2 with prokaryotic and eukaryotic IMPDHs.

Hs, *H. salinarium*; Pf, *P. furiosus*; Ec, *E. coli*; Cp, *C. parvum*; Tb2, *T. brucei* IMPDH 2; Tc2 *T. cruzi* IMPDH 2; Ld2, *L. donovani* IMPDH 2; Tb1, *T. brucei* IMPDH 1; Tc1 *T. cruzi* IMPDH 1; Ld1, *L. donovani* IMPDH 1, H2, Human Type 2. The residues that have been shown to establish contacts to substrates and inhibitors in the solved structure of the human enzyme are underlined. Residues that are conserved among all sequences are highlighted in grey. Residues shared between the Trypanosomatid IMPDH 2 and the *C. parvum* and *E. coli* sequences, but divergent from the Trypanosomatid IMPDH 1 and the human enzyme, are displayed in red. Residues shared between the Trypanosomatid IMPDH 1 and the human sequences, but divergent from the Trypanosomatid IMPDH 2 and the *C. parvum* and *E. coli* sequences are displayed in black. Analysis of the multiple sequence alignments of IMPDH 1 and 2 with other IMPDHs, shows a closer similarity of IMPDH 2 to prokaryotic IMPDHs than IMPDH 1 and the human Type 2 IMPDH.

Figure 2.4: Phylogenetic analysis reveals that the trypanosomatid IMPDH 1 and 2 are highly-divergent.

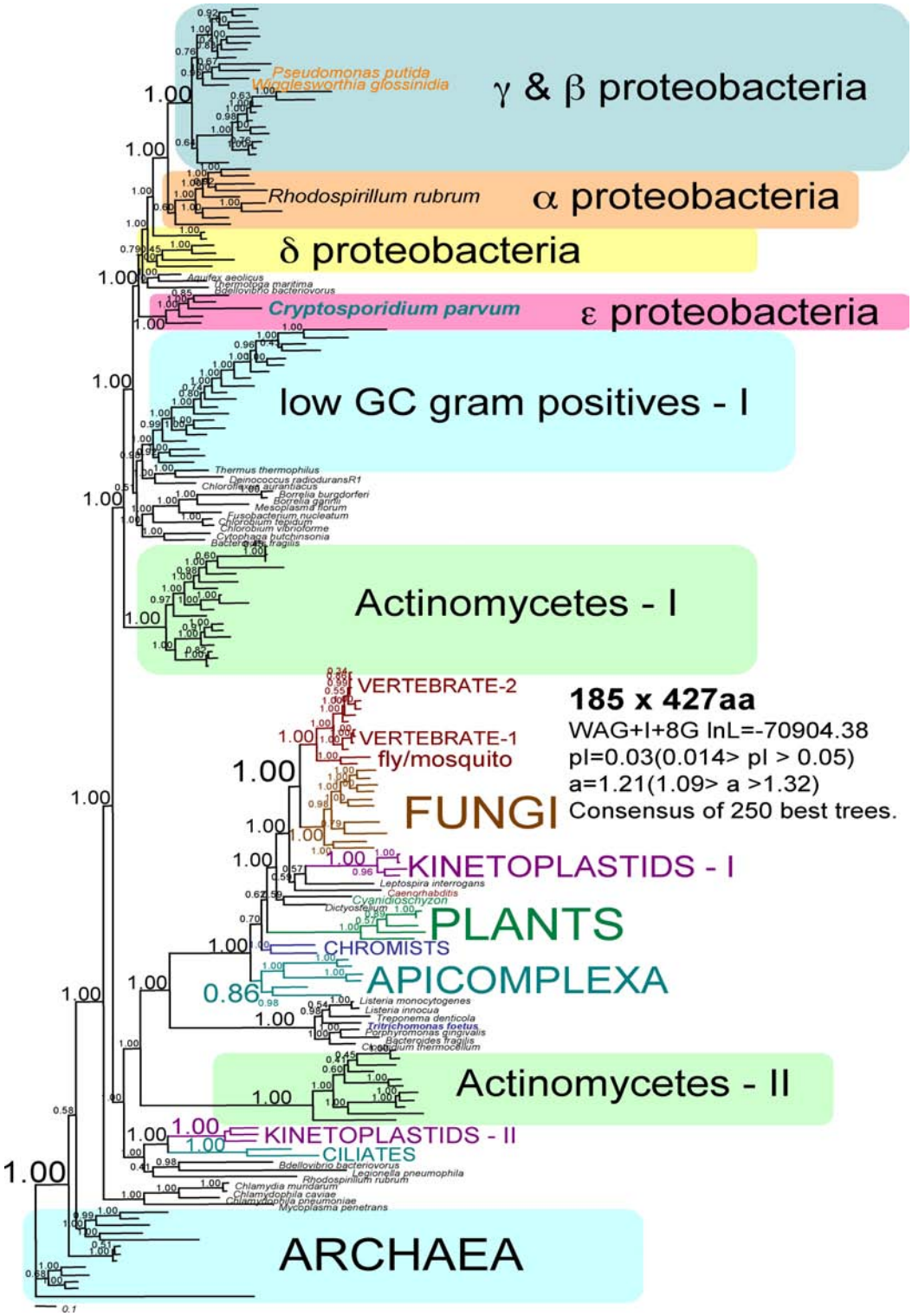
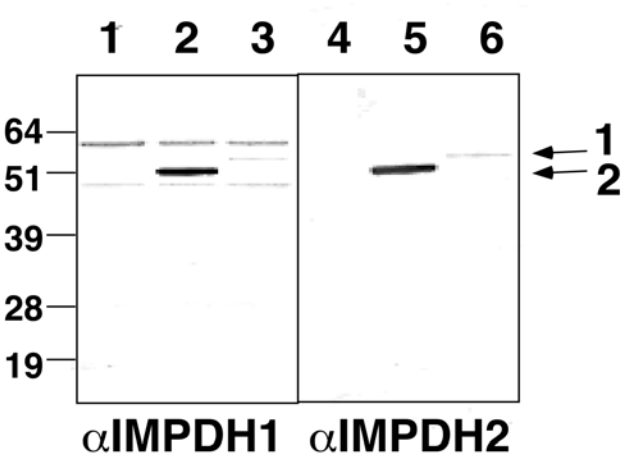


Figure 2.4: Phylogenetic analysis reveals that the trypanosomatid IMPDH 1 and 2 are highly-divergent.

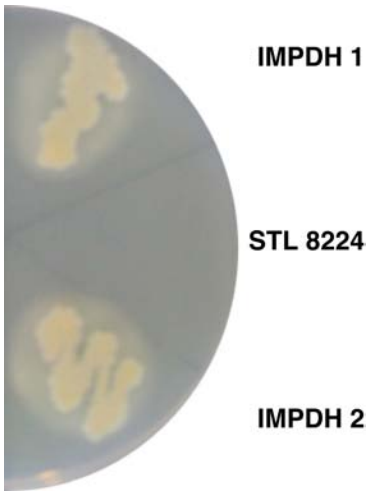
Protein sequences homologous to IMPDH in eukaryotes, bacteria and archaea were found by using the *Leishmania* IMPDH as a query sequence. Alignments of these protein sequences were subsequently prepared. Phylogenetic analysis was conducted on the resulting 185-sequence alignment of 430 amino acids and then subjected to analysis with MrBayes for 1000000 generations. The Tree was drawn using Treeview. Boot strap values of more than .40 are displayed. The phylogenetic analysis reveals that IMPDH 1 is of clear eukaryotic origin. IMPDH 2, however, groups with one of two IMPDHs of the alpha-proteobacterium, *Rhodospirillum* and with the ciliates, *Tetrahymena thermophila* and *Paramecium tetraaurelia*.

Figure 2.5: Western Blot analysis demonstrates differential expression of the IMPDH 1 and 2 in the procyclic and bloodstream forms of *T.brucei*.

A.



B.



C.

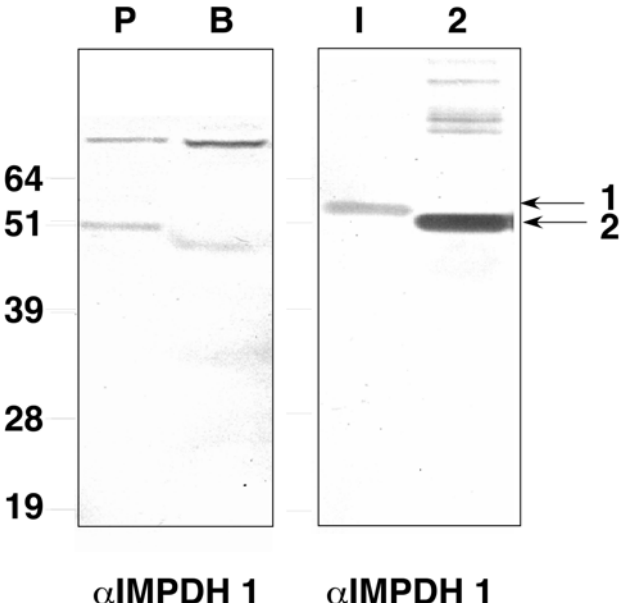


Figure 2.5:

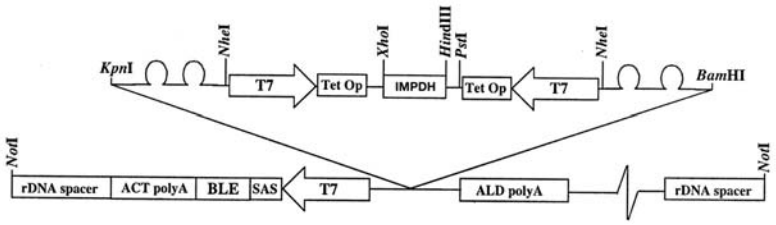
Western Blot analysis demonstrates differential expression of IMPDH 1 and IMPDH 2 in the procyclic and bloodstream forms of *T. brucei*.

A. Western Blot analysis of lysates of TX685 *E. coli* cells expressing the *T. brucei* IMPDH 1 and IMPDH 2 enzymes using antisera against *L. donovani* IMPDH 1 and IMPDH 2 enzymes. Lanes 1 & 4, TX685 cells expressing only parent expression plasmid. Lanes 2 & 5, TX685 cells expressing *T. brucei* IMPDH 1. Lanes 3 & 6, TX685 cells expressing *T. brucei* IMPDH 2. Antisera against *L. donovani* IMPDH 1 and 2 are cross-reactive and detect both the *T. brucei* IMPDH 1 and 2 proteins in lysates of bacteria expressing either IMPDH 1 or 2.
B. Complementation assay. STL8224 cells expressing either IMPDH 1 or 2 were grown on minimal media lacking guanine. Expression of IMPDH 1 or 2 in these cells rescues the mutant suggesting that these enzymes are biologically active. STL8224, parental strain that does not display endogenous IMPDH activity. IMPDH 1, STL 8224 cells expressing IMPDH 1. IMPDH 2, STL8224 cells expressing IMPDH 2.
C. Western Blot analysis of procyclic and bloodstream stage parasite lysates using antisera against *L. donovani* IMPDH 1 demonstrated the differential expression of IMPDH 1 and 2 in the life cycle stages of *T. brucei*. IMPDH 1 expression is detected in the procyclic trypomastigote stage and IMPDH 2 expression is detected in the bloodstream trypomastigote stage. B, Bloodstream. P, Procyclic. 1, Lysates of *E. coli* cells expressing IMPDH 1. 2, Lysates of *E. coli* cells expressing IMPDH 2.

Figure 2.6

Effect of IMPDH RNAi on Procyclic and Bloodstream Trypomastigotes

A.



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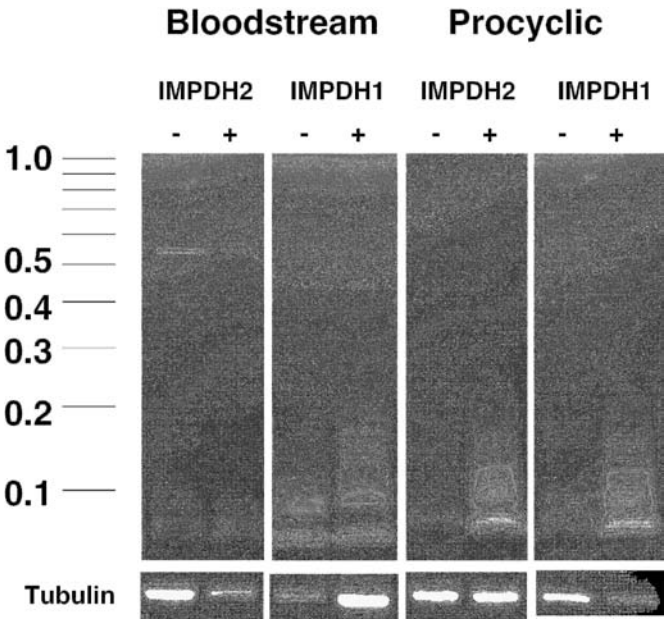

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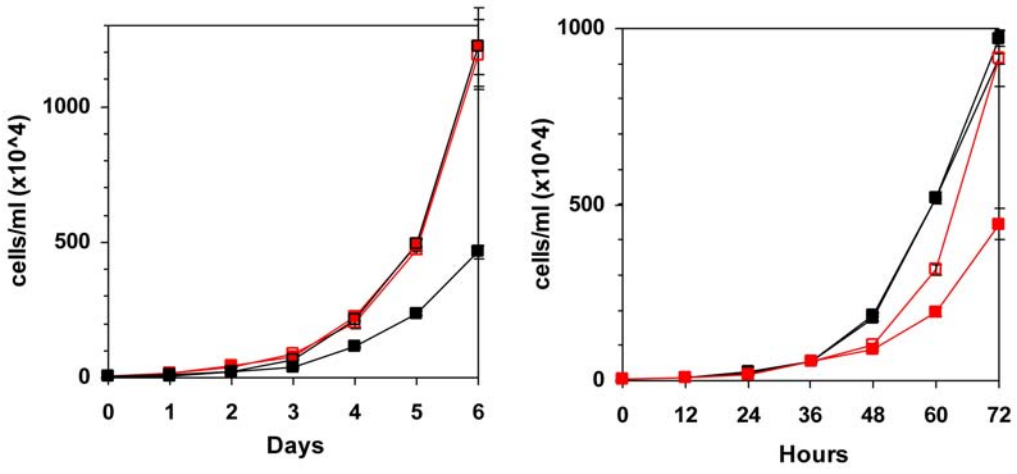
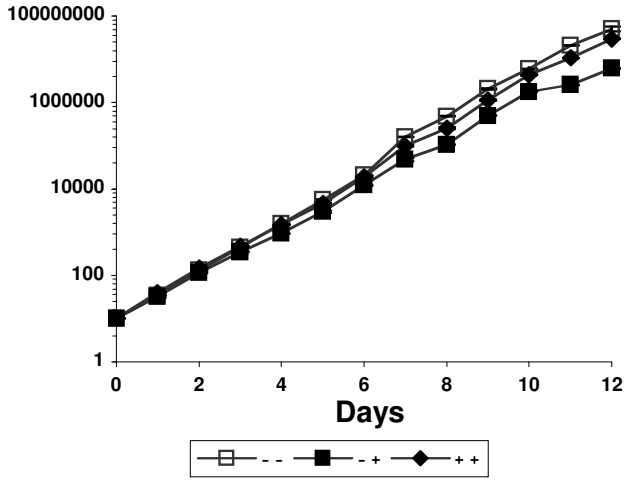


D.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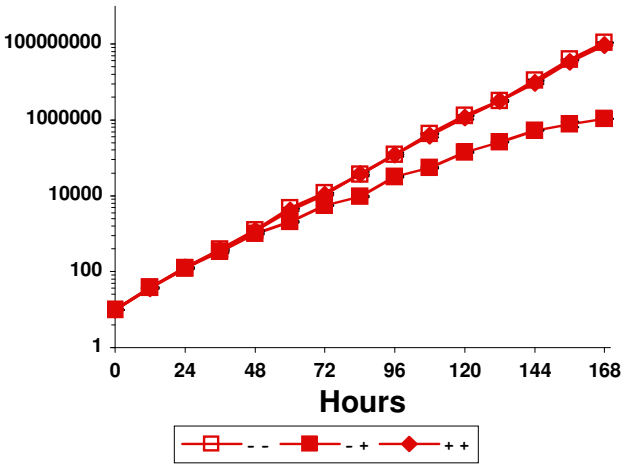
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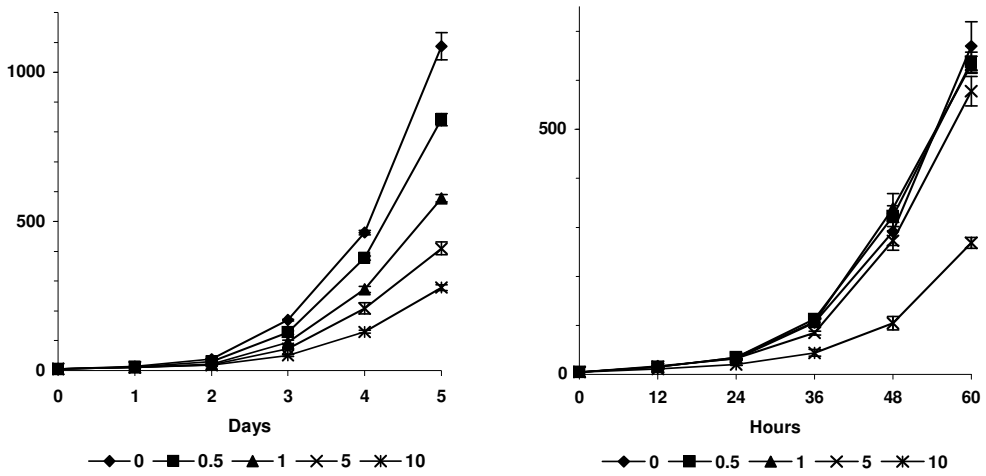
Figure 2.6: Effect of IMPDH RNAi on Procyclic and Bloodstream Trypomastigotes

A. pZJM: RNA interference vector. A 500bp coding region of the *T. brucei* IMPdH 1 or IMPDH 2 gene was amplified from genomic DNA and cloned between two T7 promoters that are under the control of a tetracycline inducible operator. The schematic represents the plasmid after linearization with *NotI* at the rDNA spacer region. B. RT-PCR analysis of tectracycline uninduced and induced procyclic and bloodstream stage parasites. RNA was isolated from uninduced and induced parasites, reverse-transcribed to obtain cDNA which was subsequently used in a PCR reaction with primers against the 500bp region of the IMPDH 1 and 2 genes that were used to clone into the pZJM vector. IMPDH 1 transcript is detected in the uninduced procyclic trypomastigotes while IMPDH 2 transcript is detected only in uninduced bloodstream trypomastigotes. No IMPDH transcript is detected in tetracycline-induced parasites. C. Effect of RNA interference of IMPDH 1 or 2 in *T. brucei*. 5x10^4 procyclic and bloodstream stage parasites were either left uninduced (open squares) or were induced (closed squares) with tetracycline and parasite counts were obtained every 24 hours (procyclic) or every 12 hours (bloodstream) for a total of 5 counts. RNAi of IMPDH 1 (Black squares) causes a growth defect only in procyclic trypomastigotes while RNAi of IMPDH 2 (Red squares) causes a growth defect only in bloodstream tryopmastigotes. D. Effect of RNA interference and complementation with guanine of IMPDH 2 in bloodstream forms and IMPDH 1 in procyclic forms. 5x10^4 procyclic and bloodstream stage parasites were either left uninduced, were induced with tetracycline or were induced as well as complemented with guanine. Parasites were counted every 24 hours (procyclic) or every 12 hours (bloodstream).

Figure 2.7

Effect of Mycophenolic Acid on Procyclic and Bloodstream Trypomastigotes

A.



B.

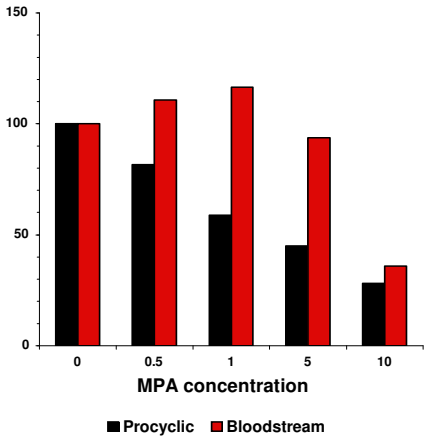


Figure 2.7: Effect of Mycophenolic Acid on Procyclic and Bloodstream Trypomastigotes

A. 5x10^4 Procyclic and Bloodstream stage trypomastigotes were treated with 0, 0.5, 1, 5 and 10uM of Mycophenolic acid. Parasite counts were obtained either every 24 hours (procyclic) or every 12 hours (bloodstream) with a cytometer. Procyclic stage trypomastigotes are more susceptible to MPA mediated inhibition than bloodstream stage trypomastigotes with an EC50 of ~1uM compared to an EC50 of ~10 uM for bloodstream trypomastigotes.
B. Parasite counts obtained from treatment of procyclic and bloodstream stage parasites with mycophenolic acid were normalized and plotted against MPA concentration.

CHAPTER 3

CONCLUSIONS

The trypanosomatids are a group of vector-borne protozoan parasites that cause serious and debilitating diseases affecting several millions world-wide. The parasites are unable to synthesize purines *de novo* and are completely dependent on the salvage of purine nucleobases and nucleosides from the host. One central enzyme in this pathway is IMPDH that catalyzes the penultimate step in guanine nucleotide biosynthesis.

Preliminary studies in our laboratory have suggested the presence of two distinct IMPDHs in the genomes of the trypanosomatids, *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania*. Phylogenetic analysis of the IMPDHs has suggested highly divergent phylogenies. The first, IMPDH 1is of clear eukaryotic origin and has been described in the insect stages of *T. brucei* and *L. donovani*. The previously unknown IMPDH 2 gene appears to have evolved from a duplication event early in trypanosome evolution. We hypothesized that evolutionary mechanisms such as horizontal gene transfer or gene duplication might have served as a mechanism to generate metabolic diversity and pre-adapted parasites for the colonization of new hosts, which exposed them to a divergent nutritional environment. In fact, our studies indicate that the two highly divergent IMPDHs are differentially expressed in the insect and mammalian life cycle stages. IMPDH 1 is expressed only in the procyclic or insect stage and IMPDH 2 is expressed only in the bloodstream or mammalian stage of the parasite. Furthermore, knock-down of IMPDH 2 in the mammalian stage causes a significant growth defect. Since IMPDH 2 is phylogenetically distant from the human enzyme and important for parasite viability, it can be

selectively targeted. Further biochemical and structural characterization of IMPDH 2, however, is essential for its development as a drug target.