Identification of a Disulfide Isomerase Protein of *Leishmania major* as a Putative Virulence Factor

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Several approaches have been previously used to elucidate the genetic basis of *Leishmania* virulence. In general, they were based on laboratory *Leishmania* clones genetically modified or grown in the presence of selecting agents. In a previous study, we demonstrated that *Leishmania major* freshly isolated from human cutaneous lesions showed significant differences in the severity of the experimental disease induced in BALB/c mice. Here, using the mRNA differential display technique, we analyzed gene expression in *L. major* promastigotes showing different levels of virulence. We have identified a novel *Leishmania* gene encoding a 477-amino-acid protein exhibiting two distinct regions that are identical to the putative active-site sequence (CGHC) of the eukaryotic protein disulfide isomerase (PDI). The recombinant protein displayed a specific PDI enzymatic activity. This *L. major* disulfide isomerase protein (LmPDI) is predominantly expressed, at both the mRNA and protein levels, in highly virulent strains. Specific PDI inhibitors abolished the enzymatic activity of the recombinant protein and profoundly affected parasite growth. These findings suggest that LmPDI may play an important role in *Leishmania* natural pathogenicity and may constitute a new target for anti-*Leishmania* chemotherapy.

Leishmaniasis constitute a heterogeneous group of diseases that affect millions of people and are due to the infection of mammalian hosts with a protozoan *Leishmania* parasite (12). The disease ranges from asymptomatic infection to self-limiting cutaneous lesions or fatal visceral forms. During their life cycle, parasites alternate between two stages: flagellated promastigotes in the midgut of the insect vector and amastigotes in the host macrophage (1). Tools available for the control of human leishmaniasis are of poor efficacy; antileishmanial drugs are toxic, and recently developed vaccine preparations induced a relatively low level of protection (23, 26, 44). This is partly explained by the complexity of the transmission cycle and insufficient knowledge about the parasite biology and the protective host immune responses. Over the past decades, several molecules playing a key role in the biology of the parasite and its infectivity have been identified. Due to their abundance and location, *Leishmania* glycoconjugates, particularly lipophosphoglycan (LPG), were shown to be important virulence factors of *Leishmania major* and *Leishmania donovani* (5, 11, 41, 45). However, LPG does not affect the virulence of *Leishmania mexicana* (19, 20). Molecules involved in the biosynthesis of the LPG, i.e., phosphomannose isomerase (15), LPG1 (45), LPG2 (10), and galactosyl transferase (9), were also associated with virulence. Other virulence factors were described, including the cysteine proteinase family (33), mitogen-activated protein kinase (51), A2 gene (58), surface glycoprotein gp63 (7), kinetoplastid membrane protein 11 (34), superoxide dismutase (36), trypanothione reductase (13), heat shock protein 100 (17), and the proteins encoded by *L. mexicana* cDNA16 (30). The characterization of these virulence factors obviously has important implications for the design of new drugs or vaccines

against *Leishmania* parasites. Previous studies were generally based on long-term in vitro-maintained laboratory clones or strains that have lost their virulence (34, 42) or on strains genetically manipulated by mutagenesis (6), overexpression (3), or knockout experiments (15, 16, 45) but not on recently established wild isolates. It is therefore unknown whether the conclusions drawn from the earlier experiments on virulence attenuation are relevant to the natural pathogenicity of the parasite in the field.

L. major is the causative agent of zoonotic cutaneous leishmaniasis (ZCL), a disease highly prevalent in North Africa and the Middle East (52). In a previous study, we have demonstrated that *L. major* parasites freshly isolated from human cutaneous lesions showed significant differences in the severity of the experimental disease that they could induce in BALB/c mice, differences which correlate with variation in in vitro parasite growth and macrophage infectivity (22). In the present study, using the mRNA differential display technique (24, 25), we analyzed gene expression in *L. major* promastigotes characterized by different levels of virulence. We could identify a novel *Leishmania* gene encoding a disulfide isomerase protein (LmPDI) which was shown to be predominantly expressed in highly virulent strains. Specific PDI inhibitors abolished the enzymatic activity of the recombinant protein and profoundly affected parasite growth. Together, these findings suggest that LmPDI may play an important role in *Leishmania* natural pathogenicity and may constitute a new target for anti-*Leishmania* chemotherapy.

MATERIALS AND METHODS

Parasites and culture conditions. *L. major* parasites (zymodeme MON25) isolated from human ZCL lesions in 1994 at El Guettar, southern Tunisia (27), were used for this study. They were cultivated on NNN medium and then adapted to RPMI medium and frozen in liquid nitrogen at a maximum of 2 weeks after isolation. Four isolates were selected among 19 strains on the basis of their

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TABLE 1. Nucleotide sequences of primers used in reverse transcription and PCR

Primer	Sequence	Length
Reverse		
VBD1	5'TTTTTTTTTTTAA3'	13 (nucleotides)
VBD2	5'TTTTTTTTTTTAG3'	13 (nucleotides)
VBD3	5'TTTTTTTTTTTAC3'	13 (nucleotides)
VBD4	5'TTTTTTTTTTTAT3'	13 (nucleotides)
VBD5	5'TTTTTTTTTTTGA3'	13 (nucleotides)
VBD6	5'TTTTTTTTTTTGG3'	13 (nucleotides)
VBD7	5'TTTTTTTTTTTGG3'	13 (nucleotides)
VBD8	5'TTTTTTTTTTTGT3'	13 (nucleotides)
VBD9	5'TTTTTTTTTTTCA3'	13 (nucleotides)
VBD10	5'TTTTTTTTTTTTCG3'	13 (nucleotides)
VBD11	5'TTTTTTTTTTTCC3'	13 (nucleotides)
VBD12	5'TTTTTTTTTTTCT3'	13 (nucleotides)
Forward		
VAD1	5'CTGATCCATG3'	10 (nucleotides)
VAD2	5'CTTGATTGCC3'	10 (nucleotides)
VAD3	5'CTGCTCTCA3'	9 (nucleotides)
VAD4	5'GGAACCAATC3'	10 (nucleotides)
VAD5	5'GGTACTAAGG3'	10 (nucleotides)
VAD6	5'GTTTTTCGCAG3'	10 (nucleotides)
VAD7	5'GATCAAGTCC3'	10 (nucleotides)
VAD8	5'GATCCAGTAC3'	10 (nucleotides)
VAD9	5'GATCTCAGAC3'	10 (nucleotides)
VAD10	5'GATCTAAGGC3'	10 (nucleotides)

experimental pathogenicity expressed in BALB/c mice: two high-virulence isolates, MHOM/TN/94/GLC94 and MHOM/TN/94/GLC67 (identified as GLC94 and GLC67, respectively), which induced large and rapidly progressing experimental lesions, and two low-virulence isolates, MHOM/TN/94/GLC32 and MHOM/TN/94/GLC07 (identified as GLC32 and GLC 07, respectively), which induced a less severe experimental disease. For the present study, parasite stocks were prepared, using the original frozen isolates, after two passages in BALB/c mice and growth on NNN medium at 26°C. Parasites were then progressively adapted to RPMI 1640 medium (Sigma, St. Louis, Mo.) containing 2 mM L-glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 10% heat-inactivated fetal calf serum (complete medium) for 3 weeks. Promastigotes were taken during logarithmic-phase culture in complete medium, adjusted to 10⁶ parasites/ml in a constant volume, and further incubated at 26°C. The stationary phase was reached after 4 to 6 days, with parasite numbers ranging from 3 × 10⁷ to 8 × 10⁷ parasites/ml. Stationary-phase promastigotes were used for protein and RNA extraction.

In some experiments, promastigotes were cultured at 10⁶/ml in complete medium supplemented with various concentrations of inhibitors of PDI function, i.e., zinc bacitracin (0.05 to 5 mM), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (0.05 to 2.5 mM), or *p*-chloromercuric benzoic acid (pCMB) (0.5 to 2.5 mM). The parasites were enumerated after 48, 72, and 96 h of culture.

mRNA isolation and differential display. Total RNA was extracted with TRIzol reagent (Gibco-BRL, Cergy Pontoise, France). Poly(A)⁺ mRNA was purified from total RNA by using a poly(A)⁺ RNA isolation kit (Amersham-Pharmacia Biotech, Glylada, Greece) according to the manufacturer's recommendations. mRNA (200 ng) was used to synthesize first-strand cDNAs in a 20-µl reaction mix containing 1 µM specific anchored primer oligo(dT)₁₁MN (where M is A, C, or G and N is A, C, G, or T) (Genset, Paris, France), 1× first-strand buffer (Gibco-BRL), 5 µM deoxynucleoside triphosphates (Amersham-Pharmacia), 10 U of RNasin (Promega, Charbonnières, France), and 200 U of reverse transcriptase (Gibco-BRL). After incubation at 37°C for 1 h, the reaction was terminated by heating at 95°C for 5 min. The cDNAs were PCR amplified using a combination of the 12 specific anchored primers and 10 arbitrary 5' primers (Table 1). The PCR was performed in a 20-µl reaction mix containing 2 µl of first-strand cDNA, 0.2 µM arbitrary 5' decamer (Genset), 1 µM 3' anchored primer (Genset), 2 µM deoxynucleoside triphosphates (Amersham-Pharmacia), 10 µCi of α-³²S-dATP (Amersham-Pharmacia), 1× *Taq* DNA polymerase reaction buffer (Amersham-Pharmacia), and 1U of *Taq* DNA polymerase (Amersham-Pharmacia). Reactions were performed in a Perkin-Elmer (Courtaboeuf, France) 9600 thermal cyclor and consisted of 40 cycles at 94°C for

30 s, 40°C for 60 s, and 72°C for 30 s and one cycle at 72°C for 6 min. PCR products were resolved on a 6% polyacrylamide DNA sequencing gel. The gel was dried onto 3MM Whatman paper under vacuum and subjected to autoradiography. Differentially expressed bands were excised from the gel, eluted, and reamplified by PCR using the same primers and conditions as described above. PCR products were then subcloned using the Blunt-Ended PCR Cloning Kit (Amersham-Pharmacia) according to the manufacturer's protocol. The samples were sequenced on an ABI 377 sequencer (Perkin-Elmer).

Northern blot hybridization analysis. mRNA (200 ng) from *L. major* stationary-phase promastigotes was denatured, separated on a 1.2% agarose-2.2 M formaldehyde gel, and transferred to a Hybond N⁺ membrane (Amersham-Pharmacia) by capillary action in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The RNA was cross-linked to the membrane by heating at 80°C for 2 h. The differentially expressed cDNA fragments and α-tubulin cDNA were labeled with [α-³²P]dCTP by using the Megaprime DNA Labeling System Kit (Amersham-Pharmacia) and hybridized overnight in 1× Denhardt's solution-6× SSC-0.1% sodium dodecyl sulfate (SDS)-0.1 mg of salmon sperm DNA ml⁻¹ at 65°C. The membranes were then washed at 65°C with a final stringency of 0.1× SSC-0.1% SDS and exposed to X-ray film.

cDNA library construction and isolation of LmPDI cDNA. An oligo(dT)-primed cDNA library from *L. major* promastigote poly(A)⁺ RNA was constructed in λZAP II according to the instructions of the manufacturer (Stratagene, La Jolla, Calif.). A total of 6 × 10⁵ plaques were screened under high-stringency conditions with a p14 cDNA probe labeled with [α-³²P]dCTP by using the random primer method (Amersham-Pharmacia). Positive plaques were picked from plates and subjected to secondary and tertiary screenings to ensure purity of the clones obtained from the screening. Positive clones were then sequenced.

Southern blot hybridization analysis. Southern blotting with genomic DNA (10 µg/lane) isolated from GLC94 promastigotes digested with the restriction enzymes indicated in Fig. 4, resolved in 0.6% agarose gel, and transferred to a Hybond N⁺ membrane (Amersham-Pharmacia) was performed. The blot was hybridized with an *EcoRI-XhoI* restriction fragment of the full-length LmPDI clone labeled with [α-³²P]dCTP according to the instructions of the manufacturer (Amersham-Pharmacia). The membranes were washed at a stringency of 0.1× SSC-0.1% SDS and exposed to X-ray film.

Expression and purification of the recombinant LmPDI protein in *Escherichia coli*. The 1,371-bp open reading frame lacking the potential signal peptide region was subcloned into the pET-22b expression vector (Novagen, Fontenay-sous-Bois, France). The BL21 *E. coli* strain harboring the recombinant plasmid was grown in Luria-Bertani medium, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h, and lysed. The recombinant LmPDI-(His)₆ was then purified by affinity chromatography over Ni-nitrilotriacetic acid resin with imidazole gradient elution (Amersham-Pharmacia), and purity was demonstrated by SDS-polyacrylamide gel electrophoresis.

Preparation of antiserum to LmPDI and Western blotting. Two rabbits were immunized by intramuscular injection of 500 µg of the purified recombinant LmPDI emulsified in incomplete Freund's adjuvant (Sigma, Steinheim, Germany). The rabbits received two additional injections with the same amount of protein emulsified in incomplete Freund's adjuvant, first by the intramuscular route 15 days after the first injection and second by intradermal injections in eight different sites at least 30 days later. The rabbits were bled starting 10 days after the final injection. Sera were separated and stored at -80°C until use. Lysates of stationary-phase *L. major* promastigotes in 1× SDS sample buffer were boiled for 10 min (10 µg of protein per well), separated on an SDS-12% polyacrylamide gel, and electrotransferred to nitrocellulose membranes. Filters were blocked with phosphate-buffered saline-0.1% Tween 20 (PBS-T) containing 5% nonfat dried milk at room temperature for 1 h and incubated with antiserum to LmPDI diluted 1:1,000 in blocking solution at 4°C overnight. The filters were then washed three times with PBS-T, incubated with goat anti-rabbit secondary antibody coupled to peroxidase for 1 h at room temperature, washed three times with PBS-T, and revealed by peroxidase activity detection with the light-based ECL system as described by the manufacturer (Amersham-Pharmacia).

PDI enzymatic assays. Activity of PDI was assayed by a method based on the catalysis of the oxidative refolding of "scrambled" bovine pancreatic RNase type III-A as described by Lyles and Gilbert (28). In brief, 8 µM reduced and denatured RNase A (Sigma) was incubated with 1.4 µM purified recombinant LmPDI protein in a buffer containing 4.5 mM cytidine 2',3'-cyclic monophosphate (Sigma), 1 mM reduced glutathione (Sigma), 0.2 mM oxidized glutathione (Sigma), 2 mM EDTA, and 100 mM Tris-HCl (pH 8). RNase activity was assayed by monitoring the rate of change of absorbance at 296 nm at 25°C during 30 min in a DU65 spectrophotometer (Beckman Instruments Inc., Champlan, France).

FIG. 1. (A) Differential display analysis of mRNAs obtained from promastigotes of *L. major* isolates expressing high (GLC94 and GLC67) or low (GLC32 and GLC07) virulence. A portion of a sequencing gel autoradiography showing the amplified products obtained with combinations of an anchored primer (*VBD6*) and an arbitrary decamer primer (*VAD6*) is shown. The arrowheads indicate the differentially accumulating PCR products, including p14. (B) Northern blot analysis of the same mRNAs used for panel A. The nitrocellulose membranes were hybridized with cloned p12, p14, and p21 probes. After autoradiography, the blots were stripped and rehybridized with a probe for *L. major* α -tubulin (α -tub).

In experiments testing the effects of inhibitors of PDI functions, zinc bacitracin (0.01 to 2 mM), tocinoic acid (0.01 to 5 mM), or pCMBa (0.01 to 2 mM) was added to the RNase refolding reaction mix.

RESULTS

Identification of differentially expressed genes in high- and low-virulence *L. major* isolates. Promastigotes from *L. major* isolates expressing either high (GLC94 and GLC67) or low (GLC32 and GLC07) virulence were cultured in complete medium until stationary phase was reached. The mRNA was then purified and reverse transcribed into cDNA by using six anchored oligo(dT)₁₁MN primers. PCR amplification was carried out with the same anchored primer used in the reverse transcription reaction combined with 10 different arbitrary primers to give a total of 60 different combinations (Table 1). Amplicons generated by the multiple primer sets were resolved on 6% polyacrylamide sequencing gels. The comparative analysis revealed that the four *L. major* isolates shared 95% of the mRNAs, expressed at similar levels. However, a thorough examination of DNA bands generated with the 60 primer pairs revealed 25 distinct bands that appeared to be differentially expressed. An example of a differential display gel is shown in Fig. 1A. The 25 differentially expressed fragments were isolated and reamplified, using the same primer combinations as in the first PCR, and subsequently cloned into the pMOS-blue

plasmid vector. The sequencing of these fragments showed that some of them were identical.

Fourteen differentially expressed fragments were selected and used as probes to analyze by Northern blotting the mRNAs purified from the four *L. major* strains. Three clones displayed clear differences in expression between high- and low-virulence isolates (Fig. 1B). One of these clones, p14, was chosen for further analysis. The p14 clone recognized an approximately 2.2-kb transcript that was preferentially expressed in the two high-virulence isolates compared to the two low-virulence isolates, as predicted from the differential display. The p14 clone was then sequenced. A comparison of the 339-bp fragment with the GenBank and EMBL databases yielded no significant match with the sequence of any known gene, a result which reflects the fact that clone p14 corresponds to a 3' untranslated region.

Cloning and sequence analysis of the full-length p14. To isolate a full-length cDNA clone, the 339-bp fragment was used as a probe to screen a promastigote *L. major* GLC94 cDNA library. Two positive clones were isolated by screening of 6×10^5 recombinants and sequenced. The nucleotide sequence of the longest clone, consisting of 2,094 bp, is shown in Fig. 2, together with the deduced amino acid sequence. We identified an open reading frame, starting from the ATG codon at position 256, which encodes a polypeptide of 477 amino acid residues with a predicted molecular mass of 52.4 kDa and an isoelectric point of 5.22. The N-terminal part of the deduced protein corresponds to a potential 20-amino-acid signal peptide. The 5' untranslated region contains a characteristic *Leishmania* splice leader sequence and the 3' untranslated region contains a poly(A) stretch.

The deduced amino acid sequence of the isolated clone exhibits an overall identity of 27 to 36% to proteins of the PDI family from several species (Fig. 3). More specifically, this protein contains two distinct regions at residues 47 to 52 and 381 to 386 that are identical to the putative active-site sequence (CGHC) of the PDI, ERp, and thioredoxin family of proteins. The C-terminal end displays a potential KDEL-type endoplasmic reticulum (ER) retention signal (EEDL) at positions 474 to 477, suggesting that, like PDI and ERp, this protein is a luminal ER protein. p14 was therefore considered a putative LmPDI.

To assess the copy number of the LmPDI gene, Southern blot analysis was carried out with ³²P-labeled LmPDI cDNA as a probe. Under stringent conditions, only a single band was revealed except when predicted internal restriction sites were present (Fig. 4A). This pattern of hybridization was consistent with LmPDI being encoded by a single-copy gene. Similarly, hybridization with genomic DNA demonstrated that a *Leishmania* PDI gene is also present in the *Leishmania infantum* and *L. donovani* genomes (Fig. 4B).

Enzymatic activity. In order to characterize the enzymatic activity of the LmPDI, the cDNA corresponding to the protein lacking the potential signal peptide was subcloned into the plasmid expression vector pET-22b and expressed in *E. coli* as a C-terminal polyhistidine fusion protein. The recombinant protein was purified by affinity chromatography over Ni-nitrilotriacetic acid resin and tested for PDI activity by using the scrambled RNase folding method. The ability to refold RNase that has been reduced, and denatured is classically used as a

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ggcaccagcggcaccagagttttctgtactttattgtctttctctattctaccacacttgccctctctgcgct 72
ctctgtgcctgtgcggcgcgcaacgtgcctttctctcgtattgccagctgtgagctgctgcctactggc 144
aacgtgtacgccattcccgtttcttgattctggtgcagtgctcagctctaccctatttgtattgataaccgtt 216
ttccttttctgttttgcgaagaaaa ATG CAG CGC TCA TTC CTT GTT TTT GTT CTG TGC GCC 276
                               M Q R S F L V F V L C A 12
CTT CTC TTC TGC GTC GCG TCC GCA GAG GTG CAG GTG GCC ACT AAG GAC AAC TTT 330
L L F C V A S A E V Q V A T K D N F 30
GAC AAG GTC GTA ATC GGG GAT CTC ACG TTG GTC AAG TTT TAT GCT CCG TGG TGC 384
D K V V I G D L T L V K F Y A P W C 48
GGC CAC TGC AAG ACA CTC GCC CCG GAG TTT GTA AAG GCC GCT GAC ATG CTG GCC 438
G H C K T L A P E F V K A A D M L A 66
GGC ATC GCG ACC CTT GCA GAG GTC GAT TGC ACC AAA GAA GAG AGC CTT GCT GAG 492
G I A T L A E V D T K E S L E 84
AAG TAC GAA AAT AAG GGG TTC CCC ACG CTG TAC ATC TTC CGT AAC GGT GAG AAA 546
K Y E I K G F P T L Y I F R N G G E K 102
GTG AAG ATC TAC GAT GGT CCC CGC ACT GCC GCC GGC ATC GCG TCG TAC ATG AAG 600
V K I Y D G P R T A G G I A S Y M K 120
GCG CAT GTC GG CCA TCG ATG AAG GCC AAT TCA ACG GCT GAA GAG CTG GAG GAG 654
A H V G P S M K A I S T A E E L E 138
CTC AAG AAG GAG ACT TTC CCG GTG TGC GTG GTG AAG ACA GCG AGC ACC GAC TCG 708
L K K E T F P V C V V K T A S T D S 156
GAG ATG GCG TCG ATG ATA ACC AAG GTG GCG GAC TCT CTC CGC TCG CAG ATG AAC 762
E M A T S M T K V A D S L R S Q M N 174
TTT GTG CTC GTG ACG GAT GCG GCC ATC TCT CCG AAT GAT GCC ATG GAG TCG GTT 816
F V L V T D A A I S P N D A M E S V 192
ACG GTG TAT CGC AAG AAT GCG GAG CGC GAG GCG TAC ACC GGC GCT ACA CCA ATG 870
T V Y R K N A E R E A Y T G A T P M 210
ACG GCA GAG TCG GTG AAG AGC TTT CTC ACG AGT GCT GTG TTG GAC TAC TTT GGC 924
T A E S V K S F L T S A V L D Y F G 228
GAG CTC GGC CAG GAG AGC TTT CAG AAG TAC ATG GAA GCG AAC AAG GAT AAA CCT 978
E L G C E E S T K Y M E A N K D A A P 246
CTT GGG TGG GTG TTC ATC GAC AAG AAC ACG GAT TCT GCG TTG AAG GGG TCA CTT 1032
L G W V F I D K N T D S A L K G S L 264
GTG GCG GTG GCG GAG AAG TAC CGC TCG CAG GTG TTG CTA ACC TAC ATT GAC GGC 1086
V A V A E K Y R Q V L L T Y I D G 282
GAT CAG TAC CGC CCC GTC TCG CGC CAG CTG GGC ATT CCT GAG GAT GCG AAG TTC 1140
D Q Y R P V S R Q L G I P E D A K F 300
CCG GCG TTT GTG GTC GAT TTC GAG CGC CGC CAT CAC GTG ATG GGG ACG GAC ACC 1194
P A T V F E R H V M G H A G G A C T T 318
CCA GTC ACC TCC GAG TCT GTC GCT AAG TTT GTG GAG AAG TAT GTC AAG GGC GAG 1248
P V T S E S V A A F V E K Y V K G E 336
ACG AAG CAG ACC GTG ATG TCC GAC GCG ATT CCC GCT AAG GAG ACG GTG AAC GGC 1302
T K Q T V M S D A I P A K E T V N G 354
CTC ACA ACG GTG GTG GGT CAG ACT TTT GCG AAG TAC ACG GAC GGC ACA CAA AAC 1356
L T T V V G Q T F A K Y T D G G A T Q A N 372
GTG ATG CTG CTC TTC TAC GCG CCG TGG TGC GGA CAC TGC AAG AAG CTG CAC CCC 1410
V M L L F Y A P W C G H C K K K H P 390
GTC TAC GAT AAA GTA GCC AAG AGC TTC GAG TCT GAG AAT GTG ATC ATT GCG AAG 1464
V Y D K V A K S T E N V I I A A K 408
ATG GAT GCC ACG ACG AAC GAC TTT GAC CGC GAG AAG TTT GAG GTG TCT GGA TTT 1518
M D A T T N D F D R E K F E V S G F 426
CCA ACG ATT TAC TTC ATC CCA GCC GCG AAG CCG CCA ATC GTG TAC GAG GGT GGC 1572
P T I Y F A P A G G K P I V Y E G G G 444
CGC ACC GCA GAC GAA ATC CAG GTG TTT GTG AAG TCT CAC CTG ACC GCC TCC GCC 1626
R T A D E I Q V F V S G H L T A S A 462
GCT CCA TCT GGC GGC CCT TCC GGC AAC AGC GAA GAG GAT TTG TAG gactgca 1681
A P S G G P S G N S E E---E---D---L * 478
agggatgtggcgtttataggtgccttgccttcccttgcctgtttctatgacggattaggctttttttgtta 1753
tatgtggggtggtcaagagagtgccagggtccttctttatataccttgcgctttctttttattttgcttcctt 1825
gtgttgacgtctatgcatgctgtgtgcgactccttgtcaacctgcgtcctatctagtagcatcgatgt 1897
gaaaagaagagtagagggaggttaacgatgctgctgctggctgccgttttcatggcgcaatttcgagaagga 1969
aaatcggaataatggacagtagcgaaattagcgcaacgacaaggtcgtgcttctctatcggtcatta 2041
aatctctgggctttgtacaatatgaaggaggtcacacaaaaaaaaaaaaaaaaaaaaa 2094

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FIG. 2. Nucleotide and predicted amino acid sequences of the LmPDI cDNA. The nucleotide sequence is numbered on the top, and the deduced amino acid sequence is numbered on the bottom. Lowercase type represents untranslated regions. The splice leader 18-nucleotide sequence is underlined. The predicted signal peptide residues are shown in boldface. The thioredoxin/PDI/Erp active sites are double underlined, and the probable ER retention sequence (EEDL) is underlined with dashes.

FIG. 3. Alignment of the LmPDI amino acid sequence with those of PDIs from *Trypanosoma brucei* (GenBank accession number P12865), *Hypocrea jecorina* (074568), *Caenorhabditis elegans* (017908), *Chlamydomonas reinhardtii* (048949), *Drosophila melanogaster* (P54399), *Cryptosporidium parvum* (Q27553), and *Homo sapiens* (P072237). Black shading indicates identical amino acids, and grey shading indicates similar amino acids. Gaps were inserted to achieve maximum similarity and are indicated by dashed lines.

measure of protein-disulfide isomerization (28). While the spontaneous refolding of scrambled RNase did not exceed 6% after 20 min of incubation, the presence of recombinant LmPDI showed a time- and dose-dependant increase (17 and

32% after 20 min of incubation in the presence of 1.4 and 1.8 μ M, respectively) in RNase folding, whereas the control protein bovine serum albumin had no activity (Fig. 5). These data demonstrate that in addition to its sequence homology with the

FIG. 4. Southern blot hybridization analysis of the LmPDI gene locus. (A) Genomic organization of the LmPDI gene in *L. major*. Ten micrograms of *L. major* genomic DNA was digested to completion with restriction enzymes that lack a cleavage site in the LmPDI cDNA (*AvaI*, *EcoRV*, *HindIII*, *NcoI**, *PstI*, *SalI**, *EcoRI*, *SphI**, *XhoI*) and with restriction enzymes that cut once (*NcoI*, *SacI*, and *SphI* [marked with an asterisk]) and probed with the full-length cDNA of LmPDI. DNA molecular size markers are indicated on the left. (B) Hybridization analysis of the LmPDI gene using genomic DNAs from different *Leishmania* species as indicated. Two *L. infantum* isolates, viscerotropic (*L. infantum* V) and dermatropic (*L. infantum* D), were used. DNAs were digested with *PstI* and analyzed by hybridization with the same probe used for panel A.

members of PDI family, LmPDI expressed a specific enzymatic activity.

Expression of native LmPDI in *L. major* promastigotes from high- and low-virulence isolates. To characterize the native LmPDI expressed by *L. major* isolates, specific antibodies were raised in rabbits immunized with the purified recombinant protein produced in *E. coli*. Rabbit antibodies to LmPDI strongly reacted with a band of the expected size (55 kDa) in lysates of GLC94 stationary-phase promastigotes (Fig. 6A, lane 3). In addition, two other proteins were detected, one with a molecular mass that was approximately twice that of LmPDI (105 kDa) and the second with a molecular mass of 35 kDa. The 105-kDa protein is likely an LmPDI dimer since it was no longer detected when promastigote lysates were subjected to SDS-polyacrylamide gel electrophoresis under highly denaturing conditions (Fig. 6A, lane 2). The 35-kDa protein seems to be a contaminant since it did not react with affinity-purified antibodies to LmPDI (Fig. 6A, lane 2).

To compare the levels of the LmPDI expressed by the different *L. major* isolates, stationary-phase promastigotes were used for protein extraction and quantified by the Lowry method, and the same amount was resolved in 12% polyacrylamide gels. Ponceau red and Coomassie blue staining showed that extracts from *L. major* displayed similar patterns with no obvious qualitative or quantitative differences (data not shown). However, Western blot analysis of the lysates using

FIG. 5. Enzymatic activity of the recombinant LmPDI protein. Thiol isomerase activity of the purified preparation of the recombinant LmPDI was determined according to the procedures of Lyles and Gilbert (28). Native RNase was reduced, denatured, and incubated with 1.4 μM LmPDI, 2.8 μM LmPDI, 1.4 μM bovine serum albumin (BSA), or no enzyme. RNase folding was monitored at the indicated time points. Results are expressed as a percentages of the RNase folding.

rabbit antibodies to LmPDI revealed a strong band corresponding to the protein and its dimer in high-virulence isolates (Fig. 6B, lanes 4 and 5), whereas LmPDI and its dimer were barely detectable in low-virulence isolates (Fig. 6B, lanes 6 and 7). Similar amounts of the irrelevant 35-kDa protein were observed in all strains.

FIG. 6. Identification and expression levels of native LmPDI. (A) Cell lysates of stationary-phase promastigotes of *L. major* isolate GLC94 (lanes 2 and 3) and purified recombinant LmPDI (rLmPDI) (lane 1) were analyzed by Western blotting. The blot was immunostained with the rabbit anti-LmPDI antiserum (lane 3) or with affinity-purified antibodies to recombinant LmPDI (lanes 1 and 2). (B) Total protein lysates (10 μg) obtained from the promastigotes of the different *L. major* isolates (GLC94 [lane 4], GLC67 [lane 5], GLC32 [lane 6], and GLC07 [lane 7]) were analyzed by Western blotting using anti-LmPDI antiserum. Arrowheads indicate the three proteins recognized by the polyclonal antibody. Molecular mass (MW) markers are indicated on the right.

FIG. 7. Effects of PDI inhibitors on thiol isomerase activity of the recombinant LmPDI (rLmPDI). The activity of the LmPDI was determined in the presence of zinc bacitracin (BACZn), pCMBA, or tocinoic acid (TOC) or, as a control, without inhibitors. Results are expressed as the percentage of residual activity compared to the control.

Effect of PDI inhibition on LmPDI activity and *L. major* parasite growth. Specific inhibitors of PDI enzymatic activity have been previously described (29, 32, 39, 40). Some of them were used in the present study to evaluate their inhibitory effect on the recombinant LmPDI. Various concentrations of zinc bacitracin, tocinoic acid, or pCMBA were incubated with the same amount of recombinant LmPDI (1.4 μ M), and the enzymatic activity was assayed by the scrambled RNase method (Fig. 7). Total inhibition was observed with as little as 0.01 mM pCMBA. For zinc bacitracin, 50% inhibition was obtained with 0.1 mM and 100% inhibition was obtained with 2 mM. Tocinoic acid had only a partial effect, i.e., less than 30% inhibition at 2 mM.

The effects of PDI inhibitors on the kinetics of in vitro parasite growth were also checked. The virulent GLC94 isolate was grown in complete medium with increasing amounts of zinc bacitracin, pCMBA, and DTNB, another inhibitor of PDI. Promastigotes were then enumerated after 48, 72, and 96 h of incubation. A significant inhibition of parasite growth was observed at 1.5 mM zinc bacitracin, and a complete inhibition was observed at 2 mM (Fig. 8A). Parasite growth was also totally blocked by pCMBA at 0.5 mM (Fig. 8C). However, DTNB had no effect at up to 2.5 mM (Fig. 8B). These experiments indicate that some, but not all, PDI inhibitors may exert profound inhibition of in vitro *L. major* parasite growth.

DISCUSSION

In the present study, we identified a new *Leishmania* gene coding for a PDI, which is probably involved in parasite natural pathogenicity and may constitute a new target for chemotherapy.

Over the last decade, several approaches have been used to elucidate gene functions, oriented towards the identification of

FIG. 8. In vitro *L. major* parasite growth in the absence or the presence of various concentrations of zinc bacitracin (BACZn) (A), DTNB (B), and pCMBA (C). The *L. major* GLC94 isolate was used. Parasites were inoculated at 2×10^6 /ml at time zero, and the parasite growth was measured after 48, 72, and 96 h of culture.

parasite virulence factors. These include genetic studies such as the complementation of *Leishmania* mutants (6), the use of gene disruption to target stage-regulated genes (15, 16, 45), or the analysis of drug-resistant parasites generated in the laboratory (21, 35). These studies led to the identification of im-

portant parasite proteins that may represent targets for new drugs (31, 43, 49), be used as subunit vaccines (8, 38, 48), or allow the development of mutants to be used as attenuated live vaccines (2, 47). The majority of *Leishmania* virulence studies were based on laboratory clones. It is therefore unknown whether the conclusions drawn from these studies on *Leishmania* virulence are relevant to the natural pathogenicity of the parasite in the field.

We used an alternative approach based on the analysis of parasites freshly isolated from human ZCL lesions. We think that this strategy may be suitable to track new genes whose products would be more relevant to the parasite variability occurring in naturally infected hosts. Therefore, wild *L. major* isolates with contrasting pathogenicities in BALB/c mice were selected for the present study. We hypothesized that genes differentially expressed between these isolates may constitute candidate parasite virulence genes. In a previous study, we failed to detect any significant difference in the mRNA levels of eight genes that have been reported by others as virulence genes (i.e., the genes for LPG1, LPG2, kinetoplastid membrane protein 11, cysteine proteinases, HSP100, GP63, and gene B) (22). Therefore, we used a broader screening technique, namely, the mRNA differential display, to try to track mRNA species that are differentially expressed. Since *Leishmania* parasites produce polycistronic mRNAs and control gene expression at the level of mRNA processing and stability (46), one may ask how differential display PCR works to track differentially expressed mRNAs. If the overexpression of an mRNA is due to its higher stability, it will then be amplified by differential display, while a less stable (and hence less abundant) mRNA will be detected to a lesser extent. Three transcripts preferentially expressed in the two high-virulence isolates were identified in the present study. One of the cDNAs was fully characterized and shown to be a member of the eukaryotic disulfide isomerase family of proteins. This newly identified *Leishmania* gene was designated LmPDI for the following reasons: (i) it contains two distinct CGHC active domains characteristic of this family; (ii) the native or recombinant protein can organize in an oligomeric structure; (iii) most importantly, the recombinant protein expresses a PDI activity; and (iv) this enzymatic activity could be inhibited by specific PDI inhibitors. Interestingly, outside conserved stretches around the active sites, this protein strongly diverged from other described PDIs (14).

LmPDI is encoded by a single gene copy which appeared to be structurally conserved among the three *Leishmania* species tested so far, namely, *L. major*, *L. donovani*, and *L. infantum*.

PDI is a member of the thioredoxin superfamily, which is composed of several redox proteins playing a key role in disulfide bond formation, isomerization, and reduction within the ER, and it displays chaperone activity (14). Its structure encompasses two double-cysteine, redox-active sites, each within domains with high sequence similarity to thioredoxin. It is a highly abundant ER luminal protein in mammalian cells and in yeast. These molecules are essential for assisting unfolded or incorrectly folded proteins to attain their native state.

Recently, several studies indicated a role for disulfide bond A (DsbA), a bacterial homologue of PDI, in the pathogenicity of some microorganisms (4, 53). Thus, inactivation of the gene for DsbA affects the intracellular survival and virulence of

Shigella flexneri (53–55). Moreover DsbA is involved in the biogenesis of the enterotoxin and the toxin-coregulated pili of *Vibrio cholerae* (37, 56). DsbA is also important for pathogenic *E. coli*: in uropathogenic species, it catalyzes disulfide bond formation in a pilin-specific chaperone, PapD (57), and in enteropathogenic species, it is required for the stability of bundle-forming pili (18, 50).

Our results indicate that, as it does in bacteria, PDI may play a role in virulence of *L. major*. Thus, some inhibitors of the PDI activity had a profound negative effect on *Leishmania* growth in liquid medium. In addition, we could demonstrate, in preliminary experiments, that zinc bacitracin, a known PDI inhibitor, attenuates disease progression when locally applied as an ointment on the parasite inoculation site in BALB/c mice (M. Chenik et al., unpublished data). One cannot exclude, however, the possibility that these inhibitors may act on other members, not yet identified, of the *Leishmania* PDI family.

Despite having 30% identity with human PDI, mainly restricted to the functional sites of the protein, LmPDI may constitute a new target for drug development. Its three-dimensional model structure may help in the design of new specific drugs, especially because LmPDI is also highly expressed at the amastigote stage of the parasite which multiplies in the infected mammalian hosts (data not shown). Even though the potential drug may have an inhibitory effect on human PDI, its use as an ointment may not constitute a serious problem in the treatment of cutaneous leishmaniasis.

The mechanism by which LmPDI may affect parasite virulence is presently unknown. One may suggest a role in the optimal folding of proteins important for parasite multiplication that are either secreted or expressed at the parasite surface. This may play a role in host-parasite interactions and intracellular survival. Gene invalidation or overexpression experiments are obviously needed to fully confirm the involvement of LmPDI in *Leishmania* virulence. LmPDI amino acid sequence variation does not account for differences in virulence, since the four isolates used in this study showed strictly identical PDI sequences (data not shown). However, we cannot exclude the possibility that a polymorphism of the regulatory regions may account for these differences.

In conclusion, the newly identified LmPDI may play an important role in *Leishmania* natural pathogenicity and may constitute a new target for anti-*Leishmania* chemotherapy.

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