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A unique, highly conserved secretory invertase is differentially expressed by promastigote developmental forms of all species of the human pathogen, *Leishmania*

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Abstract

Leishmania are protozoan pathogens of humans that exist as extracellular promastigotes in the gut of their sand fly vectors and as obligate intracellular amastigotes within phagolysosomes of infected macrophages. Between infectious blood meal feeds, sand flies take plant juice meals that contain sucrose and store these sugars in their crop. Such sugars are regurgitated into the sand fly

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anterior midgut where they impact the developing promastigote parasite population. In this report we showed that promastigotes of all *Leishmania* species secreted an invertase/sucrase enzyme during their growth in vitro. In contrast, neither *L. donovani* nor *L. mexicana* amastigotes possessed any detectable invertase activity. Importantly, no released/secreted invertase activity was detected in culture supernatants from either *Trypanosoma brucei* or *Trypanosoma cruzi*. Using HPLC, the *L. donovani* secretory invertase was isolated and subjected to amino acid sequencing. Subsequently, we used a molecular approach to identify the *LdINV* and *LmexINV* genes encoding the ~72 kDa invertases produced by these organisms. Interestingly, we identified high fidelity *LdINV*-like homologs in the genomes of all *Leishmania* sp. but none were present in either *T. brucei* or *T. cruzi*. Northern blot and RT-PCR analyses showed that these genes were developmentally/differentially expressed in promastigotes but not amastigotes of these parasites. Homologous transfection studies demonstrated that these genes in fact encoded the functional secretory invertases produced by these parasites. Cumulatively, our results suggest that these secretory enzymes play critical roles in the survival/growth/development and transmission of all *Leishmania* parasites within their sand fly vector hosts.

Keywords

Leishmania; Protozoan pathogen; Human parasite; Invertase; Secretory enzyme

Introduction

Leishmania sp. are a group of trypanosomatid protozoan pathogens which are transmitted to their mammalian hosts by the bite of infected female sand fly vectors. Collectively, in humans, these parasites cause over 2 million new cases of cutaneous, mucocutaneous, and fatal visceral diseases (i.e., leishmaniasis) per year worldwide [1]. All *Leishmania* parasites have a developmental life cycle that includes two major parasite developmental stages: (1) an extracellular, flagellated promastigote form that resides and multiply in the alimentary tract of their sand fly vector hosts and (2) an obligate intracellular, amastigote form which resides and multiplies within phagolysosomal compartments of infected mammalian macrophages [2]. Following their uptake in an infectious blood meal, *Leishmania* amastigotes transform within ~24–48 h into extracellular promastigotes within the sand fly vector midgut. These promastigotes subsequently rapidly divide and multiply, and undergo a complex series of transformations that result in the generation of mammalian-infective metacyclic promastigotes [3–6]. During this entire developmental process, members of the promastigote population continue to differentiate and migrate anteriorly in the sand fly gut culminating with the arrival of infectious metacyclics in the sand fly vector mouth parts. It is important to point out that all of these activities and developmental processes require an exogenous source of energy which the parasites must salvage/scavenge from the nutrients present within the insect vector midgut. To sustain themselves between their infectious blood feeds, female sand flies characteristically take plant juice meals including sucrose and other complex polysaccharides [7]. Following ingestion, such sugar meals are shunted and stored within the crop compartment of the insect's gut. When needed, these sugar meals are regurgitated back into the sand fly anterior midgut where they would impact/interface with the anterior-migrating population of *Leishmania* parasites. To harness the energy present in

these complex polysaccharide sugar meals (e.g., sucrose) the sand fly must hydrolyze them into smaller transportable monosaccharide units. To that end, it has been reported that sand fly guts are capable of synthesizing and secreting invertase-like activities which are capable of hydrolyzing sucrose into glucose and fructose [7]. Interestingly, Blum and Opperdoes reported that *Leishmania donovani* promastigotes also produced and released/secreted an extracellular soluble sucrase (i.e., invertase) enzyme activity during their growth in vitro [8]. Further these authors suggested that this enzyme may play "an important role in the nutrition and development of the promastigotes in the insect gut,…" [*sic*] [8]. Subsequently, these observations were verified by Gontijo et al. who reported that promastigotes of all New and Old World *Leishmania* species which they tested, also produced extracellular/released invertase activities [9]. In light of the published observations above, we initiated our studies toward identifying, characterizing, and episomally expressing in situ the gene(s) encoding these putative leish-manial released/secretory invertase-like enzymes.

Results

The first report of a released/secreted sucrase/invertase activity by any *Leishmania* sp. was reported from culture supernatants of *L. donovani* promastigotes (MHOM/SD/62/1S/CL2D) by Blum and Opperdoes [8]. Subsequently, these observations were confirmed and extended to a variety of promastigotes of Old and New World *Leishmania* sp. by Gontijo et al. [9]. These two reports served as the basis for the current report concerning the identification and characterization of the gene(s) encoding these potentially important parasite enzymes.

Identification of secreted/released invertase activities by in vitro grown *Leishmania* promastigotes

As indicated above, invertase-like activity was detected in culture supernatants of *L. donovani* promastigotes [8, 9]. To verify and extend those observations, concentrated culture supernatants from *L. donovani* (1SCL-2D) promastigotes were tested for invertase activity using our agarose-gel-triphenyltetrazolium chloride (TTC) reaction product capture assay as described below. Results of these qualitative assays demonstrated that this *L. donovani* promastigote isolate synthesized and released/secreted significant quantities of soluble invertase activity into its culture supernatant which accumulated during growth in vitro (Fig. 1a). No invertase activity was detected in similarly processed unused parasite growth medium controls (Fig. 1c). The gel shown in Fig. 1 is representative of the results obtained from numerous gel assays which were carried out in these experiments. Results of parasite growth experiments indicated that the accumulation of such soluble invertase activity in these parasite cell cultures was in relative proportion to total parasite cell number (i.e., parasite cell growth). It is important to point out that the "soluble" invertase activity measured in these parasite culture supernatants was not enhanced by the addition of non-ionic detergents (i.e., showed no detectable compartmentalized latency, data not shown). Results similar to those shown in Fig. 1a were also obtained from in vitro short-term release assays carried out with *L. donovani* (1SCL-2D) promastigotes (data not shown). Both concentrated (20×) and unconcentrated (1×) culture supernatants and aliquots of short-term release assays were also analyzed for their invertase activity using the glucose oxidase (GO) spectrophotometric assays described below. Results of a typical experiment obtained from

unconcentrated (1×) culture supernatants from *L. donovani* promastigotes are shown in Fig. 1d. Approximately 20-fold higher levels of invertase activity were obtained in these assays when 20× concentrated parasite culture supernatants were used. Thus, in our agarose gel assays we determined that we needed at least 100 GO spectrophotometric units to readily detect invertase activity. Results of various assays demonstrated that ~1 × 10^7 *L. donovani* promastigotes produced ~100 units of invertase activity as determined by GO spectrophotometric assays. Overall, the results of our quantitative assays were in very good agreement with our qualitative results obtained in our agarose-based invertase assays. Having demonstrated that *L. donovani* 1SCL-2D promastigotes constitutively produced considerable quantities of invertase activity, we decided to assess whether any other Old World *Leishmania* strains and species might also produce similar invertase activities. To that end, concentrated culture supernatants were obtained from an Indian isolate of *L. donovani* (LRC-751); two strains of *L. infantum*, one from Spain (FVM10001JL) and one from Israel (LRC-639); one isolate from Israel of *L. major* (Friedlen) and one isolate from the Soviet Union of *L. tropica* (WR683). In addition, similar culture supernatants were obtained from a single Old World saurian strain of *Leishmania*, *L. tarentolae* (Jena 101), which does not cause disease in humans. Such culture supernatants were assayed using our agarose-gel-TTC reaction product capture assay. Interestingly, results of those assays showed that each of these Old World *Leishmanias* including *L. tarentolae* constitutively produced and released/secreted similar levels of invertase activity (data not shown) to those produced by the *L. donovani* (1SCL-2D) parasites. Similar results were also obtained with aliquots of in vitro short-term release assays from each of these Old World *Leishmania* species (data not shown). Results of spectrophotometric assays with samples derived from all of these Old World *Leishmanias* demonstrated that all of them produced comparable levels of invertase activity (i.e., ~10^7 promastigotes produce ~100 units of invertase activity/ml; data not shown). Taken together, results of these various assays demonstrated that all of the Old World *Leishmanias* tested appear to constitutively produce/release significant quantities of soluble invertase activity (i.e., non-latent, as indicated above). Having found this, it was of interest to determine whether any New World *Leishmania* species might also produce invertase activities. To test this hypothesis, concentrated culture supernatants obtained from *L. mexicana* (M379) promastigotes were tested in our agarose-gel-TTC reaction product capture assays. Results of those assays showed that *L. mexicana* (M379) promastigotes did in fact produce invertase activity but at qualitatively lesser amounts than that produced by similar numbers of *L. donovani* (1SCL-2D) promastigotes (*c.f.* Fig. 1a, b). No invertase activity was detected in similarly processed unused parasite growth medium controls (Fig. 1c). As above, the gel depicted in Fig. 1 is representative of one example of the results obtained from numerous gel assays which were carried out in these experiments. Results similar to those shown in Fig. 1b were also obtained from in vitro short-term release assays carried out with *L. mexicana* (M379) promastigotes (data not shown). Similarly, culture supernatants and aliquots of short-term release assays from *L. mexicana* (M379) promastigotes were also analyzed for their invertase activity using the GO spectrophotometric assay. Results of those quantitative assays indicated that ~1 × 10^7 *L. mexicana* (M379) promastigotes produced ~10 units of invertase activity as determined by GO spectrophotometric assays. Thus, the combined results of both our qualitative and quantitative assays indicated that *L. mexicana* (M379) did in fact produce invertase activity

but at lower levels than that produced by any of the other Old World *Leishmanias* tested. These observations suggested that it would be of interest to assess whether any other New World species of *Leishmania* also produced invertase activity. To test this hypothesis, concentrated culture supernatants were obtained from in vitro grown promastigotes of a second *L. mexicana* isolate (BEL21, from a patient in Belize); an isolate of *L. braziliensis* (WR668, from a Brazilian patient); two isolates of *L. amazonensis* (PH8 and GML584, from a vector in Brazil and a patient in Panama, respectively); one isolate of *L. pifanoi* (WHOLL1, from a Venezuelan patient); two isolates of *L. panamensis* (WR470 and WHO594 isolated from several individual patients in Brazil); and an isolate of *L. guyanensis* (M4147, from a patient in Brazil). Such culture supernatants and aliquots of release assays obtained from each of these New World species were assayed for their invertase activity using our agarose-based-TTC capture method. Results of those assays showed that each of the New World *Leishmanias* tested constitutively produced and released/secreted similar levels of invertase activity (data not shown) to those produced by the *L. mexicana* (M379) promastigotes (re: Fig. 1b). Results of these qualitative assays were fully supported by those obtained from our GO spectrophotometric invertase assays (data not shown). Taken together, results of all of these assays demonstrated that each of the New World species tested did in fact produce soluble (non-latent) invertase activity, similar to each other, but none of them produced invertase at levels equivalent to that produced by any of the Old World *Leishmania* species tested above.

The cumulative results of our invertase assays for the Old and New World *Leishmania* sp. are summarized in Table 1A. While not tested in the current report, it is of interest to point out that similar sucrase/invertase activities were reported previously by Gontijo et al. for the non-*Leishmania*, monoxenic insect trypanosomatids (i.e., *Crithidia fasciculata*, *Herpetomonas samuelpessoai*, and *Leptomonas seymouri*) as well as a *Phytomonas* sp. (Table 1B). In contrast to the foregoing, despite numerous attempts, neither we nor Gontijo et al. were able to detect any invertase activity in the culture supernatants of either African or American *Trypanosoma* sp. (i.e., *T. brucei and T. cruzi*; Table 1B).

Isolation of the *L. donovani* (1SCL-2D) invertase

Results of our qualitative and quantitative invertase assays indicated that the *L. donovani* (1SCL-2D) promastigotes, as virtually all other Old World species tested, produced significant quantities of invertase activity. Based on those observations it was deemed practical to attempt to isolate the *Leishmania* invertase synthesized and released/secreted by *L. donovani* (1SCL-2D) promastigotes. In preliminary experiments, we attempted to isolate invertase from concentrated culture supernatants of *L. donovani* (1SCL-2D) promastigotes using a variety of affinity-based chromatographic methods. Unfortunately, none of these methods proved successful because during the concentration steps, culture medium components tended to aggregate and precipitate out of solution (i.e., hemin aggregates/ppts). Subsequently, we found that this problem could be obviated by using samples obtained from short-term release assays (as described in “Experimental procedures” section below) using *L. donovani* (1SCL-2D) promastigotes. In that regard, dialyzed cell-free release assay supernatants obtained from *L. donovani* (1SCL-2D) promastigotes were loaded on to a Mono-Q column and subjected to fast protein liquid chromatography (FPLC). During

fractionation, eluates were scanned at $A_{220\ nm}$ and $A_{280\ nm}$. Aliquots of these samples were collected and subjected to SDS-PAGE and silver staining. An example of one such chromatographic separation obtained with these samples is shown in Fig. 2a. Results of such silver staining demonstrated that one of these fractions (INV) contained a single protein band with an apparent molecular mass of approximately 70 kDa (Fig. 2b). Subsequently, an aliquot of this selected fraction was separated by SDS-PAGE, blotted onto a polyvinylidene fluoride (PVDF) membrane and stained with Coomassie Blue. The resulting single Coomassie Blue-stained protein band was excised from such blots and subjected to *N*-terminal End Edman Amino Acid Sequencing.

Identification and characterization of the *L. donovani* INV gene

Results of *N*-terminal End Edman Amino Acid Sequencing of the 70 kDa *Ld*INV protein revealed that it contained KDGVPYE amino acid sequence. This sequence was used to do a BLAST*P* search of GeneBank. That search revealed that the *LdINV* sequence fragment was common to numerous annotated invertases. Results of these BLAST*P* searches showed that many of these invertases contained a conserved consensus sequence of FYASKTFYD. Based on these two target sequences, oligonucleotide primers were designed and used in PCR reactions with *L. donovani* (1SCL-2D) genomic DNA as template. The resulting 858 bp PCR product was cloned into *p*CR®2.1-TOPO and the resulting plasmid *LdINV*-PCR858 was subjected to nucleotide sequencing. Results of such sequencing showed that *LdINV*-PCR858 had very high sequence identity (~96 %) with that of an *L. major* Friedlin gene sequence (gi|15027087|emb|AL389894.4|LMFLCHR4A) which was annotated as an invertase (β-D fructofuranosidase). While this *L. major* sequence was annotated as an invertase no evidence of its functional activity was provided. Subsequently, the *LdINV*-PCR858 plasmid was DIG labeled and used to screen an *L. donovani* (1SCL-2D) cosmid library. A positive cosmid clone (16.3) was isolated and sequenced in the region of the putative *LdINV* gene. Results of such sequencing demonstrated that the *LdINV* consisted of an open reading frame (ORF) of 1932 bp including a methionine start codon of ATG at the 5′ end and a stop codon of TAG at its 3′ end. As indicated in the “Experimental procedures” section below, the 5′ splice leader (SL) acceptor site of the *LdINV* gene was mapped using a *Leishmania* SL primer in an RT-PCR reaction. Results of the latter confirmed the authenticity of the methionine start codon of the *LdINV* ORF. Further, the insert nucleotide sequence of the *LdINV*-PCR858 plasmid was found to have 100 % identity to that contained within the *LdINV* ORF. Sequence analyses showed that the composition of the *LdINV* ORF was GC-rich, which is consistent with the overall GC content of the *Leishmania* genome [10].

Furthermore, such analyses showed that the *LdINV* ORF encodes a polypeptide of 644 amino acids with a calculated molecular mass of 72,290 Da and a pI of 5.18. The nucleotide sequence of the *LdINV* ORF was used in multiple sequence alignments of invertases from various organisms (e.g., yeast, plant, fungi, etc.). Analyses of the *LdINV*-deduced protein using the Pfam database indicated that it belongs to the glycohydrolase family 32 of proteins [11, 12]. Characteristic features of this family of hydrolases include an *N*-terminal five-bladed beta propeller and a *C*-terminal beta sandwich structure [12]. Each of these domains was mapped on to the *LdINV*-deduced protein sequence. Typical members of this family

hydrolyse the glycosidic bond between two or more carbohydrates. One of the major sub-groups of the glyco hydro family 32 includes the invertases (i.e., protein/enzymes which can hydrolyze sucrose into glucose and fructose).

The hydrophobic, *N*-terminal, 28 amino acids of the *LdINV*-deduced protein constitutes a putative signal peptide based on the SignalP 3.0 algorithm [13]. Cleavage at this site, presumably in the endoplasmic reticulum (ER) of the parasite, would result in a mature protein with Lys^{27} as its *N*-terminal amino acid residue of the mature protein (Fig. 3). This hypothesis was fully supported by the results obtained of *N*-terminal sequencing of the ~70 kDa *LdINV* protein obtained from culture supernatants of FPLC fractionations (Fig. 3). Further the deduced amino acid sequence of the *LdINV* gene (KDGVPYE) is identical to that obtained from FPLC-isolated LdINV protein above (Fig. 3). Thus, cleavage at this site would result in a mature protein consisting of 618 amino acids with a calculated molecular mass of 69.6 kDa and a theoretical pI of 5.19. The *LdINV*-deduced protein was also analyzed using other structural algorithms. Those analyses indicated that this parasite enzyme lacked any apparent hydrophobic transmembrane domains or glycosylinositol phosphate anchor signature sequences [14]. Similarly, no KDEL or analogous ER retention sequences [15] or any other intracellular organelle specific-targeting sequences were identified in the *LdINV*-deduced protein. Based on its overall hydrophilicity, the presence of an *N*-terminal signal peptide and the absence of both membrane anchors and ER retention motifs suggest that the *Ld*INV represents a soluble/released protein. These deduced structural features are in good agreement with our experimental observations, which demonstrated that the endogenous wild-type invertase was constitutively released/secreted by *L. donovani* parasites during their growth in vitro.

The *LdINV*-deduced protein was also analyzed for potential N- and O-linked glycosylation and phosphorylation sites using NetNGlyc, NetOGlyc, and NetPhos web-based tools. Results of such analyses indicated that the *Ld*INV possessed six potential N-linked glycosylation sites at Asn120, Asn282, Asn396, Asn439, Asn480, and Asn628. These predictions are consistent with our earlier preliminary observations, which showed that the native, wild-type parasite (promastigote) released/secreted invertase, was a mannose-containing glycoprotein (i.e., was bound to concanavalin A beads, and such binding was inhibited with α-methylmannoside; data not shown). Results of NetOGlyc analyses failed to identify any potential O-linked glycosylation sites in the *LdINV*-deduced protein. In contrast, results of NetPhos analyses showed that *Ld*INV contained at least 24 potential sites for phosphorylation by several different mechanisms (e.g., casein kinase II, protein kinase C, etc.). These include 8 potential phosphorylation sites on serines (i.e., Ser5, Ser219, Ser269, Ser423, Ser447, Ser476, Ser514, and Ser567), 15 on threonines (i.e., Thr59, Thr84, Thr92, Thr129, Thr253, Thr284, Thr299, Thr311, Thr326, Thr355, Thr386, Thr408, Thr502, Thr522, and Thr581), and one on tyrosines (i.e., Tyr634).

As indicated above, results of our bioinformatic searches indicated that invertases in general possessed a conserved block of 14 amino acids including a critical Asp residue near their *N*-terminal end which define their substrate binding and hydrolytic site [11]. It is important to point out that this conserved invertase signature sequence was readily apparent in the *N*-terminal end of the *Ld*INV-deduced protein (Fig. 3).

Identification of LdINV homologs in other *Leishmania* parasites

Since *L. donovani* is a human pathogen it was of interest to ascertain whether an *LdINV*-like gene might also be present in the genomes of other related trypanosomatid parasites. In that regard, the *LdINV* sequence was used as our reference to examine the TriTryp/*Leishmania* genomes at www.tritrypdb.org. Results of such searches showed that *LdINV* homologs existed in the genomes of *L. infantum* (LinJ.04.0300), *L. major* (LmjF.04.0310), *L. tarentolae* (LtaP04.0290), *L. mexicana* (LmxM.04.0310), and *L. braziliensis* (LbrM. 04.0350). To validate whether such *LdINV* homologs were actually present in these various *Leishmania* species they were subjected to PCR analyses. For these assays, forward and reverse primers based on the *LdINV* ORF were used in conjunction with gDNA as template from *L. donovani* (1S-CL2D and LRC-751), *L. amazonensis*, *L. mexicana*, *L. tropica*, *L. major*, *L. infantum*, *L. braziliensis*, and *L. tarentolae*. Results of these assays confirmed the presence of an 850 bp product which was amplified from each of these species indicating that they all possessed an *LdINV* homolog. The products of these PCR reactions were cloned and subjected to nucleotide sequencing. Results of such sequencing confirmed that each of these species possessed an *LdINV* homolog. All of the above data are in agreement with our experimental results which showed that the culture supernatants of each of these species possessed secreted/released invertase-like activities. Having identified these *LdINV* homologs, it was of interest to compare their amino acid sequences. This was done using the MAFFT alignment algorithm as referenced below. A portion of this alignment analysis is shown in Fig. 3. Further, results of those analyses showed that each of the four annotated genomes of Old World *Leishmania* (i.e., *L. donovani*, *L. infantum*, *L. major*, and *L. tarentolae*) showed an overall identity of 82 % between amino acid 1 and 60 of their sequences. This included a putative signal peptide sequence of approximately 28 amino acids (*c.f. L. donovani* aa 1–29; Fig. 3). Signal peptidase cleavage at this putative site for *Ld*INV would result in a mature protein with KDGVPYE as an amino acid sequence at its *N*-terminal end. This prediction was verified by *N*-terminal end amino acid sequencing of the *Ld*INV FPLC-purified protein. It is of interest to point out that cleavage at this site in each of the *Ld*INV homologs would result in mature proteins with a conserved *N*-terminal 10 amino acid sequence [i.e., virtually identical with that of *Ld*INV (KDGVPYEPIF)]. Of equal or greater significance, is the immediate downstream conservation of a 14 amino acid glyco/hydrolase 32 subfamily invertase signature sequence among all of the *Ld*INV homologs (Fig. 3). It is also of significance to point out that the critical aspartic acid residue, which is the critical aa residue within the active/hydrolytic site of all invertases, is structurally conserved in all of the *Ld*INV homologs (Fig. 3). In addition, this high level of amino acid sequence conservation among all of the *Ld*INV homologs ensues downstream of this 14 aa “INV family signature block” for a total of 324 amino acids (Fig. 4). This 324 amino acid block represents an *N*-terminal end conserved domain among all of the invertase members of the glyco/hydrolase 32 protein family. The purported three-dimensional structure of this region has been characterized as a “five-bladed beta propeller” structure [12]. Also preserved downstream from this region in all of the *Ld*INV homologs is a 71 aa sequence characteristically conserved among all invertases of the glyco/hydrolase 32 protein family (Fig. 4). This “invertase family” *C*-terminal domain has a purported three-dimensional structure composed of a sandwich of beta-pleated sheets (Fig. 4) [12]. Interspersed between and connecting these two invertase family signature domains is a 142

amino acid sequence (i.e., aa 363–505) which appears to be common to all of the *Ld*INV homologs (Fig. 4). In addition, each of the *Ld*INV homologs also possess a common *C*-terminal extension of 67 amino acids (i.e., aa 576–643) terminating with a non-polar aliphatic amino acid residue (e.g., Val^{643} for *Ld*INV; Fig. 4). As indicated above, results of Net*N*Glyc analysis indicated that *Ld*INV possessed six potential N-linked glycosylation sites at Asn120, Asn282, Asn396, Asn439, Asn480, and Asn628 (Fig. 4). Among these, only two N-linked glycosylation sites (Asn396 and Asn628) were present in all of the *Ld*INV homologs (Fig. 4). Interestingly, these two N-linked glycosylation sites are restricted to the two amino acid domains that were unique to only the *Ld*INV homologs. Of the other four potential N-linked glycosylation sites, Asn120 was present in all of the human *Ld*INV homologs (Fig. 4). Similarly, Asn282 and Asn480 were present in all of the *Ld*INV homologs except *L. braziliensis* (Fig. 4). The remaining potential N-linked glycosylation site at Asn439 was conserved in all of the Old World *Ld*INV homologs and was positionally conserved near this amino acid in the New World *Ld*INV homologs (i.e., −2 aa *L. braziliensis*, +6 aa *L. mexicana*). The identification of N-linked glycosylation sites in *Ld*INV is consistent with our experimental results which showed that it is a released/secreted glycoprotein, as described above. It is conceivable that such N-linked glycosylation might be a common functional characteristic of each of the *Leishmania* secreted/released invertase activities which we demonstrated above.

Taken together, the general conservation in protein structure among the *Ld*INV homologs is in good agreement with our experimental results which showed that each of these *Leishmania* species actually released/secreted functional invertase activities. Further, this high level of conservation among *LdINV* homologs predicts that these secreted/released invertase enzymes must play important roles in the biology of all of these *Leishmania* parasites within their insect vectors.

As mentioned above, Gontijo et al. showed that various *Leishmania* sp. produced soluble invertase activities. Interestingly, they also showed that a variety of non-*Leishmania*, monoxenic insect trypanosomatids (i.e., *C. fasciculata*, *H. samuelpessoai*, and *L. seymouri*) also produced/released soluble sucrase/invertase activities. In addition these authors showed that the pathogenic plant trypanosomatid *Phytomonas* sp. also produced sucrase/invertase activity during their growth in vitro. In light of those observations, we searched various databases and found that multiple *LdINV*-like homologs were annotated (18 and 9, respectively) in the genomes of the non-*Leishmania* trypanosomatids *C. fasciculata* and *Phytomonas* sp. (i.e., HART-1 strain). These observations lend further support to the putative evolutionary relationships between *Leishmania* sp. and their insect trypanosomatid relatives. In contrast to the latter observations, it is important to point out that in the current report we were unable to detect any invertase activity, similar to the observations of Gontijo et al. in either African or American *Trypanosoma* sp. (i.e., *T. brucei* and *T. cruzi*). Congruent with these findings, despite multiple database searches, we were unable to identify any annotations for invertase in the genomes of any African or American *Trypanosoma* species. These data are compared and summarized in Table 1B.

Identification of an INV message during parasite development

Northern blot analyses were performed to evaluate the expression of *LdINV* mRNA during the parasite developmental life cycle. To that end, equivalent amounts of total RNA from both *L. donovani* promastigotes and axenic amastigotes were separated in agarose/formaldehyde gels (Fig. 5b), blotted onto nylon membranes, and hybridized with the *LdINV*-DIG858 probe. Results obtained from such blots showed that the *LdINV* probe specifically hybridized to two distinct messages, predominantly to one of approximately 4.4 kb and to a lesser extent to one greater than 7.5 kb, in both promastigote and axenic amastigote developmental forms (Fig. 5a). In that regard, the ~4.4 kb message is sufficiently large enough to encode the entire ~72 kDa *L. donovani* invertase protein, and the >7.5 kb message could represent its precursor. Alternatively, the >7.5 kb mRNA might reflect hybridization of the *LdINV*-DIG858 probe with some other structurally related genomic sequence(s). This remains a possibility due to the fact that during our genomic database searches for *LdINV* homologs we discovered a larger beta-fructofuranosidase-like gene (LdBPK_350650.1 which encodes ~120 kDa protein) that contained a partial homologous sequence of the *LdINV* gene. Interestingly, even though equivalent amounts of mRNA from each parasite developmental form were probed (Fig. 5b), the overall hybridization signals obtained in these blots appeared to be significantly more intense with RNA isolated from promastigotes (i.e., the insect vector stage of the parasite) than from axenic amastigotes (i.e., the developmental stage that produces disease in mammals). While these results indicated that the *LdINV* gene was transcribed throughout the parasite developmental life cycle, they also suggested that this gene's expression might be developmentally regulated. The latter hypothesis is in full agreement with our experimental results which consistently showed that *L. donovani* promastigotes, but not axenic amastigotes, produced/secreted *Ld*INV enzymatic activity (data not shown). Thus, our Northern results showed that *LdINV* mRNA was transcribed in both developmental forms but these results could not demonstrate that said message was actually translatable into protein. To investigate this further, it was of interest to determine whether the *LdINV* gene was indeed transcribed into mature mRNAs by both parasite developmental forms. To examine this, RT-PCR analyses were performed. It is important to point out that in trypanosomatid parasites all of their mature, translatable mRNAs are capped at the 5′ end with a conserved 39 nt, "splice leader" (SL) sequence [16]. Thus, for these experiments cDNAs were reverse transcribed from total RNA isolated from both *L. donovani* developmental forms. Aliquots of such cDNAs were used as template in PCR amplifications. For these reactions we used the 5′ forward primer corresponding to a portion of the SL sequence (SpliceFwd, see "Experimental procedures" section) and a Reverse primer corresponding to an internal sequence within the *LdINV* ORF (i.e., INV Rev, see "Experimental procedures" section). Results of these RT-PCR analyses showed that an approximately 500 bp product was amplified from both *L. donovani* promastigote and axenic amastigote developmental forms (data not shown). These results indicated that each of the parasite developmental forms did in fact produce a mature translatable *LdINV* mRNA from the *LdINV* gene. As anticipated from our Northern results, considerably more of the 500 bp RT-PCR product was detected in promastigotes than in axenic amastigote samples. Taken together with our Northern results, these RT-PCR results further suggest that *LdINV* protein expression is presumably post-transcriptionally regulated in these parasites.

Having made these observations above for *L. donovani* (i.e., our archetypical Old World *Leishmania* causing visceral disease in humans); it was deemed of interest to determine whether this phenomenon also pertained to *L. mexicana* (i.e., our prototypical New World *Leishmania* causing human cutaneous disease). To test this hypothesis, RT-PCR analysis was carried out using total RNA isolated from both *L. mexicana* promastigotes and axenic amastigotes. These reactions were carried out using a SL primer and an internal *LmexINV* primer (R*Lmex*INVRT) similar to those done with *L. donovani* above. Results of these assays showed that an approximately 500 bp product was amplified from both *L. mexicana* promastigote and axenic amastigote developmental forms (Fig. 5c). Thus, these RT-PCR results in conjunction with those from *L. donovani* above, strongly suggest that the expression of these parasite enzymes must be developmentally regulated, presumably involving post-transcriptional mechanisms. The latter hypothesis would account for the absence of detectable invertase activity in axenic amastigotes of either of these parasite species.

Reactivity of the anti-*E. coli*-recombinant LdINV antibody

As indicated, the *Ld*INV ORF was used to generate a recombinant *Ld*INV protein that was expressed in *E. coli*. Aliquots of purified *E. coli*-recombinant *Ld*INV (*rLd*INV) were subjected to SDS-PAGE, blotted onto PVDF membranes and tested for invertase activity. Cumulative results of these assays demonstrated that this single ~72 kDa *E. coli*-recombinant protein possessed invertase enzymatic activity. Having shown that this recombinant protein actually possessed functional invertase activity, we used it to generate a rabbit anti-*E. coli rLd*INV antibody (α-*rLd*INV). Subsequently, Western blot analyses were done to determine whether the α-*rLd*INV antibody would recognize the endogenous, wild-type *L. donovani* invertase. To examine this, concentrated culture supernatants from wild-type *L. donovani* promastigotes and aliquots of purified *E. coli rLd*INV protein were separated in SDS-PAGE gels and transblotted onto PVDF membranes. Such membranes were probed with our rabbit α-*rLd*INV antibody or its control NRS. In such blots the α-*rLd*INV antibody reacted specifically with a single ~72 kDa protein present in the *L. donovani* culture supernatants (Fig. 6a). As anticipated, this antibody reacted very strongly with a major ~70 kDa protein present in the purified *E. coli rLd*INV preparations. Presumably the difference in molecular mass between the ~72 kDa *Ld*INV and the ~70 kDa *E. coli rLd*INV reflects post-translational modifications (e.g., N-linked glycosylations and phosphorylations) to the parasite protein but not the bacterial protein. In addition, this α-*rLd*INV antibody also reacted with several other proteins of lower molecular mass in the purified *E. coli rLd*INV preparations. These bands presumably reflect potential protein degradations of the purified *rLd*INV. A similar conclusion pertains to the minor ~50 kDa protein observed in native *Ld*INV preparations. In parallel, NRS blots showed no reactivity with the native *Ld*INV or *E. coli rLd*INV samples (data not shown). Further, the α-*rLd*INV antibody showed no reactivity in control blots containing lysates of non-transfected *E. coli* or unused parasite culture medium (data not shown). Results of these Western blots demonstrated two significant points: (1) that our *E. coli rLd*INV expressed protein actually possessed functional invertase activity and (2) that the antibody we made against it specifically recognized the native *Ld*INV enzyme produced/secreted by promastigotes during their growth in vitro. Taken together these observations indicate the *Ld*INV gene that

we identified actually encodes a secreted/released *Ld*INV parasite enzyme. Results of our preliminary studies showed that the native released/secreted *Ld*INV enzyme is a mannose-containing glycoprotein which was in agreement with our NetNGlyc predictions above. Presumably such N-linked glycosylations aid in secretion and potential stability of the parasite enzyme. In light of our observations with the *E. coli rLd*INV protein, such N-linked glycosylations are not necessary for invertase enzymatic activity per se.

Results of our molecular searches above demonstrated that an *LdINV* homolog was present in *L. mexicana*. Further, we showed that this homolog had high structural identity with the *Ld*INV-deduced protein and that *L. mexicana* promastigote developmental forms made mature mRNA for this gene product. Moreover, these observations are in good agreement with results of our qualitative invertase gel assays which showed that *L. mexicana* promastigotes produced/secreted functional invertase activity during their growth in vitro. In light of these observations, it was of importance to determine whether the antibody that we made against the *rLd*INV-expressed protein (α-*rLd*INV) would also recognize the native released/secreted invertase produced by *L. mexicana* promastigotes. To examine this, aliquots of 1× culture supernatants from both *L. donovani* and *L. mexicana* promastigotes and unused culture medium controls were separated by SDS-PAGE, transblotted onto PVDF membranes and probed with the α-*rLd*INV antibody (Fig. 6b). As anticipated, results of these assays showed that the α-*rLd*INV antibody reacted with only a single ~72 kDa protein present in the homologous *L. donovani* culture supernatants. More importantly, this antibody specifically reacted with only a single ~72 kDa protein present in the culture supernatants of *L. mexicana* promastigotes (Fig. 6b). The unused culture medium controls showed no activity with the α-*rLd*INV antibody. To extend our observations, cell-free culture supernatants from additional *Ld*INV homologs [*L. donovani* (LRC-751), *L. amazonensis*, *L. tropica*, *L. major*, *L. infantum*, *L. braziliensis*, and *L. tarentolae*] promastigotes were tested in Western blots against the rabbit α-*rLd*INV antibody. Results of these assays showed that the α-*rLd*INV antibody reacted with only a single ~72 kDa protein present in the culture supernatants of each of these *Leishmania* species (data not shown). Taken together, these results in conjunction with our molecular observations strongly suggest that in addition to the native released/secreted *L. donovani* and *L. mexicana* invertase enzymes, each of the Old and New World species tested in this report all possess invertase enzymes with common structural features recognized by the α-*rLd*INV antibody.

Transfection of *Leishmania* promastigotes with epitope-tagged INV gene constructs

Having shown that the α-*rLd*INV antibody specifically recognized both the wild-type *L. donovani* and *L. mexicana* released/secreted invertases, it was deemed necessary to determine whether the *LdINV* and *LmexINV* genes in fact encoded functional invertase activities. To test this, a homologous expression system was devised. For these experiments, two separate chimeric constructs were generated: one contained the complete ORF of the cloned *LdINV* gene and the second contained the complete ORF of the cloned *LmexINV* gene. Each of these ORFs were fused, in-frame, at their 3′-end with a sequence encoding a 9-aa HA epitope of the influenza virus (all as described in the “Experimental procedures” section). Subsequent to ligation into the *pKSNEO* leishmanial expression vector, these constructs (designated subsequently as *LdINV-HA* and *LmexINV-HA*) were used to transfect

L. donovani and *L. mexicana* promastigotes, respectively. Promastigotes of both species were transfected with the *pKSNEO* vector alone and these served as controls in all subsequent transfection experiments. Following drug selection, the growth kinetics of these transfected promastigotes were compared as described below. Results of those in vitro assays showed that promastigotes transfected with either the *LdINV-HA* or the *LmexINV-HA* chimeric constructs had very similar growth kinetics to their *pKSNEO*-transfected controls (data not shown). Furthermore, non-transfected ("wild type") *L. donovani* and *L. mexicana* promastigotes, grown in complete medium lacking G418, displayed growth kinetics identical to those obtained with the transfectants above (data not shown). Taken together, our observations indicated that these episomal transfectants did not appear to alter the characteristic growth kinetics of the parental *L. donovani* or *L. mexicana* promastigote cell lines. Subsequently, such transfected promastigotes were analyzed for their expression of either the *LdINV-HA* or *LmexINV-HA* chimeric proteins using Western blots, immunoprecipitations (IPs), in situ enzyme activity gels, and spectrophotometric invertase enzyme assays.

Expression of the LdINV-HA and LmexINV-HA chimeric proteins in transfected parasites

Western blot analyses were performed to determine whether *LdINV-HA* and *LmexINV-HA* transfected promastigotes actually synthesized and released/secreted their respective chimeric protein during their growth in vitro. For these experiments, the transfected promastigotes were grown in chemically defined media, and their unconcentrated cell-free culture supernatants were subjected to IP as indicated below with either the α-*rLd*INV antibody or the rabbit α-HA antibody. The resulting immunoprecipitates were subjected to SDS-PAGE, transblotted onto PVDF membranes, and probed appropriately with either the respective α-*rLd*INV or α-HA antibodies. Western blots of samples immunoprecipitated with the α-HA antibody were probed with our α-*rLd*INV antibody. Results of such blots showed that the α-*rLd*INV antibody specifically reacted with only a single ~72 kDa *Ld*INV-HA or *Lmex*INV-HA-expressed protein in these α-HA immunoprecipitated samples (Fig. 7a). As anticipated, similar immunoprecipitates obtained from *L. donovani pKSNEO* and *L. mexicana pKSNEO* control transfectants showed no reactivity with our α-*rLd*INV antibody (Fig. 7a). Similarly, Western blots of culture supernatants immunoprecipitated with our α-*rLd*INV antibody were probed with the α-HA antibody. Results of such assays showed that the α-HA antibody specifically reacted only with an ~72 kDa *Ld*INV-HA or *Lmex*INV-HA-expressed protein in these immunoprecipitated samples (Fig. 7b). Immunoprecipitates obtained from *L. donovani pKSNEO* and *L. mexicana pKSNEO* control transfectants showed no reactivity with the α-HA antibody (Fig. 7b).

Taken together, results of these Western blot experiments demonstrated the following: (1) that the *LdINV-HA* and *LmexINV-HA* chimeric gene constructs were readily transcribed and translated into single ~72 kDa *Ld*INV-HA and *Lmex*INV-HA chimeric proteins in each of the transfected parasite species, respectively, (2) that these chimeric proteins were actually released/secreted by the transfected parasites during their growth in vitro, and (3) that the rabbit α-*rLd*INV antibody (i.e., made against the recombinantly expressed *L. donovani* invertase in *E. coli*) in fact recognized and immunoprecipitated both the *Ld*INV-HA and the *Lmex*INV-HA-expressed recombinant leishmanial invertases. Cumulatively, these

observations showed that both the native parasite released/secreted invertases and their HA epitope-tagged episomally expressed recombinant proteins (i.e., encoded by the *LdINV* and *LmexINV* genes) possessed common structurally conserved antigenic epitopes.

Results of our IP and Western blot analyses above showed that both the *Ld*- and *Lmex-INV-HA*-transfected parasites synthesized and released/secreted their respective chimeric proteins. Therefore, it was relevant to determine whether these chimeric proteins in fact possessed functional invertase activity. For those assays, unconcentrated (1×) culture supernatants from *Ld*INV-HA and *Lmex*INV-HA transfected promastigotes and their *pKSNEO* controls were assayed for invertase activity in our qualitative agarose-gel-TTC reaction product capture assay. Results of those qualitative assays demonstrated that the *Ld*INV-HA-transfected promastigotes synthesized and released/secreted significantly enhanced levels of invertase activity versus their *pKSNEO* controls (Fig. 8a, b). The latter observations were verified by results obtained with these culture supernatants in our GO spectrophotometric invertase assays (Fig. 8e). The enhanced levels of invertase activity produced by the *Ld*INV-HA transfectants (i.e., ~10,000 units of invertase activity/10^7 parasites/ml) presumably reflect an additive contribution of both their wild-type activity and that of the expressed chimeric enzyme (*c.f.* Figs. 1d, 8e). In contrast, the activity observed with culture supernatants from *pKSNEO* control transfectants (i.e., ~100 units of invertase activity/10^7 parasites/ml) is equivalent to the endogenous levels of invertase activity produced and released by "wild-type" promastigotes (*c.f.* Figs. 1d, 8e). As anticipated, very similar results were obtained in these assays using culture supernatants from *Lmex*INV-HA-transfected promastigotes and their *pKSNEO* controls (Fig. 8c, d). The gel shown in Fig. 8 is representative of the results obtained from numerous gel assays which were carried out in these experiments. Conclusions drawn based on our results from the *L. donovani* transfectants above similarly apply to those obtained with the *L. mexicana* transfectants. Results of those assays showed that the *L. mexicana INV-HA* transfectants did in fact produce and secrete the chimeric invertase activity but interestingly only at qualitatively lesser amounts than that produced by similar numbers of *L. donovani* transfectants (*c.f.* Fig. 8a, c). The latter observations mirror those which we obtained above with wild-type parasites from both of these species (*c.f.* Figs. 1, 8). Taken together, these observations further support the hypothesis which we stated above suggesting that the New World species produce invertase activity but at overall lower levels than that produced by Old World parasites. These observations also suggest that even though driven by episomal expression, synthesis and release/secretion of invertase by the New World *L. mexicana* parasites must involve some level of regulation.

Discussion

Species of the protozoan parasite *Leishmania* cause over 2 million new cases of human cutaneous, mucocutaneous, and fatal visceral disease per year worldwide [1]. All *Leishmania* parasites undergo a digenetic life cycle which includes differentiation, development, and transmission between a sand fly vector and a mammalian host. Within these hosts, *Leishmania* parasites reside and multiply in highly restricted microenvironments (i.e., as extracellular, flagellated, promastigote forms within the alimentary tract of their sand fly vectors, and as obligate intracellular amastigote forms within the phagolysosomal

compartments of infected macrophages) [2]. In this report we chose to use *L. donovani* (1S/CL2D) as an archetypical example of an Old World *Leishmania* which can cause fatal human visceral disease. Conversely, we chose to use *L. mexicana* (M379) in this study because it is an important protozoan pathogen of humans throughout Central and South America and that it is the major causative agent of human cutaneous leishmaniasis in the New World [17].

It is of relevance to point out that the focus of our laboratory concerns the identification and characterization of both cell surface membrane and released/secreted enzymes produced by these pathogenic *Leishmania* parasites. The rationale for our studies is to address how these parasites access essential nutrients from both their mammalian and insect vector hosts to facilitate their survival, multiplication, differentiation, and transmission throughout their developmental life cycle. With that in mind, we were motivated to initiate the current study based on the report of Blum and Opperdoes [8] which described for the first time, the identification of a released/secreted sucrase/invertase-like activity from *L. donovani* promastigotes. In that report, those investigators showed that a soluble sucrase/invertase-like enzyme activity was constitutively produced and released/secreted by *L. donovani* promastigotes (i.e., the 1S/CL2D strain) during their growth in vitro. Further in their discussion, Blum and Opperdoes concluded: “The sucrase that is secreted by promastigotes is likely to play an important role in the nutrition and development of the promastigotes in the insect gut,…” [*sic*]. Subsequently, the results of Blum and Opperdoes were verified and extended by Gontijo et al. [9]. In that report, those authors showed that promastigotes of a geographically distinct isolate of *L. donovani* from India (MHOM/80/DD8) also produced and released/secreted sucrase/invertase-like enzyme during their growth in vitro. Further, they showed that promastigotes of several other Old World *Leishmanias* also produced and released/secreted sucrase/invertase-like activities [i.e., *L. aethiopica, L. infantum, L. major, L. (Sauroleishmania) tarentolae,* and *L. tropica*]. Of equal or greater significance, Gontijo et al. demonstrated that such sucrase/invertase-like activities were also produced and released/secreted by promastigotes of at least six different New World *Leishmanias* (i.e., *L. amazonensis*, *L. braziliensis*, *L. chagasi*, *L. guyanensis*, *L. mexicana*, and *L. panamensis*). Cumulatively, the results of Gontijo et al. indicated that the production and release of sucrase(s)/invertase-like activities by promastigotes is a common biological characteristic of both Old and New World *Leishmanias*.

In light of those reports, we initiated our studies toward the identification, characterization and expression of the gene(s) encoding these putative leishmanial secretory/released invertase-like enzymes. In preliminary experiments, we devised an agarose-gel-TTC reaction product capture assay to qualitatively measure the invertase activity produced and released/secreted by *L. donovani* promastigotes in vitro. The invertase activities of such promastigote culture supernatants were also quantitated using the GO spectrophotometric assay. Results of these assays were in full agreement with the observations reported by Blum and Opperdoes concerning *L. donovani*. In subsequent experiments, we demonstrated that invertase activity was also produced and released/secreted by promastigotes of numerous different Old and New World species of *Leishmania*. Thus, our current results confirmed and extended the observations of Gontijo et al. and provide strong evidence showing that the

synthesis and release/secretion of invertase-like activities is a common feature of promastigotes of all *Leishmania* species. It is of interest to note, that the results of our quantitative assays showed that promastigotes of each of the Old World *Leishmania* species tested typically produced at least 100 units of invertase activity per 1×10^7 cells. In contrast, promastigotes of each of the New World species tested produced only ~10 units of invertase activity per 1×10^7 cells. The dichotomy in invertase production observed between Old and New World *Leishmania* parasites presumably reflects their individual adaptation for growth and survival within the alimentary tracts of their geographically unique sand fly vector hosts.

As indicated above, the cumulative results of our qualitative and quantitative invertase assays demonstrated that promastigotes of virtually all of the Old World *Leishmania* species tested produced significant quantities of invertase activity. In that regard, experiments were devised to isolate the released/secretory invertase produced by *L. donovani* (1SCL-2D) promastigotes. Following Mono-Q FPLC fractionation and SDS-PAGE separation, a single ~70 kDa *L. donovani* putative invertase (*Ld*INV) was isolated. Subsequently *N*-terminal end Edman Amino Acid Sequencing of this ~70 kDa *Ld*INV protein revealed that it contained an amino acid sequence KDGVPYE. Results of BLAST*P* searches of GeneBank revealed that the *Ld*INV sequence fragment was common to numerous annotated invertases. Further, many of these invertases also contained a downstream conserved consensus sequence of FYASKTFYD. Oligonucleotide primers of these two target sequences were used in PCR reactions with *L. donovani* gDNA as template. The resulting ~850 bp PCR product served as a probe to identify an *L. donovani* cosmid clone (16.3) for our subsequent isolation of the gene encoding the *Ld*INV. Nucleotide sequencing of this cosmid clone revealed that the *Ld*INV ORF consisted of 1932 bp. Analyses showed that this sequence was identical to that present on chromosome 4 of the *L. donovani* TriTryp genome database which was recently annotated as a putative/potential invertase enzyme (LdBPK_040300.1). Translation of the *Ld*INV ORF showed that it encodes a polypeptide of 644 amino acids with a calculated molecular mass of 72,290 Da. Analysis of the *Ld*INV-deduced protein using various structural algorithms indicated that it possessed features typical of a soluble/secreted glycoprotein (e.g., the presence of a putative *N*-terminal signal peptide for targeting to the ER; six potential N-linked glycosylation sites, overall hydrophilicity; absence of both, membrane anchor domains and ER retention motifs). These predicted structural properties are in good agreement with our experimental data demonstrating that the native wild-type ~70 kDa *Ld*INV was glycosylated (i.e., mannosylated) and that it was constitutively released/secreted by *L. donovani* promastigotes during their growth in vitro. Further, these observations are in agreement with the earlier studies of Gontijo et al. who isolated an ~70 kDa released/secretory invertase-like (*sic* sucrase) protein from culture supernatants of *L. amazonensis* promastigotes. Results of *Pfam* analysis of the *Ld*INV-deduced aa sequence showed that it had high homology with, and characteristics of, the glyco/hydrolase 32 protein family. Moreover, within this family, the *Ld*INV possessed characteristic signature sequences of the invertase subfamily (i.e., proteins/enzymes which can hydrolyze sucrose into glucose and fructose).

Results of our molecular analysis indicated that we identified a gene which putatively encoded the *L. donovani* released/secreted invertase enzyme. The *LdINV* sequence was subsequently used as our reference to identify its homologs in several other *Leishmania* species [i.e., *L. infantum* (LinJ.04.0300), *L. major* (LmjF.04.0310), *L. tarentolae* (LtaP04.0290), *L. mexicana* (LmxM.04.0310), and *L. braziliensis* (LbrM.04.0350)] annotated within the TriTryp genome database (www.tritrypdb.org). We experimentally validated the presence of all of these *LdINV* gene homologs using PCR analysis and nucleotide sequencing. Further, genomic analyses showed that all of these *LdINV* homologs possessed similar high levels of structural synteny within their respective genomes. Thus, each of the *LdINV* homologs has been annotated respectively to chromosome number 4 (www.tritrypdb.org). Moreover, our analyses showed a very high level of conserved synteny across this region of chromosome 4 in which each upstream and downstream ORF was also conserved among the *Leishmania* species examined. The cumulative data of our alignments and TriTryp database annotations demonstrated that the *LdINV* gene homologs appear to be structurally conserved in the genomes of both Old and New World *Leishmania* species. The prevalence of the *LdINV* homologs among all *Leishmanias* presumably reflects parasite adaptation for survival, growth, and transmission within their sand fly vector hosts (e.g., *Phlebotomus* sp. and *Lutzomyia* sp.). In parallel to expression in all *Leishmania species* that have been tested, a variety of monoxenic insect and plant kinetoplastids have also been shown to constitutively release/secrete functional invertase activities (i.e., *C. fasciculata*, *H. samuelpessoai*, *L. seymouri*, and *Phytomonas* sp.). As anticipated, we found several *LdINV*-like homologs in the published genomes of two of these organisms [i.e., *C. fasciculata* and *Phytomonas* sp. (HART-1 strain)]. It contrast to the foregoing, it is significant to point out that no *LdINV* homologs have been identified/annotated within the genomes of either the pathogenic Old World African trypanosomes (i.e., *T. brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense*, *T. congolense*, or *T. vivax*) nor the New World human pathogen *T. cruzi*. The absence of *LdINV* homologs from all of the *Trypanosoma* species suggests that this gene product is not necessary for their survival within their tsetse fly or triatoma insect vector hosts.

Results of *Pfam* analyses indicated that the *Ld*INV-deduced protein contained a 28 aa signal peptide and cleavage at this site would result in the ~70 kDa secreted/released protein having an *N*-terminal end sequence of KDGVPYE. These predictions are in full agreement with our experimental results below. MAFFT alignment analyses showed that an analogous-predicted signal peptide was also present in each of the other leishmanial *LdINV* homologs. Those predictions are in good agreement with our experimental results which demonstrated that promastigotes of each of the *Leishmania* species tested produced released/secreted invertase activity during their growth in vitro.

Moreover, MAFFT analyses further showed that each of the *LdINV* homologs possessed a conserved 14 amino acid signature sequence characteristic of the glyco/hydrolase 32 protein subfamily of invertases, including the critical aspartic acid residue within the active site. In addition, each of the *Ld*INV-deduced protein homologs possessed both a characteristic *N*-terminal domain (i.e., a 324 amino acid block) and a *C*-terminal domain (i.e., a 71 amino acid block) typically found in members of the glyco/hydrolase 32 protein (invertase) family.

Interestingly, all of the *Ld*INV-deduced protein homologs also possessed a common 142 amino acid sequence which is inserted between the two invertase family signature domains above. Further, all of the *Ld*INV-deduced protein homologs also possessed a common 67 amino acid *C*-terminal extension sequence, terminating with a valine residue. While these two "inserted leishmanial sequences" were present in all of the *Ld*INV-deduced protein homologs, neither sequence was present in any other known member of the glyco/hydrolase 32 protein family. Results of Net*N*Glyc analyses indicated that all of the *Ld*INV-deduced proteins possessed six potential N-glycosylation sites. However, of these, only two were present in all of the *Ld*INV-deduced protein homologs and those were restricted to the two amino acid domains unique to the *Leishmania* invertases (i.e., Asn396 and Asn628). The cumulative results of these structural analyses demonstrated a very high level of conservation in protein structure among all of the *Ld*INV homologs. Presumably such structural conservation must be required for the function of this secretory enzyme. Further, this would suggest that this enzyme's function must be critical to the survival and development of all of these *Leishmania* species within their insect vector hosts.

In light of the foregoing observations, it was necessary to actually demonstrate that a mature *LdINV* mRNA was in fact synthesized by these parasites. In that regard, the cumulative results of our Northern blot and RT-PCR analyses with *L. donovani* demonstrated that a mature message for the *LdINV* gene was in fact readily transcribed by promastigotes and to a much lesser degree by axenic amastigote developmental forms of this parasite. Interestingly, virtually identical Northern and RT-PCR results were obtained concerning the presence of mature message for *LmexINV* in *L. mexicana* promastigotes and axenic amastigotes. Thus, these results demonstrated that mature, translatable mRNA was transcribed from both the *LdINV* and *LmexINV* genes. Further, these data suggested that each of these genes was differentially/developmentally up-regulated in promastigote developmental forms in each of these species. These findings are in good agreement with results of our enzymatic assays which showed that promastigotes but not axenic amastigotes produced significant quantities of soluble/secretory invertase activity. Taken together, these observations underscore the significance of the idea that this enzyme must play an important role(s) in the survival, development, and transmission of *Leishmania* promastigotes within their insect vector host(s).

In this report, we generated a recombinant *Ld*INV protein (*rLd*INV) in *E. coli* to determine whether this gene encoded a functional invertase activity. Results of enzymatic assays demonstrated that this *rLd*INV protein did in fact possess potent invertase activity. Subsequently, we generated a rabbit antibody (α-*rLd*INV) against the functional *rLd*INV protein. Results of Western blot analyses demonstrated that this α-*rLd*INV antibody specifically recognized both the ~70 kDa *rLd*INV protein as well as the ~72 kDa native released/secreted invertase produced by *L. donovani* promastigotes. Of equal importance were our results which showed that this α-*rLd*INV antibody also recognized the ~72 kDa native released/secretory invertase produced by *L. mexicana* promastigotes. Thus, these results showed that the *Ld*INV and *Lmex*INV proteins possessed common antigenic epitopes. Moreover, these cumulative results demonstrated that the *LdINV* and *LmexINV* genes actually encode the native secretory invertases produced by promastigotes of both of

these parasite species. Further, it is of significance to point out that this antibody (α-*rLd*INV) also specifically recognized and reacted with the native ~72 kDa secretory invertases produced by a second geographically distinct *L. donovani* isolate (LRC-751) and in addition to multiple individual isolates of *L. amazonensis*, *L. tropica*, *L. major*, *L. infantum*, *L. braziliensis*, and *L. tarentolae* promastigotes used in this study. These experimental results are fully congruent with our molecular analyses which demonstrated the very high level of structural conservation noted among the various *LdINV* homologs analyzed in this report (i.e., LinJ.04.0300, LmjF.04.0310, LtaP04.0290, LmxM.04.0310, and LbrM.04.0350).

While the foregoing conclusions were compelling, we felt it was necessary to demonstrate that the *L. donovani* and *L. mexicana INV* genes in deed produced functional parasite secretory invertases. To do this, we generated two separate chimeric constructs: one contained the *LdINV* ORF and the second contained the *LmexINV* ORF. The 3′ end of each of these constructs contained the 9-aa HA epitope of the influenza virus. Promastigotes of both *L. donovani* and *L. mexicana* were episomally transfected with their homologous constructs. Subsequently, aliquots of culture supernatants from these transfectants were subjected to coupled IP and Western blotting analyses with rabbit α-HA and our rabbit α-*E. colirLd*INV antibodies. Taken together, the results of these experiments showed that: (1) the *LdINV-HA* and *LmexINV-HA* chimeric gene constructs were readily transcribed and translated into single ~72 kDa *Ld*INV-HA and *Lmex*INV-HA chimeric proteins in each of the transfected parasite species, respectively, (2) these chimeric proteins were actually released/secreted by the transfected parasites during their growth in vitro, and (3) the rabbit α-*rLd*INV antibody (i.e., made against the recombinantly expressed *L. donovani* invertase in *E. coli*) in fact recognized and immunoprecipitated both the *Ld*INV-HA and the *Lmex*INV-HA-expressed recombinant leishmanial proteins. Results of our qualitative agarose-gel-TTC reaction product capture assays demonstrated that the *Ld*- and *Lmex*INV-HA-transfected promastigotes synthesized and released/secreted significantly enhanced levels of invertase activity versus their *pKSNEO* controls. These observations were verified and extended by results of our quantitative GO spectrophotometric assays. The latter showed that the *LdINV-HA* transfectants produced about ~100-fold higher levels of invertase activity than their *pKSNEO* control transfectants (i.e., ~10,000 vs. ~100 units of invertase activity). The levels of invertase activity observed with *pKSNEO* controls were found to be equivalent of those of non-transfected “wild-type” *L. donovani* promastigotes. Similar conclusions apply to the invertase activities observed with our *LmexINV-HA* and *pKSNEO* control transfected parasites. Cumulatively, these results demonstrated that the *LdINV* and *LmexINV* genes did in fact encode functional secretory invertases produced by these two parasite species. As indicated above, results of our preliminary experiments showed that all of the “wild-type” Old World *Leishmania* species characteristically produced ~10-fold higher levels of invertase activity compared to any of the New World species tested. Interestingly, this ~10-fold difference in invertase production between Old and New World *Leishmania* was also maintained and manifestly evident from the results of our over-expression studies using *Ld*- and *Lmex*INV-HA-transfected parasites. These observations strongly suggest that even though driven by episomal expression, synthesis, and release/secretion of invertase by New World *L. mexicana* parasites must involve some level of endogenous/innate regulation.

As indicated above, the studies of Blum and Opperdoes were the first to report that *L. donovani* promastigotes produced and released/secreted an invertase-/sucrase-like activity [8]. Subsequently, Gontijo et al. reported that such invertases/sucrases (*sic*) were produced by promastigotes of a variety of New and Old World *Leishmania* species [9]. The results of the current study have confirmed those previous observations and extended them to include the molecular and structural characterization of the genes encoding these parasite secretory invertases. It is of significance to note that both the reports of Blum and Opperdoes and Gontijo et al. suggested that such secreted parasite invertases/sucrases might play important roles in the survival and development of promastigotes within the gut of their insect vector host(s). It is of relevance to point out that both Old and New World *Leishmania* parasites are transmitted to their respective phlebotomine sand fly vectors (i.e., *Phlebotomus* sp. in the Old World and *Lutzomyia* sps. in the New World) when these insects take a blood meal from an infected mammalian host. Within these infected insect vectors, *Leishmania* parasites multiply and move anteriorly in the sand fly alimentary tract. In order to sustain this anterior migration, these parasites must obtain nutrients/energy sources derived from the insect host gut. Interestingly, between their infectious blood meals, female sand flies characteristically feed on plant juice sugars including sucrose and other complex polysaccharides [7]. Following ingestion, such sugar meals are shunted and stored within the crop compartment of the insect's gut. Hence, between infectious blood meal feeds, such sugars are regurgitated back into the sand fly anterior midgut where they would impact/interface with the anteriorly migrating *Leishmania* parasites. Within this milieu, the parasite extracellular secretory invertase could readily hydrolyze such plant sugars rendering them available for uptake by the parasites as nutrients/energy sources. Such uptake is imminently likely since *Leishmania* promastigotes possess multiple cell surface membrane sugar transporters (e.g., the highly active glucose/H^+ symport system) [18, 19]. Thus, within this context, the secretory invertases would fulfill a vital role(s) in the survival, growth, development, and transmission of *Leishmania* parasites within their insect vector hosts.

It is tempting to speculate that these secretory invertases might also be part of a parasite chemi/osmotic sensory system which could stimulate/facilitate the anterior migratory behavior typically observed in *Leishmania* parasites within the insect vector gut. In that regard, *Leishmania* promastigotes have been shown experimentally to directionally orient and migrate within sugar and osmotic/chemical gradients similar to those potentially present in the insect vector midgut [20]. It is important to point out that the *Leishmania* apical flagellum is responsible for such directional orientation and movement of these parasites. Interestingly, two *Leishmania* cell surface transport proteins have recently been characterized [i.e., the *L. mexicana* glucose transporter 1 (LmxGT1) and a *L. major* aquaglyceroporin channel (LmjAQP1)] and shown to be localized to the flagellar membranes of their respective *Leishmania* sps. [21–23]. In these reports it was speculated that their respective specific proteins were involved with chemical/environmental sensing and transport regulation. Thus, in such a model, the hydrolysis products of the parasite secretory invertases, described herein, would change the osmotic microenvironment and could readily be sensed and transported by the latter flagellar transport systems to facilitate parasite motility and mobility within the insect vector gut.

As mentioned above, it has been reported that both Old and New World species of sand flies produce their own gut invertase-like activities [7]. It has been suggested that such endogenous gut invertases would aid the sand fly to digest complex polysaccharides they imbibed from their plant juice meals. Thus, in an infected (female) sand fly the leishmanial secretory invertase could be nutritionally beneficial to both the parasite and its insect vector. Presumably, the production of the parasite secretory invertases represents a symbiotic evolutionary adaptation to the plant juice feeding behavioral habits of their insect vector hosts. In that context, the ~10-fold difference observed in invertase production in the current study between Old and New World *Leishmania* species must reflect a symbiotic adaptation to the plant feeding preferences and habits of their respective geographically distinct sand fly vector hosts (i.e., *Phlebotomus* sps. vs. *Lutzomyia* sps.). The foregoing hypothesis seems logical within the context of the symbiotic relationships between the *Leishmania* parasites and their sand fly vector hosts. In addition to the secretory/released invertases described herein, a novel sucrose/H^+ (proton) symport system has been recently reported to be present in the surface membranes of *L. donovani* promastigotes [24]. Presumably, the secretory invertases and this sucrose symport system would work in concert to fulfill the energy needs of *Leishmania* promastigotes within their insect vector gut. In contrast to *Leishmania*, no parallel host/parasite relationships can be espoused for either of their African or American *Trypanosoma* sp. relatives. In that regard, to date, no biochemical or molecular evidence exists concerning the presence of invertase or invertase-like enzymes or genes in any African or American *Trypanosoma* species.

In the current report, we have demonstrated that promastigotes of both Old and New World *Leishmania* species constitutively synthesized and released functional invertase enzyme activity into their cell culture supernatants. We assume that such invertase activity is released from these parasites via their endogenous secretory pathways similar to that described previously for their leishmanial secretory acid phosphatases [25, 26]. Recently; however, exosomes have been reported to exist as an alternative mechanism for the release of endogenous constituents by *Leishmania* parasites [27, 28]. While we cannot rule out that at least some invertase activity might leave these parasites via exosomes, the majority of the invertase activity that we measured in this study showed no functional latency (i.e., as mentioned in the “Results” section above).

In summary, results of the current study have shown that promastigotes of both Old and New World *Leishmania* species universally synthesize and release invertase enzymes during the course of their developmental life cycles. Further, we have identified the genes encoding these enzymes, shown that they are differentially/developmentally expressed and demonstrated the functional activity of their expressed proteins. The ability to now homologously over-express these genes in various *Leishmania* parasites should facilitate future studies concerning the role(s) of this enzyme in the developmental life cycles of these important human pathogens. Since invertases appear to be universally expressed by the promastigote (i.e., insect vector) forms of all *Leishmania species* tested, but not by amastigote forms (i.e., the parasite stage producing disease in humans), this enzyme might represent a logical target for disrupting their developmental life cycle and hence their transmission by their insect vector hosts.

Experimental procedures

Reagents

All chemicals used, unless specified, were of analytical grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Enzymes used for molecular studies were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA); DNA and RNA molecular mass standards were from Life Technologies (Grand Island, NY, USA), and protein molecular mass standards were purchased from GE Healthcare Biosciences (Pittsburg, PA, USA).

Nomenclature

The designations used in this report for genes, proteins, and plasmids follow the nomenclature for *Trypanosoma* and *Leishmania* as outlined by [29].

Parasite cell lines

Multiple *Leishmania* cell lines which cause either visceral or cutaneous disease were used in this report. These included two visceral strains of *L. donovani* 1S-CL2D (WHO designation MHOM/SD/62/1S/CL2D originally isolated from a patient in Sudan) and LRC-751 (WHO designation MHOM/IN/93/BI2302/LRC-751 isolated from a visceral patient in India); two strains of *L. infantum* Spain (WHO designation MCAN/SP/00/FVM10001JL) and *L. infantum* LRC-639 (WHO designation MCAN/IL/94/Robi/LRC-639 isolated from a dog in Israel); two isolates causing cutaneous disease in the Old World: a single Old World isolate of *L. major* Friedlen (WHO designation MHOM/IL/81/Friedlin isolated from a patient with cutaneous disease); *L. tropica* WR683 (WHO designation MHOM/SU/58/OD/WR683); and various strains causing cutaneous disease in the New World: i.e., two cutaneous isolates of *L. mexicana* M379 (WHO designation MNYC/BZ/62/M379 an isolate from a rodent in Belize) and BEL21 (WHO designation MHOM/BZ/82/BEL21 isolated from Belize); one isolate of *L. braziliensis* WR668 (WHO designation MHOM/BR/76/LTB/300); two isolates of *L. amazonensis* PH8 (WHO designation VEC/BR/67/PH8) and GML584 (WHO designation MHOM/P/GML/584); one isolate of *L. pifanoi* WHOLL1 (WHO designation MHOM/VE/57/LL1); two isolates of *L. panamensis* WR470 (WHO designation MHOM/BR/470) and WHO594 (WHO designation MHOM/BR/L/594); and one isolate of *L. guyanensis* M4147 (WHO designation MHOM/BR/75/M4147), and a single isolate of *L. tarentolae* (Jena 101, Jena Bioscience, Jena, Germany an isolate of a gecko).

In vitro culture of parasites

Promastigote developmental forms of all of the above *Leishmania* species were grown at 26 °C in Medium M199 (Life Technologies), supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA). In contrast, axenic amastigote forms for *L. mexicana* and *L. donovani* were grown at 32 and 37 °C, respectively, in medium RPMI 1640 supplemented with 20 % (v/v) FBS, as described previously [30]. For isolation of nucleic acids and proteins, both promastigote and axenic amastigote parasite cultures were harvested at ~mid-log phase (~1–2 × 10^7 cells ml^{-1}) by centrifugation at 2100×*g* for 15 min at 4 °C. The resulting cell pellets were washed twice in

ice-cold phosphate buffered saline (PBS, 10 mM sodium phosphate, 145 mM NaCl, pH 7.4) by centrifugation and finally re-suspended in the appropriate buffers for isolation of nucleic acids and proteins.

Short-term release assay for secretory proteins

In order to obtain useable quantities of proteins released/secreted by promastigotes during their growth in vitro, promastigotes of the various strains were cultured at 26 °C in a macromolecule-free, chemically defined medium (M199+) [31]. When such cultures reached a density of ~1.5×10^7 cells ml^{-1}, the cells were examined by phase contrast microscopy to ensure >99.9 % cell viability and subsequently harvested by centrifugation at ~2100×*g* for 20 min at 4 °C. The cell pellets were washed (3×) by centrifugation as above using ice-cold HEPES/NaCl pH 7.4 [25] and resuspended at a density of 4×10^8 cells ml^{-1} in HEPES/NaCl pH 7.4 containing 1 mM glucose (as an energy source). Such cell suspension was incubated for 3 h at 26 °C on a rocking platform. During this period, secretory proteins previously synthesized by the cells were released into the buffered medium. At the end of incubation the cells were re-examined by phase contrast microscopy to ensure cell viability and subsequently harvested by centrifugation as described above. Such supernatants were finally recentrifuged at high speed (6000×*g* [25]) to ensure complete removal of cells. The resulting supernatants from several release assays (from each of the organisms tested) were pooled, dialyzed against several changes of buffer (50 mM Tris pH 7.4), and frozen for further analysis. One of these pools was subsequently subjected to anion exchange chromatography.

FPLC fractionation of released/secreted proteins

Dialyzed cell-free release assay supernatants obtained from *L. donovani* (1SCL-2D) promastigotes were loaded on to a Mono-Q column fitted onto a FPLC system (GE Healthcare Life Sciences). The column was extensively washed with buffer (50 mM Tris pH 7.4) and, subsequently, bound proteins were eluted using a Tris–NaCl gradient. Four ml fractions were collected, dialyzed, and concentrated. Aliquots from each fraction were separated on 10–15 % NUPAGE gels (Life Technologies) and silver stained using SilverXpress® silver staining kit (Life Technologies) according to manufacturer's recommendations. Results of silver staining demonstrated that one of these fractions contained a single protein band with an apparent molecular mass of approximately 70 kDa. An aliquot of this selected fraction was separated by SDS-PAGE, blotted onto a PVDF membrane (Life Technologies), and stained with Coomassie Blue as described previously [32]. Subsequently, a single Coomassie Blue-stained protein band was excised from such blots and subjected to *N*-terminal Edman Sequencing at the Research Technologies Branch facility of the NIAID, NIH, Rockville, MD.

Generation of cell-free culture supernatants

In order to analyze, the secreted/released proteins (enzymes) produced by promastigotes of the various *Leishmania* species listed above were grown in a macromolecule-free, chemically defined medium (M199+) [31]. Such cultures were initiated as follows: mid-log phase promastigotes were inoculated into culture medium to a final density of ~1×10^6 ml^{-1}

and allowed to grow for ~72 h at 26 °C. When such cultures reached a density of ~1.5 × 10^7 cells ml^{-1} they were examined by phase contrast microscopy to ensure >99.9 % cell viability and subsequently harvested by centrifugation at ~2100×*g* for 20 min at 4 °C. Following a high-speed re-centrifugation step, to ensure the complete pelleting of cells [33], the resulting cell-free (1×) culture supernatants were concentrated by ~100-fold (100×) using pressure ultrafiltration as described previously [34]. To evaluate the kinetics of the invertase enzyme activity produced/released by parasites during their in vitro growth, cultures as above were harvested at different time points during their growth. These were concentrated as described above and such samples were evaluated for their total enzyme activities.

Agarose-based qualitative assay for invertase

We developed an agarose-based activity assay in order to qualitatively determine the presence of invertase activity produced/secreted by parasites grown in vitro. Briefly 20 ml of a pre-warmed solution of 1 % (w/v) agarose (Sigma Aldrich) containing 0.2 M sodium acetate buffer (pH 5.0) and 25 mM sucrose was pipetted into a 100 mm petri dish. After cooling to RT, small wells were cut and the agarose plugs were extracted by vacuum. Aliquots of concentrated parasite culture supernatants from above were added to the wells of such plates and the plates were incubated for 4 h at 37 °C. The premise for these assays was based on the hypothesis that parasite released/secreted invertase activity would hydrolyze the sucrose substrate present in the agarose gel producing glucose and fructose as end products. One of the products of this reaction (fructose) was visualized in these plates by using the TTC (Sigma Aldrich) capture reagent. This was accomplished by flooding the plates with 0.1 % (w/v) TTC in 1 N NaOH and incubating them for 10–20 min at 45 °C. The resulting invertase activity was visualized in these agarose plates by the presence of a dark red–purple-colored ring of TTC-captured product radiating from the sample wells. Controls in these assays consisted of aliquots of unused parasite growth medium or short-term release assay buffer. Digital images of such plates were capture using a Nikon Coolpix 3500 camera (Nikon, Melville, NY, USA). Images were processed using Adobe Photoshop CS2 (Adobe Systems, Inc.).

Spectrophotometric assay of invertase activity

Invertase activity present in parasite culture supernatants was measured using sucrose as the substrate. The premise for these assays was also based on the hypothesis that parasite released/secreted invertase activity would hydrolyze the sucrose substrate into glucose and fructose. In these assays, we could quantitate the resulting glucose product. Briefly, aliquots of parasite culture supernatants or short-term release assays were incubated with 25 mM sucrose (final concentration) in a total volume of 0.5 ml in 0.1 M sodium citrate buffer, pH 5.5 at 37 °C. Following incubation, aliquots of the reaction mix (50–100 μl) were assayed for their glucose content using the GO assay kit (Sigma Aldrich) as per manufacturer's instructions. Enzyme activity in these assays was expressed as μg of glucose produced by *x* number of parasites/ml per unit time. It was necessary to express activity in this fashion because the total amount of invertase (protein) actually secreted by these parasites into their culture medium was below current detection methods. Controls in these assays consisted of aliquots of unused parasite growth medium or short-term release assay buffer.

Oligonucleotide primers, PCR, and probe preparation

Degenerated oligonucleotide primers: a forward primer (PCR-Fwd) was designed based on the *N*-terminal sequence (aa: KDGVPYE) of the FPLC-purified *L. donovani* (1SCL-2D) ~70 kDa protein fraction, and a reverse primer (PCR-Rev) was designed to a relatively conserved consensus sequence (aa: FYASKTFYD, GenBank) common to numerous invertases [35]. These primers (PCR-Fwd: 5′-AAG GAC/TGGC/T/G GTG/C CCG/C TAC GAG-3′ and PCR-Rev: 5′-GTC GTA GAA C/GGT CTT C/G/AGA C/GGC GTA GAA-3′) were synthesized by β-cyanoethyl phosphoramidite chemistry using an Expedite™ nucleic acid synthesis system (PE Applied Biosystems: Eurofins MWG Operon, Huntsville, AL, USA) and used in PCR amplifications with *L. donovani* (1SCL-2D) genomic (g) DNA as a template. It is important to note that is was possible to directly use gDNA as template since trypanosomatid protozoans generally do not possess introns within the coding region of their ORFs [36, 37]. After an initial “hot start” at 94 °C for 2 min, the conditions used for amplification were 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min (35 cycles), and a final step at 72 °C for 5 min. The 858 bp amplified product was cloned into the *p*CR®2.1-TOPO vector (Life Technologies) and the resulting plasmid (*LdINV*-PCR858) was subjected to nucleotide sequencing. Analyses of the sequence data obtained from the *LdINV*-PCR858 clone showed that it had very high sequence identity (~96 %) with the *L. major* Friedlin chromosome #4 left end sequence, obtained in the genome sequencing project (gi|150270 87|emb|AL389894.4|LMFLCHR4A). It is of pertinence to note that this *L. major* sequence was annotated as invertase (β-D fructofuranosidase).

Subsequently, the *LdINV*-PCR858 cloned PCR fragment was labeled with digoxigenin-dUTP (DIG) using the PCR Dig-Labeling Kit according to manufacturer’s instructions (Roche). The resulting DIG-labeled probe (*LdINV*-DIG856) was used to screen an *L. donovani* (1SCL-2D) cosmid library [38]. A positive cosmid clone (cosmid 16.3) was isolated and sequenced in the region of the putative *LdINV* gene. Results of such sequencing demonstrated that the *LdINV* consisted of an ORF of 1932 bp including a methionine start codon of ATG and a stop codon of TAG at the end. It is important to note that the *LdINV*-PCR858 sequence was found to have 100 % identity to that contained within the *LdINV* ORF.

The nucleotide sequence of the *LdINV* ORF was used in multiple sequence alignments of invertases from various organisms. These alignments were conducted using the MacVector 7.0 program and MAFFT [v6.860b] (myhits. isb-sib.ch) to determine cluster relationships among those sequences and to construct dendrograms representing cluster relationships. More importantly, such analyses were extended to assess whether an *LdINV*-like gene might also be present in the genomes of other related trypanosomatid parasites. To that end the *LdINV* sequence was used as our reference to examine the TriTryp/*Leishmania* genomes at www.tritrypdb.org.

Isolation of genomic DNA and Southern blot analysis

Total genomic gDNA was isolated from log-phase promastigotes of each parasite cell line using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI, USA) according to manufacturer’s recommendations. For Southern blot analysis, such

gDNA was digested with several restriction endonucleases and separated on 1 % agarose gels, transferred to positively charged Nylon membranes (Roche), and cross-linked to the membranes by UV irradiation using a Stratalinker® 2400 (Stratagene, Stratagene La Jolla, CA, USA). Subsequently, blots were hybridized under high stringency using a DIG-labeled DNA probe (i.e., *LdINV*-DIG856), corresponding to a portion of the *L. donovani* invertase ORF (i.e., nt 85–942). After high stringency washing (0.1× SSC, 0.1 % SDS at 65 °C), the hybridized fragments were visualized using an anti-digoxigenin-alkaline phosphatase conjugated antibody in conjunction with a chemiluminescent reagent (CSPD) according to manufacturer's instructions (Roche). Images were captured from such blots using BIOMAX™-MR X-ray film (Kodak, Rochester, NY, USA) and processed using Adobe Photoshop CS2 (Adobe). In addition, DNA was also isolated from the *Ld*INV-16.3 cosmid clone and similarly digested with several different restriction endonucleases. Such preparations were subsequently subjected to Southern blot analysis as above.

Isolation of RNA and Northern blot analysis

Total RNA was isolated from log-phase promastigote and axenic amastigote cultures using RNA STAT-60 according to the manufacturer's instructions (Tel-Test, Inc., Friendswood, TX). Total RNA (10 μg) was separated in 1.2 % agarose gels using the formaldehyde method [39], transferred onto nylon membranes, and cross-linked by UV irradiation as above. Blots were hybridized under high stringency conditions using a DIG-labeled probe (*Ld*INV-DIG858). After high stringency washing, such blots were subjected to immunological detection using anti-digoxigenin antibody conjugated to alkaline phosphatase and developed using the CSPD chemiluminescent reagent as described above.

Mapping of the 5′ spliced leader acceptor site

As indicated above, trypanosomatids generally do not possess introns within their ORFs. However, the pre-mRNAs in these organisms are joined to a 39 nt conserved spliced leader at their 5′-end by trans-splicing to generate mature, translatable mRNAs. To identify the 5′-splice acceptor site in the *LdINV* gene, RT-PCR analysis was performed essentially as described previously [40]. For these reactions, cDNA was generated from total RNAs isolated from both *L. donovani* 1S-Cl2D promastigotes and axenic amastigotes. Such cDNAs were used as template in PCR with a forward primer (i.e., SpliceFwd) based on the *Leishmania*-spliced leader sequence [40] 5′-AACGCTATATAAGTATCAGTTTCTGTACTTTATTG-3′ and an internal reverse primer (INV Rev: 5′-CCGTTGGGGTTG TACTGCATGTACAGGTGAATTTT-3′). The resulting PCR-amplified products obtained from these reactions (i.e., using cDNAs from both promastigote and axenic amastigotes) were cloned into the *p*CR®2.1-TOPO plasmid vector (Life Technologies), and sequenced using the vector-encoded M13 forward and reverse primers. Sequence data obtained from these two parasite developmental forms were compared. The results obtained from such analyses were also used to further verify the authenticity of the start site of the *L. donovani* invertase ORF.

Escherichia coli-recombinant LdINV expressed proteins and antibody production

An *E. coli* expression plasmid containing the wild-type *LdINV* gene [minus the *N*-terminal sequence (nt 1–75) encoding signal peptide] was made. To that end, the *LdINV* gene lacking a sequence encoding the putative signal peptide was amplified by PCR using the *LdINV*-16.3 cosmid as template and two primers. These included Primer-3 (5′-AAA**TGGATCC**Agccgctcgcaaaggacggcgtgc-3′) which contained a *Bam*HI restriction site (bold), and an extra nt A (underlined) to facilitate in-frame cloning in the expression vector, followed by 23 nucleotides of the *LdINV* gene sequence (lowercase), and Primer-5 (5′-ACAA**CTCGAG**gac ggcgatgagggtgtcgagtaga-3′) which contained an *Xho*I restriction site (bold) followed by the last 25 nts of the *LdINV* gene sequence (excluding the stop codon, lowercase). The sequence of the resulting PCR product was cloned into *p*CR®2.1-TOPO (Life Technologies). The sequence of the cloned fragment was confirmed by nucleotide sequencing using the M13 forward and reverse primers. Subsequently the *L. donovani* invertase encoding fragment was excised from this plasmid by *Bam*HI and *Xho*I digestion, purified and ligated into the *Bam*HI and *Xho*I site of the pETBlue-2 expression plasmid (Novagen, Merck KGaA, Darmstadt, Germany). The resulting expression plasmid encoded the recombinant *Ld*INV protein plus a *C*-terminal 6-His tag (*rLdINV-His*). The BL21DE3 pLacI *E. coli* host cells (Novagen) were transformed with the *rLdINV-His* containing plasmid from above and clones were selected following induction with 1 mM isopropylthio-β-galactoside. One such clone was selected for further analysis. As anticipated, results of such analysis demonstrated that the bulk of the expressed *rLd*INV-His protein was found to aggregate into inclusion bodies. The purification of the *rLd*INV-His proteins was performed under denaturing conditions and the denatured expressed proteins were bound to, and eluted from, nickel–nitrilotriacetic acid Superflow beads (Qiagen) in an FPLC (Amersham) according to manufacturer's recommendations. The purified r*Ld*INV-His protein was used to immunize a New Zealand White rabbit according to a standard protocol by Spring Valley Laboratories (Woodbine, MD, USA; i.e., primary immunization followed by three boosts). The resulting antiserum (i.e., the *rLd*INV antibody) was shown to specifically react against both the *E. coli* recombinant and the native parasite LdINV by Western blotting. This antibody was also used in subsequent immuneprecipitations and Western blot experiments as described below. Pre-immune serum (NRS) from this rabbit served as a control in all experiments.

Assessment of invertase activity of the *E. coli*-recombinant LdINV protein

Aliquots of recombinant (*r*) *Ld*INV protein produced by *E. coli* were separated in 10 % SDS-PAGE gels and transferred on to PVDF membranes. Such membranes were washed extensively in several changes of Tris–HCl buffer, pH 7.4, containing 1 % (v/v) Triton X-100 (protein grade, Pierce Chemical, Thermo Fisher Scientific, Rockford, IL, USA) to remove bound SDS and re-nature the expressed *rLd*INV protein. Subsequently, such membranes were incubated for 1 h in 0.2 M acetate buffer pH 5.0 containing 25 mM sucrose as enzyme substrate. Activity of the *rLd*INV was demonstrated by the presence of its enzymatic reaction product (fructose) which was visualized by placing the gels in 0.1 % TTC in 1 N NaOH for 10–20 min at 45 °C. Subsequently, the reaction product demonstrating invertase activity appeared as a single brilliant red band at approximately 72

kDa. Images were captured from such blots using a Nikon Coolpix 3500 camera (Nikon, Melville, NY, USA) and processed using Adobe Photoshop CS2 (Adobe) as above.

Generation of an epitope-tagged LdINV expression construct

In this report *pKSNEO*, *a* leishmanial vector [41], was used to express a construct encoding an *Ld*INV-hemagglutinin-tagged (-HA) chimeric protein in *L. donovani* (1SCL-2D) as well as analogous constructs in *L. mexicana* (M379). This vector has been used previously to express a variety of homologous and heterologous genes in both promastigotes and axenic amastigotes in several different species of *Leishmania* parasites [41–44]. For initial studies, a chimeric construct was designed that contained the complete ORF of the *L. donovani* invertase gene (including its 5′-end encoding the signal peptide) joined, at its 3′-end, with a sequence encoding a nine amino acid epitope of the influenza virus HA (Roche). For those studies, the *LdINV*-Cos16.3 cosmid was used as template in PCR reaction with a forward primer 5′-GAT**ACTAGT**ATGGTGCAGAGGAGCTCACTT-3′ (containing an *Spe*I restriction endonuclease site shown in bold); and the reverse primer: 5′-CAA**ACTAGT*TCA***CGCGTAGTCCGGCACGTCGTACGGGTATAGATCGCGGTC-3′ [containing an *Spe*I restriction endonuclease site shown in bold; stop codon in bold italics; and a -HA epitope tag (underlined sequence)]. The resulting amplified product was gel purified and cloned into the *p*CR2.1-TOPO vector (Life Technologies) to generate a *pCR2.1::LdINV-HA* plasmid. The sequence of the *pCR2.1:: LdINV-HA* construct was verified by nt sequencing. The insert was excised from this plasmid using *Spe*I endonuclease treatment. Subsequently, the excised fragment was ligated into the *pKSNEO* plasmid (linearized with *Spe*I) to generate the *pKSNEO::LdINV-HA* plasmid construct. The orientation of *LdINV-HA* in *pKSNEO* was verified using restriction endonuclease analysis and nt sequencing of the construct.

Transfection of pKSNEO expression plasmids into *L. donovani* parasites

Leishmania donovani (1SCL-2D) amastigotes were isolated from infected hamsters as described previously [30]. These in vivo derived amastigotes were allowed to transform into promastigotes at 26 °C in vitro, as described above. The promastigotes (i.e., in their second in vitro passage after transformation) were subsequently used in transfection experiments. Log-phase *L. donovani* promastigotes were transfected with either the *pKSNEO* control plasmid or the *pKSNEO::LdINV-HA* construct by electroporation using methods derived from Shakarian et al. 2002 [26]. To that end, *L. donovani* promastigotes were harvested, washed two times with 1×PBS by centrifugation (2100×*g*, 10 min at 4 °C), and re-suspended in ice-cold electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose, pH 7.0) at 10^8 cells ml^{-1}. 500 µl aliquots of such cell suspensions were added to 2-mm gap electroporation cuvettes (BTX®Harvard Apparatus, Holliston, MA) to which 20 µl of purified plasmid DNA (at 1 µg/µl) in sterile 10 mM Tris, 2 mM EDTA, pH 8.0 (Quality Biological, Inc., Gaithersburg, MD) was added. The cells were electroporated using a single pulse (conditions: 475 V, 800 microfarads and 13 ohms) in a BTX ECM-600 electroporation system (BTX® Harvard Apparatus). Electroporated cells were incubated on ice for 10 min, transferred into 5 ml of complete culture medium (M199 containing 10 % [v/v] FBS) as described above and incubated at 26 °C for 24 h. Following recovery, the transfected cells were harvested by centrifugation as above and re-suspended

in the same culture medium containing Geneticin® (G418, Life Technologies; at 15 μg ml^{-1} final concentration). Following initial drug selection, drug-resistant parasites were grown in increasing concentrations of G418 up to 50 μg/ml. Such cells were routinely maintained in culture using this antibiotic concentration or frozen and stored in liquid nitrogen. For some experiments, such transfected promastigotes were placed under conditions which permitted them to transform and grow as axenic amastigotes in the presence of G418 (i.e., in pH 5.5 medium at 37 °C), as described by [30]. Growth kinetics of both *pKSNEO* control and *pKSNEO::LdINV-HA*-transfected parasites were monitored at regular intervals during the time course of their growth in vitro. To that end, aliquots of such cultures were diluted appropriately with an isotonic buffer (ISOTON-II, Beckman–Coulter Particle Characterization, Miami, FL) and counted using a Model Z1 Coulter Counter (Beckman–Coulter) essentially as described by [30]. Both control and *Ld*INV-HA transfectants were basically tested for their ability to produce/express *Ld*INV-HA chimeric invertase proteins. In addition, cell lysates and culture supernatants from both *pKSNEO* and *pKSNEO::LdINV-HA* were tested for their functional invertase activity using both our agarose-based and spectrophotometric invertase assays as described above.

Identification of LdINV homologs in other *Leishmania* species

The TriTryp genomic database (www.tritrypdb.org) was examined to assess whether any other trypanosomatid parasites possessed any homologs of the *LdINV* gene. These databases were examined using the *LdINV* gene sequence as a prototype in various multiple sequence alignments conducted in MacVector 7.0 program and MAFFT [v6.860b] (myhits.isb-sib.ch). These analyses indicated that gene homologs were present in several *Leishmania* species (i.e., *L. infantum*, *L. major*, *L. tarentolae*, *L. mexicana*, and *L. braziliensis*). To validate whether such *LdINV* homologs were actually present in these various *Leishmania* species they were subjected to PCR analyses. For these assays, a forward oligonucleotide primer was synthesized reflecting the 5′ end of the *LdINV* ORF (i.e., from nt 85) and a reverse oligonucleotide primer was synthesized reflecting an internal sequence (i.e., from nt 850 within the *LdINV* ORF). PCR reactions were carried out with these oligonucleotides using gDNAs as templates isolated from *L. donovani* (1S-CL2D and LRC-751), *L. amazonensis*, *L. mexicana*, *L. tropica*, *L. major*, *L. infantum*, *L. braziliensis*, and *L. tarentolae* all as designated above. Products of these PCR reactions were separated on agarose gels and stained with ethidium bromide. An ~850 bp product was obtained for each of these reactions. Subsequently, each of these PCR products was cloned and subjected to nucleotide sequencing.

It is of importance to point out that *L. infantum* and *L. major* are the causative agents of human visceral and cutaneous disease, respectively, in the Old World; whereas, *L. tarentolae* is an Old World saurian (lizard) *Leishmania*. In contrast, *L. mexicana* and *L. braziliensis* are agents of human cutaneous and mucocutaneous diseases in the New World. It is of significance to note that results of preliminary experiments showed that the Old World *LdINV* gene encoded a functional invertase activity. In light of that, it was of interest, to ascertain whether one of these New World *Leishmania* gene homologs would also encode a similar functional enzyme activity. In that regard, we decided to investigate those

possibilities using the *L. mexicana LdINV* homolog (*LmexINV*) as an example of an invertase enzyme present in an important New World *Leishmania* species.

Identification of an *L. mexicana* INV gene

As indicated above, the sequence of the *LmexINV* homolog was obtained from the TriTryp genome database (www.tritrypdb.org; GeneID: LmxM.04.0310). Using this sequence, we generated oligonucleotide primers reflecting the 5′ and 3′ end of this sequence: Forward Primer FLmex INVORF 5′-ATGCGCCGCGGGGTCATTCTGCTCCTCGTGGC-3′ and Reverse Primer RLmexINVORF 5′-CTAGACGTCGATGAGGGTGTCGAGTAGCGTGT-3′. These primers were used in PCR reactions with *L. mexicana* M379 promastigote gDNA as target using conditions similar to those as described above for the *LdINV*. Results of such reactions generated a 2 kb-amplified product which was cloned into the *p*CR®2.1TOPO vector and transfected into chemically competent TOP-10 cells (Invitrogen). Ten individual clones from those transfectants were randomly selected for nt sequencing. The resulting plasmid preparations from these were nt sequenced (Macrogen Corp., Rockville, MD) using vector encoded M13-F and M13-R primers. Results of these analyses showed that these plasmids contained an insert consistent with that of the *LmexINV* gene present in the Tri-Tryp genomic database.

Identification of mature mRNA encoding the LmexINV

Total RNA was obtained from ~10^7 *L. mexicana* M379 promastigotes or axenic amastigotes using STAT-60 (Tel-test, Inc., Friendswood, TX, USA) and processed according to the manufacturer's recommendations [38]. The presence of mature *LmexINV* mRNA was assessed using RT-PCR analyses. As indicated above, trypanosomatid parasites generally do not possess introns within their ORFs. Further, pre-mRNAs in these organisms possess a 39 nt conserved SL at their 5′-end to generate mature translatable mRNAs [16, 45]. For these experiments, 1.5 μg of total RNA (pre-treated with DNase) from *L. mexicana* M379 Pro- and AxAm were reverse transcribed into cDNA using the Superscript® First Strand Synthesis System for RT-PCR (Invitrogen) and oligo d(t) primers as per manufacturer's instructions. Subsequently, such cDNA was used as template in PCR with the forward *L. mexicana* SL sequence (i.e., nt 5–29 of the 39 nt SL) [46] primer 5′-AACGCTATATAAGTATCAGTTTCTG-3′) and an internal reverse primer based on a portion of the *LmexINV* ORF (i.e., R*Lmex*INVRT 5′-CGCCAACTGCAGCGCCGAGTGC-3′). Controls for the RT-PRC reactions included no reverse transcriptase, no RNA, no SL primer and no internal invertase primer. The resulting PCR-amplified products obtained from these reactions using both Pro- and AxAm cDNAs were cloned into the *p*CR®2.1 TOPO plasmid vector (Invitrogen), nt sequenced, and analyzed. The sequence data for *LmexINV* obtained from each of the parasite developmental forms (i.e., promastigotes and axenic amastigotes) were compared.

Generation of an epitope-tagged construct for LmexINV

To examine the functional activity of the *LmexINV*, we generated an epitope-tagged construct of this gene and expressed it using the *pKSNEO* homologous expression system described above. To that end, an episomal construct was designed that contained the

complete ORF of the *LmexINV* gene including the 5′-end encoding the putative signal peptide. A nine aa sequence encoding the influenza virus HA epitope was joined at the 3′-end of the above construct. This construct was generated by PCR using one of the *p*CR®2.1*LmexINV* clones above as template. The forward primer for this reaction was F*Lm*exINVHA 5′-GATCACTAGTATGCGCCGCGGGGTCATTCTGC-3′ and the reverse primer was R*LmexINV*HA 5′-GATCACTAGTCTAGGCGTAGTCGGGGACGTCGTCGTAGGGGTAGACGTCGATGAGGGTGTCGAGT-3′. The PCR conditions used in these reactions were identical to those outlined above for the LdINV-HA reaction. The resulting 2 kb product obtained from these reactions was gel purified and cloned into *p*CR®2.1TOPO (Invitrogen) to generate *pCR®2.1::LmexINV-HA*. The insert was excised from the latter using *Spe*I digestion. Subsequently, this excised fragment was ligated into the *Spe*I site of the *p*KSNEO expression vector [41] to generate the *pKSNEO::LmexINV-HA* plasmid construct. The orientation of this plasmid was verified by restriction enzyme analysis and the nucleotide sequence was confirmed by DNA sequence analysis.

Transfection of pKSNEO expression plasmid into *L. mexicana* parasites

Leishmania mexicana wild-type M379 promastigotes were grown to mid-log phase and transfected with *pKSNEO::L-mexINV-HA* or a *pKSNEO* control plasmid construct by electroporation using the same conditions as described above. Subsequently, viable transfectants were selected in vitro using increasing drug concentrations of G418 up to 50 μg/ml as above. These transfectants were routinely maintained in culture using this antibiotic concentration or frozen and stored in liquid nitrogen. Further the growth of both the *pKSNEO* control and the *pKSNEO::LmexINV-HA* transfectants were monitored for their growth in vitro as above. For some experiments, such transfected promastigotes were placed under conditions which permitted them to transform and grow as axenic amastigotes in the presence of G418 (i.e., in pH 5.5 medium at 32 °C), as described by Joshi et al. 2007 [38]. Similar to the *L. donovani* transfectants above, the growth kinetics of both *pKSNEO* control and *pKSNEO::LmexINV-HA*-transfected parasites were monitored at regular intervals during the time course of their growth in vitro. In addition, the LmexINV-HA transfectants were tested for their ability to synthesize and release/secrete LmexINV-HA chimeric invertase protein.

Immunoprecipitations (IPs)

Lysates of *pKSNEO* control and transfected (*pKSNEO::L-dINV-HA* or *pKSNEO::LmexINV-HA*) promastigotes and their cell-free culture supernatants were subjected to IP assays with pre-washed rabbit anti-HA (Sigma Aldrich Co., St. Louis, MO, USA) antibody-bound Dynabeads® Protein A (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. Briefly, 25 μl pellets of Dynabeads® Protein A were washed three times in 1× PBS/0.05 % Tween-20 (v/v) at room temperature (RT). Aliquots (25 μl pellet) of the washed Dynabeads were re-suspended in 100 μl of a 1:10 dilution of rabbit anti-HA antibody (Sigma) in 1× PBS/0.05 % Tween-20. Following agitation on an orbital rocker for 40 min at RT, the beads were washed five times with 1× PBS/0.05 % Tween-20. Subsequently, the anti-HA-bound Dynabeads were reacted with 100 μl of either parasite cell lysate or with an aliquot of 100× concentrated cell-free parasite culture supernatant. Such samples were incubated at 4 °C

overnight on an orbital rocker. Subsequently, the Dynabeads–IP complexes were washed five times in 1× PBS/0.05 % Tween-20 at RT, re-suspended in 1× SDS-PAGE buffer, and heated to 90 °C for 10 min prior to separation in 10 % SDS-PAGE gels (Novex). Similar IP assays were also carried out with these samples using the *rLdINV* rabbit antibody and its NRS pre-immune serum in conjunction with Protein A Dynabeads under conditions similar to those used above. Immunoprecipitates obtained from these assays were further analyzed by SDS-PAGE and Western blotting as described below.

SDS-PAGE and western blotting

Log-phase promastigotes and axenic amastigotes of wild-type *L. donovani* (1SCL-2D) and *L. mexicana* (M379) were harvested and washed in PBS by centrifugation as above, and the resulting cell pellets were lysed in SDS-PAGE sample buffer and prepared for SDS-PAGE as previously described [40]. Prior to use, the protein concentrations of these samples were determined as previously described [40]. Aliquots of proteins (15 μg) were analyzed by SDS-PAGE, transferred onto nitrocellulose, and processed for Western blot analysis with the anti-*Ld*INV antibody or the NRS control as described previously [30, 40]. In addition, aliquots of parasite culture supernatants, parasite release assays, and *E. coli rLd*INV proteins were prepared and subjected to SDS-PAGE and Western blot analysis. In addition to the above, cell lysates, parasite culture supernatants and immunoprecipitates obtained from both the *pKSNEO::LdINV-HA* and *pKSNEO::LmexINV-HA* transfectants and their respective controls were also subjected to SDS-PAGE analysis using 10 %, pre-cast, Tris–glycine polyacrylamide gels (Novex®, Invitrogen). Subsequently, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using the iBlot® system (Invitrogen) as per manufacturer's instructions. Membranes were blocked in a 5 % non-fat dry milk-TBST solution [0.05 % (v/v) Tween-20, 20 mM Tris–HCl, 150 mM NaCl, pH 8.0] and washed as previously described [43]. Such blots were reacted with either our rabbit anti-*Ld*INV antibody, its NRS control or a mouse anti-HA.11 (Covance Research Products, Berkeley, CA, USA) or an appropriately matched purified mouse IgG1 κ chain control immunoglobulin (Sigma). Subsequently, blots were washed in TBST and incubated either with biotinylated goat anti-rabbit or goat anti-mouse antibodies (Invitrogen). After second set of washes with TBST, membranes were reacted with Q-Dots® (625 streptavidin conjugate nanoparticles, Invitrogen) for 30 min on an orbital shaker, washed three times at RT with TBST, and imaged using an Epichemi^3 Darkroom (UVP Bioimaging Systems, Upland, CA, USA). The images were processed with Adobe Photoshop CS2 (Adobe Systems, Inc.).

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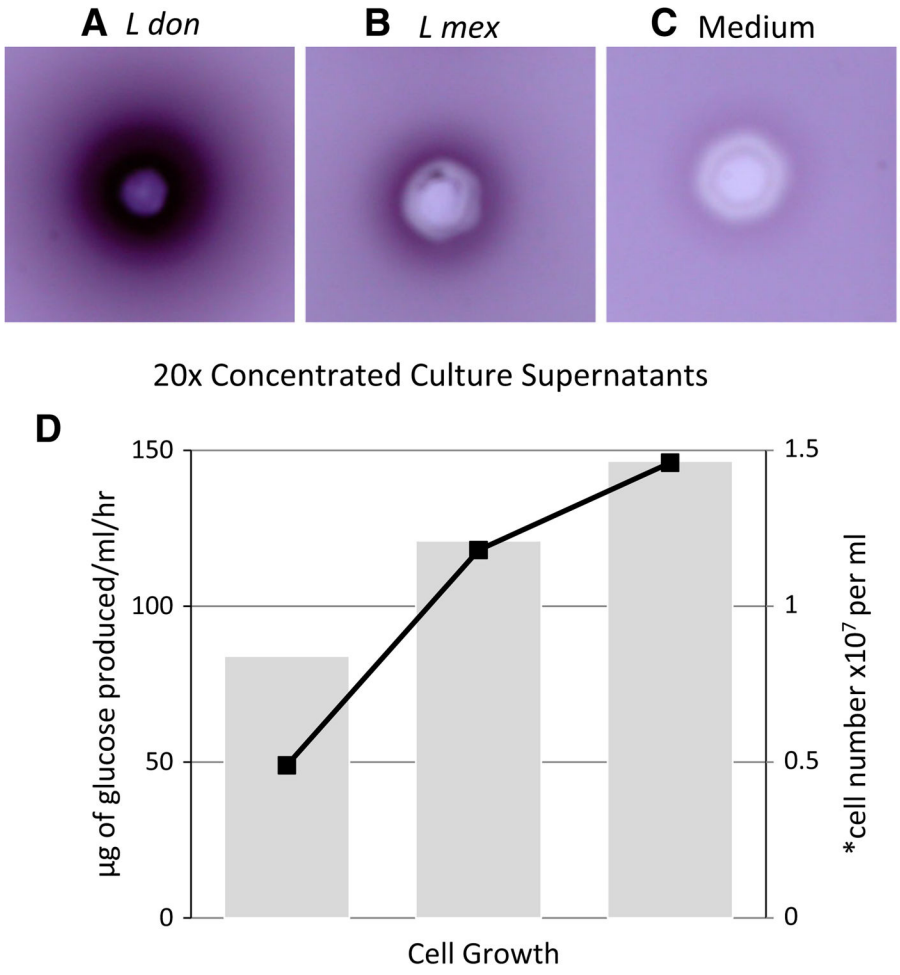

Fig. 1.

Detection of invertase activities produced by *Leishmania* promastigotes in vitro. **a–c** Agarose-gel assays of invertase activity. Aliquots of ×20 concentrated culture supernatants from *L. donovani* (1SCL-2D) and *L. mexicana* (M379) promastigotes and unused parasite culture medium were loaded to precut wells of an agarose gel containing 25 mM sucrose as substrate and allowed to diffuse into the gel for 4 h at 37 °C. Following incubation, the resulting hydrolysis product (fructose) was visualized in these plates using triphenylte-trazolium chloride (TTC) as a capture reagent. The resulting invertase activity is shown in these *plates* by the presence of a *dark red*/*purple* colored ring of TTC-captured product radiating from the sample wells. The gel shown here is one example of the results obtained from numerous gel assays which were carried out in these experiments. **d** Quantitation of invertase activity released/secreted by *L. donovani* promastigotes during their growth in vitro. Invertase activity produced by *L. donovani* promastigotes was measured using sucrose as substrate in a glucose oxidase spectrophotometric assay. Aliquots of culture supernatants were taken at various times during cell growth and assayed for invertase activity. The *bars* represent invertase activity expressed as µg of glucose produced by × number of cells/ml/h. The *solid squares* reflect the parasite cell number/ml prior to harvesting their culture supernatants. (Color figure online)

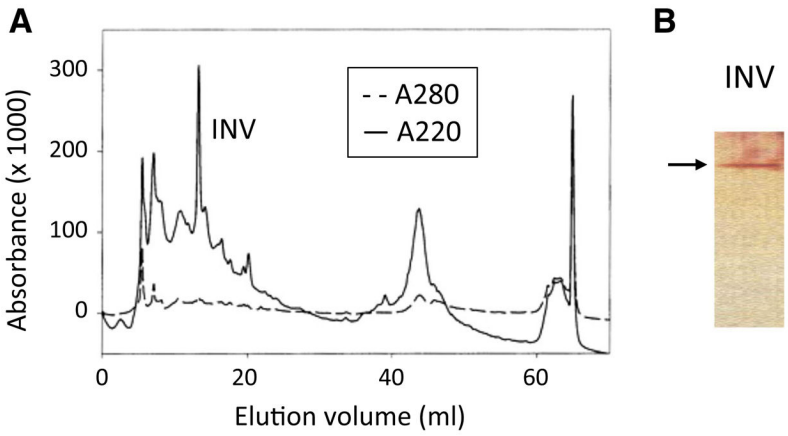

Fig. 2.

Isolation of the *L. donovani* (1SCL-2D) invertase using FPLC fractionation. Concentrated supernatants from *L. donovani* (1SCL-2D) promastigote release assays were subjected to FPLC on a Mono-Q column. Individual fractions from this column were collected, optically scanned and their absorbance at 220 and 280 nm were plotted (**a**). One of these fractions (INV) when subjected to SDS-PAGE and silver staining (**b**) contained only a single ~70 kDa protein band (*arrow*). (Color figure online)

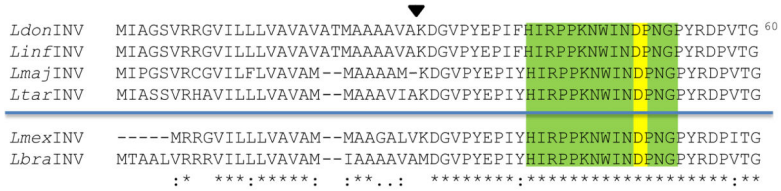

Fig. 3.

N-terminal end sequence alignment of LdINV and related *Leishmania* sp. homologs. The *N*-terminal end-deduced amino acid sequence of the *LdINV* gene (*Ldon*INV) was compared to those of several *Leishmania* species present in the TriTryp genome database [*L. infantum* (*Linf*INV: LinJ.04.0300), *L. major* (*Lmaj*INV: LmjF.04.0310), *L. tarentolae* (*Ltar*INV: LtaP04.0290), *L. mexicana* (*Lmex*INV: LmxM.04.0310), and *L. braziliensis* (*Lbra*INV: LbrM.04.0350)]. Met^{1} through Ala^{28} represents the putative signal peptide of the *Ldon*INV. The *arrowhead* shown above the *Ldon*INV sequence designates the putative signal peptidase cleavage site of this protein. The KDGVPYE gene-deduced amino acid sequence was verified by *N*-terminal end AA sequencing of the FPLC-isolated *Ld*INV protein. The *area highlighted* in *green* designates the conserved 14 amino acid signature sequence characteristically found in all members of the glyco/hydrolase 32 subfamily of invertases. The *yellow-highlighted aa* denotes the critical aspartic acid residue within the active/hydrolytic site of this invertase family. The *asterisks* (*) designate the conservation of amino acid residues among all six *Leishmania* species; the periods (.) and colons (:) represent amino acids that are semi-conserved substitutions and conserved substitutions, respectively. The *blue line* denotes geographical separation between the Old World (*above*) and New World (*below*) *Leishmania* species. (Color figure online)

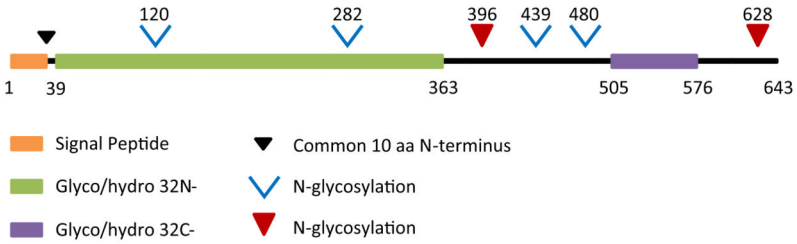

Fig. 4.

Diagrammatic representation of the features common to LdINV and its associated *Leishmania* homologs (*c.f.* Fig. 3). The *orange bar* represents a signal peptide present in all of the *Ld*INV homologs. The *black arrow* delineates the unique 10 amino acid *N*-terminal sequence present in all the *Ld*INV homologs. The *green bar* (glyco/hydro 32 N-; aa 39–aa 363) represents the 324 *N*-terminal amino acid sequence characteristic of all invertases in the glyco/hydrolase 32 protein family including its characteristic 14 amino acid active site (aa 39–aa 53). This domain has a purported 3-D structure characterized as a five-bladed beta propeller. The *purple bar* (glyco/hydro 32 C-; aa 505–aa 576) represents the 71 *C*-terminal amino acid sequence characteristic of all invertases in the glyco/hydrolase 32 protein family. This domain has a purported 3-D structure composed of a sandwich of beta- pleated sheets. The *black bars* (i.e., aa 363–aa 505 and aa 576–aa 643) represent aa sequences common/unique to all of the *Ld*INV homologs. The *red arrows* represent N-linked glycosylation sites common to each of the *Ld*INV homologs. The *blue chevrons* designate potential N-linked glycosylation sites in *Ld*INV and most of its homologs. (Color figure online)

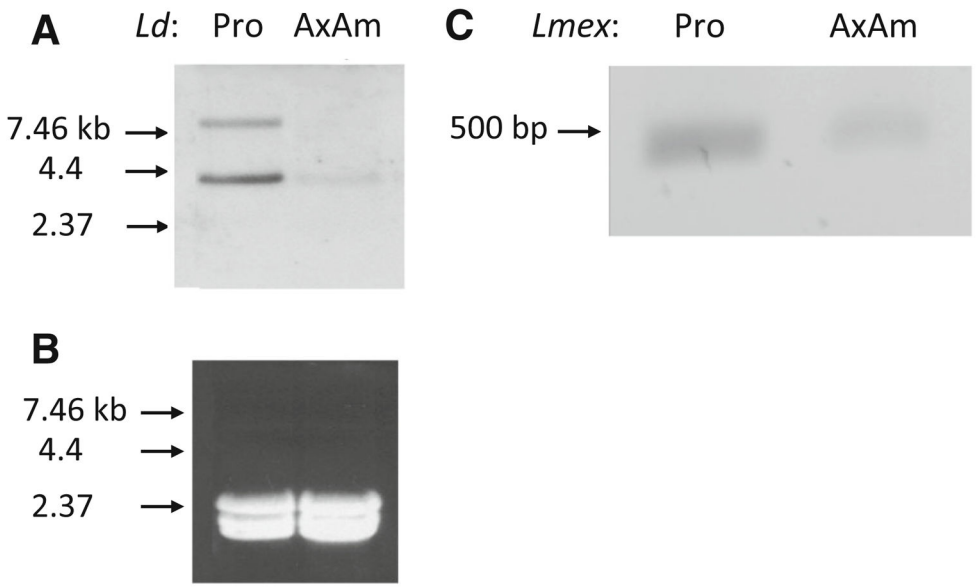

Fig. 5.

Detection of mRNAs encoding invertase in both *Leishmania* sp. promastigote and axenic amastigote developmental forms. **a** Northern blot of total RNA isolated from *L. donovani* (*Ld*) promastigotes (Pro) and axenic amastigotes (AxAm) probed with the *LdINV*-DIG858 probe. Molecular mass markers in kb are shown at the *left* (*arrows*). **b** Ethidium bromide stained gel showing equivalent amounts of Pro and AxAm RNA were used to generate the Northern blot shown in **a** above. Molecular mass markers in kb are shown at the *left* (*arrows*). **c** Ethidium bromide-stained agarose gel showing the ~500 bp product (*arrow*) obtained from RT-PCR reactions using RNA isolated from *L. mexicana* (*Lmex*) promastigotes (Pro) and axenic amastigotes (AxAm). This image was inverted using Adobe Photoshop CS2 to accentuate the RT-PCR product obtained in these reactions

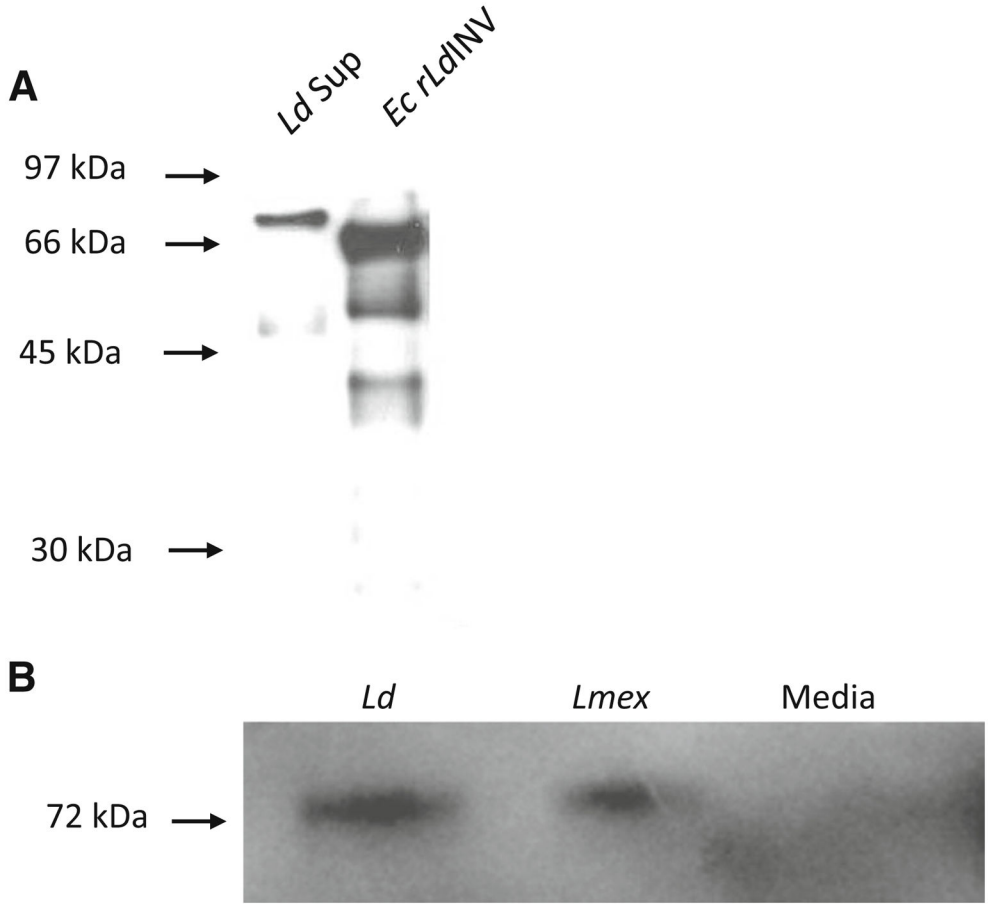

Fig. 6.

Western blots demonstrating the reactivity of our rabbit anti*E. coli*-recombinant LdINV antibody. **a** Western blot showing the immunoreactivity of our rabbit anti-*E. coli*-recombinant *Ld*INV antibody (α-*rLd*INV) with concentrated *L. donovani* culture supernatants (*Ld* Sup) and purified *E. coli*-recombinant *Ld*INV protein (*Ec rLd*INV) separated in SDS-PAGE, blotted onto PVDF membranes, and probed with the α-*rLd*INV antibody. Protein standards in kDa are shown at the *left* (*arrows*). **b** Western blot demonstrating the immuno cross-reactivity of our rabbit α-*rLd*INV antibody with the native *L. donovani* (*Ld*) and *L. mexicana* (*Lmex*) released/secreted invertases. Culture supernatants (×1) from *L. donovani* and *L. mexicana* promastigotes and unused culture media controls were separated in SDS-PAGE, blotted onto PVDF membranes, and probed with the α-*rLd*INV antibody. Only a single ~72 kDa (*arrow*) band of INV protein was detected in culture supernatants from each of these parasite species but not present in their media controls

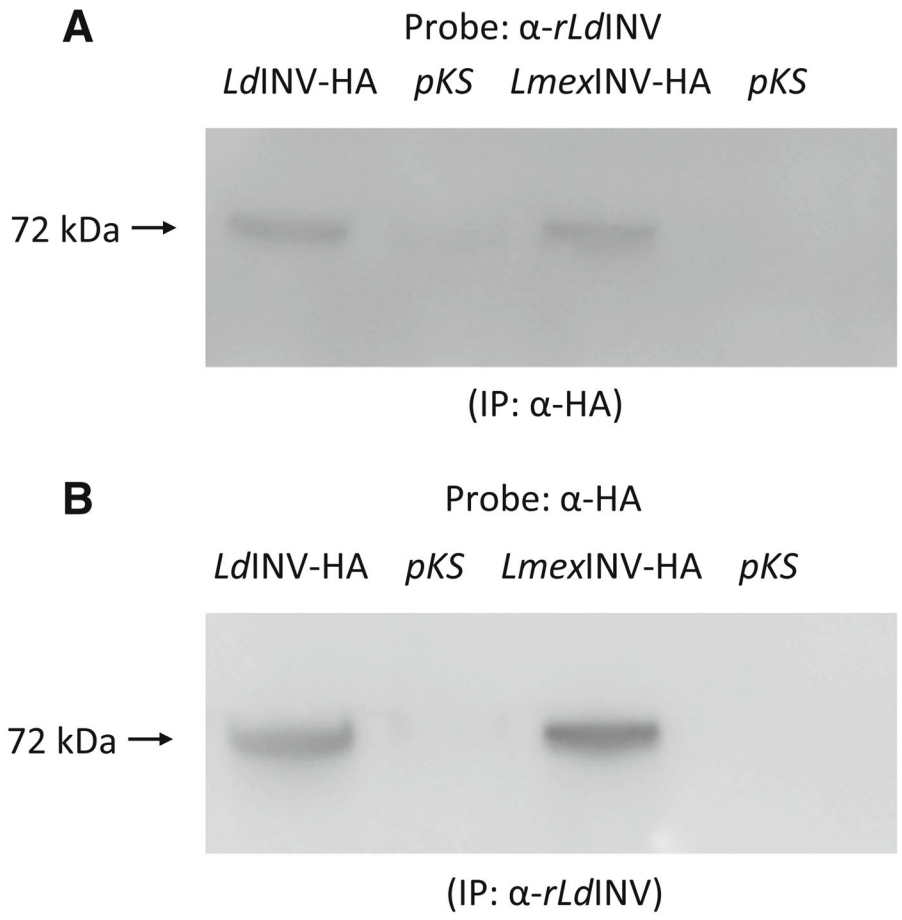

Fig. 7.

Expression of the LdINV-HA and LmexINV-HA chimeric proteins in transfected parasites. **a** Western blots of samples immunoprecipitated with the α-HA antibody and probed with our α-*rLd*INV antibody. Unconcentrated (×1) culture supernatants from *Ld*INV-HA and *Lmex*INV-HA-transfected promastigotes and their *pKSNEO* controls were immunoprecipitated with the α-HA antibody. Western blots of such immunoprecipitates were probed with our α-*rLd*INV antibody. The latter antibody only reacted with a single ~72 kDa (*arrow*) HA-abeled protein in the culture supernatants from *Ld*INV-HA and *Lmex*INV-HA promastigotes but not with their *pKSNEO*-transfected controls. **b** Western blots of samples immunoprecipitated with our α-*rLd*INV antibody and probed with the α-HA antibody. Unconcentrated (×1) culture supernatants from *Ld*INV-HA and *Lmex*INV-HA transfected promastigotes and their *pKSNEO* controls were immunoprecipitated with our α-*rLd*INV antibody. Western blots of such immunoprecipitates were probed with our αHA antibody. This α-HA antibody only reacted with a single ~72 kDa (*arrow*) protein in the culture supernatants from *Ld*INV-HA and *Lmex*INV-HA promastigotes but not with their *pKSNEO*-transfected controls

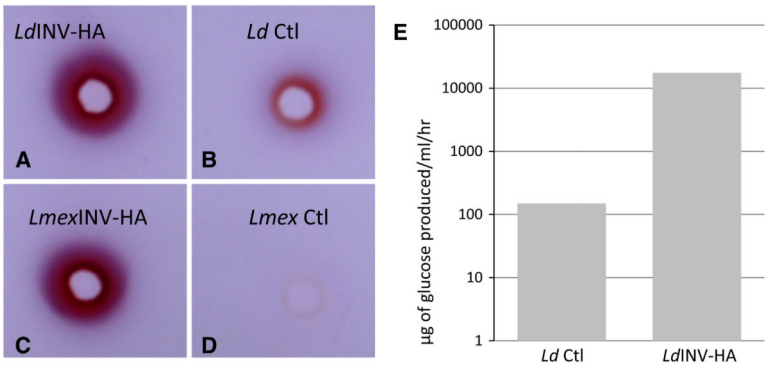

Fig. 8.

Detection of invertase activity produced by INV-HA transfected promastigotes. Aliquots of unconcentrated (1×) culture supernatants from *L. donovani LdINV-HA* (**a**) and *L. mexicana LmexINV-HA*-(**c**) transfected promastigotes and their respective *pKSNEO* controls (**b** *Ld* Ctl and **d** *Lmex* Ctl, respectively) were loaded to precut wells of an agarose gel containing 25 mM sucrose as substrate and allowed to diffuse into the gel for 4 h at 37 °C. Following incubation, the resulting hydrolysis product (fructose) was visualized in these plates using triphenyltetrazolium chloride (TTC) as a capture reagent. The resulting invertase activity is shown in these *plates* by the presence of a *dark red*/*purple*-colored ring of TTC-captured product radiating from the sample wells. The gel shown here is one example of the results obtained from numerous gel assays which were carried out in these experiments. **e** Quantitation of invertase activity produced by *LdINV-HA*-transfected promastigotes and their *pKSNEO* controls. Aliquots of unconcentrated (1×) culture supernatants from *LdINV-HA* and *pKSNEO* control promastigotes (*Ld* Ctl) were assayed using sucrose as substrate in a glucose oxidase spectrophotometric assay. Aliquots of culture supernatants were taken and assayed for invertase activity at a cell density of ~10^7 parasites/ml. The invertase activity is shown as µg of glucose produced by 10^7 parasites/ml/hour. (Color figure online)

Table 1

Detection of invertase enzyme activities and the presence of LdINV-like homologs in various kinetoplastid parasites

	Invertase activity	Possess *LdINV*-like homologs
Production of invertase activity and the presence of LdINV-like homologs of various Old World and New World *Leishmania* species		
Old World *Leishmania* sp.		
L. donovani	+++	ü
L. infantum	+++	ü
L. major	+++	ü
L. tropica	+++	ü
L. tarentolae	+++	ü
New World *Leishmania* sp.		
L. mexicana	+	ü
L. braziliensis	+	ü
L. amazonensis	+	ü
L. pifanoi	+	ü
L. panamensis	+	ü
L. guyanensis	+	ü
Production of invertase activity and the presence of LdINV-like homologs in various non-*Leishmania* kinetoplastids		
Non-*Leishmania* kinetoplastids		
C. fasciculata[a]	+	ü
Phytomonas sp.[a]	+	ü
H. samuelpessoai[a]	+	NI
Leptomonas seymouri[a]	+	NI
T. brucei	−	None
T. cruzi	−	None

Invertase activity denoted as (+++) above reflects ≥100 units of invertase activity produced by 10^7 promastigotes/ml/h and (+) denotes ~10 units of invertase activity. The (ü) designates the presence of one or more *LdINV*-like homologs in this parasite's genome

NI no *LdINV*-like homologs annotated in this partially sequenced genome, *None* there was no *LdINV*-like homologs annotated in this fully sequenced genome

[a]Data from Gontijo et al.