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ORIGINAL PAPER

Ddi1-like protein from *Leishmania major* is an active aspartyl proteinase

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Abstract Eukaryotic cells respond to DNA damage by activating damage checkpoint pathways, which arrest cell cycle progression and induce gene expression. We isolated a full-length cDNA encoding a 49-kDa protein from *Leishmania major*, which exhibited significant deduced amino acid sequence homology with the annotated *Leishmania* sp. DNA damage-inducible (Ddi1-like) protein, as well as with the Ddi1 protein from *Saccharomyces cerevisiae*. In contrast to the previously described Ddi1 protein, the protein from *L. major* displays three domains: (1) an NH2-terminal ubiquitin like; (2) a COOH terminal ubiquitin-associated; (3) a retroviral aspartyl proteinase, containing the typical D[S/T]G signature. The function of the *L. major* Ddi1-like recombinant protein was investigated after expression in baculovirus/insect cells and biochemical analysis, revealing preferential substrate selectivity for aspartyl proteinase A_2 family substrates, with optimal activity in acidic conditions. The proteolytic activity was inhibited by aspartyl proteinase inhibitors. Molecular modeling of the retroviral domain of the Ddi1-like *Leishmania* protein revealed a dimer structure that contained a double Asp-Ser-Gly-Ala amino acid sequence motif, in an almost identical geometry to the exhibited by the homologous retroviral aspartyl protease domain of yeast Ddi1 protein. Our results indicate that the isolated Ddi1-like protein is a functional aspartyl proteinase in *L. major*, opening possibility to be considered as a potential target for novel antiparasitic drugs.

Keywords *Leishmania* · Aspartyl proteinase · Ddi1 · Ubiquitin receptor proteins · Retroviral protease · Cloning

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Introduction

Various members of the genus *Leishmania* (Kinetoplastida order, Trypanosomatidae family) cause leishmaniasis, one of the world's most neglected diseases, which mostly affects developing countries. It is estimated that 350 million people are at risk of contracting leishmaniasis and there are two million clinical cases each year, with an incidence of 25 % for the fatal visceral form (Alvar et al. 2006a, b). Current treatments are expensive and can develop undesirable side effects in patients. In addition, an increasing drug resistance of *Leishmania* species has been observed. These observations

together suggest that new and novel antiparasitic drugs are required. Therefore, the development of alternative drugs for the treatment of the different clinical forms of leishmaniasis is a priority for controlling the disease (Croft et al. 2006; Castillo et al. 2010). Great efforts have been made to identify potential chemotherapeutic targets in the genome and proteome of *Leishmania* sp., in the hope of devising more effective treatments (Myler 2008; Concu et al. 2009; Chawla and Madhubala 2010). Proteolytic enzymes or proteinases play an essential role in the parasite's survival. These molecules have been identified as promising targets for the development of a rational antileishmanial chemotherapy (Coombs et al. 2001; Klemba and Goldberg 2002; Mottram et al. 2004; McKerrow et al. 2008; McLuskey et al. 2010). It is within this context that we reported the presence of aspartyl proteinase activity in promastigotes of *Leishmania mexicana* and *Leishmania infantum* (Valdivieso et al. 2007, 2010), which is sensitive to diazoacetyl-DL-norleucin methyl ester (DAN) and the specific HIV aspartyl proteinase inhibitors, Ac-Leu-Val-Phenylalaninal, Nelfinavir (NFV), and Saquinavir (Valdivieso et al. 2010). Santos et al. (2009) also reports the inhibition by NFV and Lopinavir of certain hydrolytic activity from a *Leishmania amazonensis* extract on an HIV-1 protease substrate, which supports the hypothesis that these drugs have a direct effect on the aspartyl proteinase activity of *Leishmania* sp. In addition, we found that in the presence of aspartyl proteinase inhibitors, promastigotes and amastigotes of *L. mexicana* and *L. infantum* show antiproliferative activity with a significant percentage of binucleate cells, and in some cases multinucleate cells, indicating a blockage of cytokinesis (Valdivieso et al. 2007, 2010), and reiterating the importance of aspartyl proteinase activity for the survival of these trypanosomatids. To date, two aspartyl proteinase genes have been identified within the genome of *Leishmania* sp.: a gene encoding an aspartyl proteinase belonging to the A_{22} family (GenBank ID: XP_001682072.1) and another that encodes a protein homologous to the DNA damage-inducible-like (Ddi1-like) protein with an aspartyl proteinase domain similar to that of aspartyl proteinases from the A_2 family (GenBank ID: XP_001687454). Aspartyl peptidases belonging to the family A_{22} contain eight transmembrane domains, and have been identified as necessary for the degradation of the amyloid beta peptide deposited in the brains of people with Alzheimer's disease (Wolfe 2001). Ddi1 proteins contain a conserved domain homologous to one in retroviral aspartyl protease (RP), and it has been suggested that the corresponding protease activity could be involved in a novel, ubiquitin-dependent branch of cell cycle control (Krylov and Koonin 2001). Recently, White et al. (2011a) demonstrated that an *Leishmania major* ortholog of the yeast Ddi1 protein can complement the protein secretion phenotype of *S. cerevisiae* Ddi1 knockout, and that a number of HIV proteinase inhibitors avert the resultant complementation phenotype, suggesting that inhibition of the *Leishmania* Ddi1 ortholog might be the mechanism by which HIV proteinase inhibitor therapy mediates its antiparasitic action (White et al. 2011a).

In the present work, we report the full amino acid sequence of *L. major* Ddi1-like protein includes, an additional fragment at the N-terminal end, homologous to the ubiquitin-like (UBL) domain of this family of proteins described in other organisms, and which had not been previously reported for the *L. major* Ddi1-like protein (Krylov and Koonin 2001; White et al. 2011a). The cloning, expression, and functional characterization of the *L. major* fully recombinant Ddi1-like protein allow us to demonstrate directly for the first time and in an experimental way, the proteolytic activity of this protein family. These findings together with our previous reports open the way for future studies focused on the rational design of selective inhibitors of this protein as a novel target for antileishmanial drugs.

Materials and methods

Parasites

L. major promastigotes, strain (MHOM/SU/73/5-ASKH), were grown in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10 % heat-inactivated fetal calf serum (Gibco), 50 mg/ml gentamycin (Sigma Chemical Co., St. Louis, MO, USA) and 2 mM glutamine (Sigma Chemical Co.), and placed in a chamber at 27 °C.

Isolation of nucleic acids

Messenger RNA (mRNA) from 1×10^7 parasites, previously collected and washed with diethylpyrocarbonate (Sigma Chemical Co.), was isolated using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA, USA). Briefly, once the parasite material had been incubated with sodium dodecyl sulfate (SDS) and RNAse inhibition enzymes, the mixture was passed through an oligo (dT) cellulose column and then washed with a high concentration salt buffer to eliminate DNA contaminants, membrane debris, and proteins. The nonpolyadenylated RNA fractions were eluted from the column by washing with a low concentration salt buffer; the mRNA was isolated using a buffer with no salt. Finally, the concentration and quality were estimated using a NanoDrop spectrophotometer ND-100 (NanoDrop Products, Wilmington, DE, USA) and stored at −80 °C until use.

Construction of *L. major* cDNA collection

An *L. major* cDNA collection was prepared using the Marathon cDNA Amplification kit (Clontech, Takara Bio, WI, USA). cDNA was synthesized from the mRNA isolated previously. The method allows synthetic adaptor sequences

(AP1) to be introduced to both the 5′ and 3′ ends of the cDNAs during synthesis. Furthermore, the synthesized cDNAs contain a high percentage of the complete 5′ end of the mRNA templates, which increases the chances of finding a full-length gene. The mixtures obtained were stored at −20 °C until used.

Cloning the *L. major DDI1*-like gene

The *L. major DDI1*-like gene was obtained by PCR cloning using both specific primers and the *L. major* cDNA collection as templates. Specific primers were designed using the information corresponding to the DNA sequence extracted from the database EBI-EMBL (MI ID: AE0012749) for the complete chromosome 1 *L. major* strain Friedlin (Ivens et al. 2005), as the sequence for *L. major* Ddi1 like protein included into the database was incomplete because of the erroneous prediction of the initial methionine. The following primers were used DDI0158131 (5′ ATGGTGCAGCT CACCATCAACAACGCCAGG 3′) and 3′DDI-A (5′ TTACGTGTCGAAGAGGAGCGCTGCCGCCAC 3′). The PCRs included: 1 ng of cDNA from *L. major*, 0.2 μM DDI0158131 primer, 0.2 μM 3′DDI-1A primer and 1 U/μl of Advantage Polimerase mix (Clontech), in a final volume of 50 μl. The reaction mixtures were transferred to a programmable thermal cycler, Gene Amp PCR System 2400 (Perkin-Elmer Corporation, Norwalk, CT, USA), and incubated for one cycle at 94 °C for 60 s, followed by 25 cycles of 94 °C for 5 s and 72 °C for 180 s. The product was analyzed and purified on agarose; the amplicon was eluted with a QIAQUICK gel extraction kit (Qiagen, Chatsworth, CA, USA) and cloned in a pGEM system (Promega Corporation, WI, USA). Universal primers (D, SP6) and specific primers (DDI0158131 and 3′ DDI-A) were used to confirm the sequence of the cloned *L. major* Ddi1-like gene (CEQ 2000 DNA Analysis System; Beckman Coulter, Youngstown, OH, USA).

Subcloning, expression, and purification of the recombinant *L. major* Ddi1-like protein

Subcloning of the L. major DDI1-like gene in the destination plasmid pDEST10 (His6 tag, Invitrogen)

For amplification of the coding regions, oligonucleotides were designed that include attB sites (Att-DD1-Fw 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGTGCAGCTCACCATCAACAACGCCAGG-3′ and Att-DD1-Rv 5′-GGGGACCACTTTGTACAA GAAAGCTGGGTTTACGTGTCGAAGAG GAGCGCTGCCGCCACG-3′) to generate PCR products suitable as substrates in a Gateway® BP (Invitrogen) recombination reaction with a donor vector (pDONR221; Invitrogen) for fast cloning. The oligonucleotides were purchased from Sigma Genosys. The coding regions were amplified from the cDNA clones by PCR with Platinum® Pfx DNA polymerase (Invitrogen). For the first cloning in entry vector, 150 ng of the purified PCR product was added to 150 ng of the pDONR221 vector, with 2 μl of BP Clonase (Invitrogen) for 1 h at 25 °C. Then 1 μl of proteinase K was added for 10 min at 37 °C. The final mixture was used to transform *Escherichia coli* Top10 competent cells (Invitrogen). Every positive colony was fully sequenced to rule out any mutation due to PCR amplification or recombination. Positive colonies were used for plasmid DNA purification. Similar amounts of pENTR vector and pDEST10 destination vector (300 ng) were mixed with 1 μl of LR Clonase (Invitrogen) for 1 h at 25 °C. Then 2 μl of proteinase K was added for 10 min at 37 °C. In general, we directly transformed DH5 cells (Invitrogen). Positive colonies were confirmed by PCR and were used for plasmid DNA purification.

Bacmid production, insect cell transfections, and viral stock production

MAX Efficiency® DH10Bac™ competent cells were used to produce recombinant baculovirus molecules for the expression of Ddi1 proteins.

The *L. major DDI1* recombinant bacmid was then transfected into Sf9 insect cells (ATCC-LGC Standards S.L.U., Barcelona, Spain) using Cellfectin® II Reagent (Invitrogen) and following the manufacturer's instructions. The baculovirus titer (P1 low titer stock) was determined by limiting dilution (1×10^7 pfu/ml). This viral stock was then used for production of high titer, low-passage recombinant baculovirus stocks (P2 viral stock 1×10^8 pfu/ml). The baculovirus high-titer stock was then used to determine the optimal expression conditions. Sf9 insect cells were cultured in Grace's Insect Medium, Unsupplemented (Invitrogen) containing 10 % FBS (Gibco) and antibiotics (Invitrogen).

Expression of the recombinant L. major Ddi1-like protein in Sf9 insect cells

The optimal expression conditions were determined by comparing different multiplicities of infection (MOI) and different temperatures. The optimized conditions selected were: infection with 10 MOI, incubation at 20 °C and collection at 72 h.

Purification of the recombinant L. major Ddi1-like His6-tagged protein

The His6-tagged Ddi1 protein was purified by affinity chromatography using a HiTrapTM column (GE Healthcare, St. Giles, UK) on an ÅKTA FPLC system (GE Healthcare)

following standard procedures. Used as the binding buffer was 20 mM Tris–HCl, 0.5 M NaCl, 40 mM imidazole, pH 8.0 and used as the elution buffer was 20 mM Tris–HCl, 0.5 M NaCl, 0.5 M imidazole, pH8.0. The fusion protein was pooled and dialyzed against PBS. Fractions were analyzed on 12.5 % SDS–polyacrylamide gels stained with Coomassie Brilliant Blue (Bio-Rad, Hemel Hempstead, UK). The recombinant *L. major* Ddi1-like His6-tagged purified protein was finally dialyzed and then protein concentrations were determined by the Bradford assay (Bio-Rad) (Bradford 1976) using bovine serum albumin as a reference. The final product was stored at −80 °C until used.

Aspartyl proteinase assays

The substrates RE-(EDANS)-SQNYPIVQK-(DABCYL)-R (Sigma Chemical Co.), Bz-RGFFL-4MβNA and Bz-RGFFP-4MβNA (Bachem, Torrance, CA, USA) were used to test the aspartyl proteinase activity of the recombinant *L. major* Ddi1-like protein.

For assays undertaken with RE-(EDANS)-SQNYPIVQK-(DABCYL)-R substrate: the recombinant protein (6 μg/ml) was added to 2 μM of substrate solution in 100 mM sodium acetate buffer, 1 M NaCl, 1 mM EDTA, 1 mM DTT, 10 % DMSO, 1 mg/ml BSA, pH5. Triplicates of enzymatic and control assay mixtures were incubated at 37 °C for 2 h. The cleavage of the substrate was monitored with a fluorescence microplate reader (Victor 3, 1420 Perkin Elmer), using excitation and emission wavelengths of 340 and 490 nm, respectively.

For assays undertaken with Bz-RGFFL-4MβNA and Bz-RGFFP-4MβNA substrates, the recombinant protein (6 μg/ml) was added to 2.5 μM of substrate solution in 100 mM sodium acetate buffer, 1 M NaCl, 1 mM EDTA, 1 mM DTT, 10 % DMSO, pH4 and pH8, respectively. Triplicates of enzymatic and control assay mixtures were incubated at 37 °C for 2 h. The progressive release of the fluorochrome 4-methoxy-β-naphthylamine was monitored with a fluorescence microplate reader (Victor 3, 1420 Perkin Elmer), using excitation and emission wavelengths of 340 and 425 nm, respectively.

To determine the optimum pH for the recombinant enzyme activity against synthetic substrates, the following buffers, at a concentration of 100 mM, were used: sodium citrate, pH3.0 and 3.5; sodium acetate, pH4.5–5.5; sodium phosphate, pH6.0–7.5; and sodium borate, pH8.0 and 8.5. All the buffers contained 1 M NaCl, 1 mM EDTA, 1 mM DTT, 10 % DMSO, 1 mg/ml BSA.

For inhibition studies, the enzyme was pre-incubated with inhibitors for 10 min at 25 °C before the substrate was added to measure the residual activity. The assay was performed in the presence of 500 μ DAN (Sigma Chemical Co.), 15 μM pepstatin (Sigma Chemical Co.), 20 μM Nelfinavir (Pfizer Laboratories, Groton, CT, USA), 10 μM trans-epoxysuccinyl-L-leucylamido(4-guanidine)-butane (E-64) (Sigma Chemical Co.) and 5 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma Chemical Co.).

The Michaelis constant (K_m) and maximum velocity (V_{max}) values of recombinant *L. major* Ddi1 were determined by incubating the enzyme in the reaction mixture in the presence of increasing concentrations of various fluorogenic substrates (0.01–30 μM) at 37 °C. The initial velocity was calculated from the slope of the linear range of the fluorescence versus time curve. The K_m and V_{max} values were recorded with their standard errors derived from three independent experiments.

Proteolytic assays on natural substrates

Substrate hydrolysis was performed by the method of Anson (1938). Briefly, the substrate (1 mg/ml) was incubated in Eppendorf tubes with (6 μg/ml) the recombinant Ddi1-like *L. major* enzyme in 50 mM sodium acetate buffer pH4, in a final volume of 0.2 ml. The assay and control mixtures were incubated, in duplicate, at 37 °C for 24 h. The enzyme reaction was ended by the addition of 0.2 ml of sample buffer [50 mM Tris/HCl pH6.8, 4 % SDS, 12 % (*v*/*v*) glycerol, 0.02 % bromophenol blue] and incubation for 5 min at 100 °C. The digestion products were analyzed by 10 % SDS-polyacrylamide gel electrophoresis (PAGE) gel electrophoresis following Laemmli (1970), under nonreducing conditions.

Bioinformatics and modeling procedures

A 3D model of the dimerized RP domain of the *L. major* Ddi1-like protein was generated using homology modeling procedures and the coordinates of the dimerized RP domain of yeast Ddi1 (PDB ID: 2I1A; Sirkis et al. 2006) as a template. The sequence identity between the sequences was 45 % (length of aligned domain, 127 amino acids). The model was built using the SWISS-MODEL server (Peitsch 1996; Guex et al. 1999; Schwede et al. 2003) available at http://swissmodel.expasy.org//SWISS-MODEL.html, and its structural quality was checked using the analysis programs provided by the same server (Anolea/Gromos). Global model quality estimation scores (QMEAN4; Benkert et al. 2011) are: QMEANscore4, 0.485; *Z* score, –3.329, that are within the range of those accepted for homology based structure models. To optimize geometries, models were energy minimized using the GROMOS 43B1 force field implemented in DeepView (Guex and Peitsch 1997), using 500 steps of steepest descent minimization followed by 500 steps of conjugate-gradient minimization.

Multiple sequence alignment was carried out as follows: nucleotide and amino acid sequences of *DDI1* genes from *L.*

major, *L. infantum*, *L. mexicana*, *L. braziliensis*, *Trypanosoma cruzi_CLBrener*, *Plasmodium falciparum*, human and yeast were obtained from the EMBL-EBI server (www.ebi.ac.uk) and aligned using ClustalW (Thompson et al. 1994) and TCOFFEE (Notredame et al. 2000) on the same server.

Regarding the structure alignment: 3D structures of the RP domain of yeast Ddi1 (PDB ID: 2I1A; Sirkis et al. 2006), xenotropic murine leukemia virus-related virus (XMRV) proteinase (PDB ID: 3NR6; Li et al. 2011) and HIV-1 proteinase (PDB ID: 5HVP; Pillai et al. 2001) were aligned using standard procedures included in the Pymol Molecular Graphics System, Schrödinger, LLC.

Results

Molecular cloning of the *L. major Ddi1*-like gene

The *L. major DDI1*-like gene was cloned by PCR using specific primers and a parasite cDNA library as template. Analysis of the amplified gene revealed a 1,173-bp open reading frame coding for a 390-deduced-amino-acid sequence (MI ID: FR872380). The sequence of the *L. major* Ddi1-like protein is included in Fig. 1. The protein shows three possible domains, as other members of the Ddi1 family do: an NH2-terminal UBL domain, residues 1–57; a COOH terminal ubiquitin-associated (UBA) domain, residues 347–390; and a retroviral RP domain, residues 148–309. The RP domain has the D[ST]G signature of retroviral aspartyl proteinases.

Sequence comparison to eukaryotic Ddi1 proteins

Sequences that are homologous to the *L. major* Ddi1-like protein were obtained from the EMBL-EBI database using PBLAST and TBLASTN. Multiple sequence alignment of a representative set is shown in Fig. 1, corresponding to *L. major* (GenBank ID: XP_001687454.1), *L. infantum* (GenBank ID: XP_001462676.1), and *L. mexicana* (translation code MI ID: CBZ23045.1). As the three sequences exhibited an apparent lack of the segment corresponding to the N-terminal UBL domain of the protein, the ORF was analyzed to try to find a putative additional ATG positioned upstream in the same reading frame. In all three cases, and extra ATG was found in a position compatible to those corresponding to the complete *DDI1* sequence proposed for other organisms (see below). Using this ATG as the initial one in the three cases, the complete protein sequence was translated, now containing an extra N-terminal segment (in blue in Fig. 1), including the UBL domain in addition to the RP and UBA domains. The amino acid sequence present in the EMBL-EBI database was used directly in the case of *L. braziliensis* (GenBank ID: XP_001561444.1), *T. cruzi_CLBrener* (RefSeq. code XP_820109.1), *P. falciparum* (GenBank ID: XP_001348263.1), yeast (GenBank ID: NP_011070.1), and human (GenBank ID: NP_001001711.1) Ddi1 proteins.

As shown in the alignment, the RP domain is well conserved in all the species compared, with the UBL and UBA domains exhibiting a profile that is not as well conserved. Regarding the conserved residues at the active site, the motif "D[ST]GA" is present in all the sequences. The second position of the motif is serine in all parasitic sequences and in human Ddi1-like protein, being replaced by threonine in yeast (Fig. 1) and XRMV and HIV proteinases (Fig. 4, red letters).

Expression and functional characterization of the *L. major* recombinant Ddi1-like protein

A recombinant protein was resolved as a single band with an expected size around of 42 kDa in SDS-PAGE reducing conditions (Fig. 2a). The proteolytic activity of the *L. major* recombinant Ddi1-like enzyme was analyzed against RE-(EDANS)-SQNYPIVQK-(DABCYL)-R, a synthetic substrate for HIV proteinase. The peptide was hydrolyzed by the enzyme at pH5, with an apparent K_m of 0.575± 0.288 μM and an apparent V_{max} of 53.76±1.60 FUA/min (Fig. 2b). The synthetic substrate was hydrolyzed most thoroughly in the range pH4–6.5, with an optimum at pH5 (Fig. 2c). This result provided the first direct evidence that the Ddi1-like protein family is capable of proteolytic activity. Furthermore, proteinase inhibitors were tested against recombinant Ddi1-like *L. major* aspartyl proteinase activity. Pepstatin (15 μM) and DAN (500 μM) produced 70 and 95 % inhibition, respectively, while Nelfinavir (20 μM) inhibited by 60 % (Fig. 2d). The other inhibitors tested, E-64 (cysteine proteinase inhibitor) and AEBSF (serine proteinase inhibitor), had no effect on the *Leishmania* proteolytic activity. Hydrolysis of substrates in the aspartyl proteinase A_1 family was also studied. Two synthetic substrates specific for cathepsin D, Bz-RGFFL-4MβNA, and Bz-RGFFP-4MβNA, were hydrolyzed by the *L. major* recombinant Ddi1-like protein (Table 1) although with lower affinity than that for RE-(EDANS)-SQNYPIVQK-(DABCYL)-R. This activity was inhibited by pepstatin and DAN.

Degradation of BSA by the *L. major* recombinant Ddi1-like protein

To study the ability of the *L. major* recombinant Ddi1-like protein to degrade macromolecules, protein digestion analyses were performed. Undigested BSA (control) typically showed one main polypeptide, a 66.77-kDa band in nonreducing SDS-PAGE (Fig. 3). At pH4, the recombinant *L. major* Ddi1-like molecule degraded BSA (Fig. 3).

Fig. 1 Multiple sequence alignment of *Ddi1* sequences from *L. major*, *L. infantum*, *L. mexicana*, *L. braziliensis*, *T. cruzi_CLBrener*, *P. falciparum*, yeast, and human. Nucleotide and protein database accession codes for all sequences are given as "Electronic supplementary material". Extra N-terminal sequences newly translated for *L. major*, *L. infantum*, and *L. mexicana*, and not included in the protein databases, are colored in *blue*. Number in parentheses after the last residue in the alignment indicates the identity percentage of each sequence compared to Ddi1 of *L. major*. Secondary structure (*H* helix, *E* strand) has been predicted for *L. major* Ddi1 using homology modeling techniques for UBL, RP, and UBA domains (PDB ID: 1V5O, 2I1A and 2G3Q, respectively), and JPred3 server (42) for the "helix-rich" and "coil" domains (residues 58–147 and 310–346, respectively). Residues of the active site motif "D[ST]GA" are in *red*

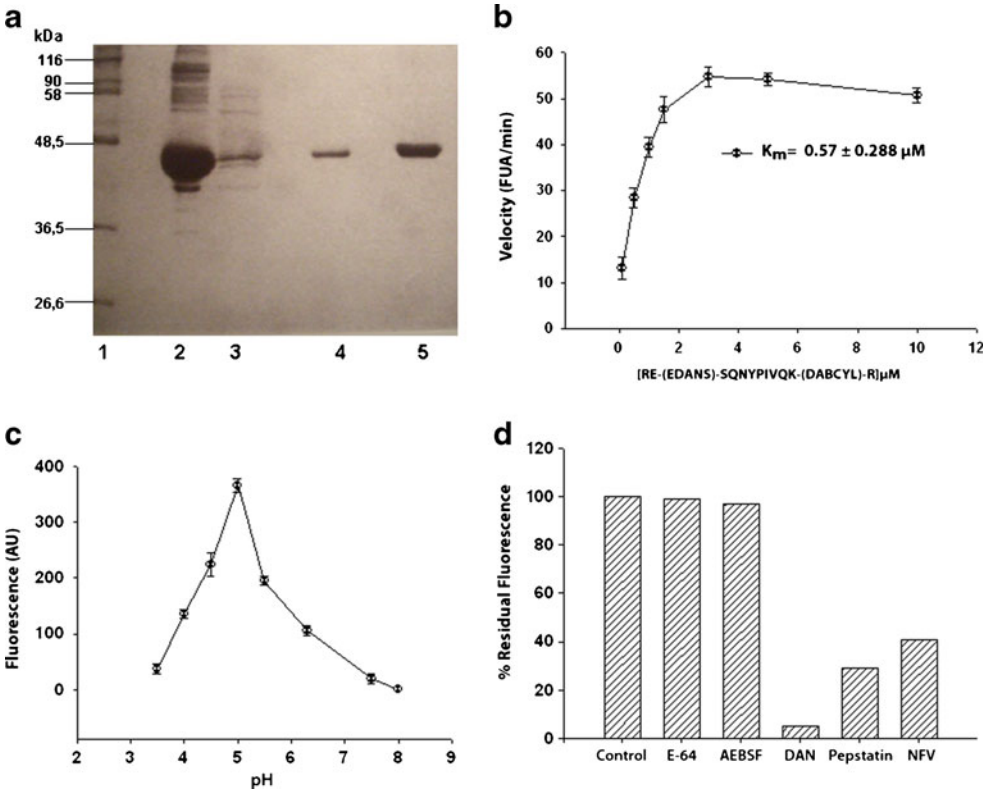

Fig. 2 Purification and characterization of the *L. major* functionally active Ddi1-like protein using the synthetic substrate RE-(EDANS)-SQNYPIVQK-(DABCYL)-R. **a** Purification of recombinant protein was monitored by 12.5 % SDS-PAGE, under nonreducing conditions Molecular weight markers (*1*); total freeze–thaw extract of insect cells infected with baculovirus carrying the *DDI1*-like gene (*2*); soluble supernatant extract of recombinant insect cells (*3*); and purified recombinant protein preparations eluted from Ni-NTA-agarose column on an ÅKTA FPLC system (*4–5*). **b** Kinetics of substrate degradation by recombinant *L. major* Ddi1-like protein. **c** Effect of pH on substrate hydrolysis by recombinant *L. major* Ddi1-like protein. **d** Effect of different proteinase inhibitors: 10 µM E-64, 500 µM DAN, 15 µM pepstatin, 20 µM Nelfinavir and 5 µM AEBSF on recombinant *L. major* Ddi1-like protein activity. Results are expressed as mean±standard deviation of three independent experiments. *FAU* fluorescence arbitrary units

Structural model of the RP domain of the *L. major* Ddi1-like protein

A 3D model for the RP domain of the *L. major* Ddi1-like protein was generated using homology modeling procedures based on sequence comparison between this protein and its close crystallized homolog, the dimerized RP domain of *Saccharomyces* Ddi1 (PDB ID: 2I1A; Sirkis et al. 2006). The modeled dimer (Fig. 4) has a double Asp-Ser-Gly-Ala amino acid sequence motif in an almost identical geometry to that exhibited by the homologous RP domain of yeast Ddi1, and also by the related structures of the XMRV proteinase (PDB ID: 3NR6; Li et al. 2011) and HIV-1 proteinase (PDB ID: 5HVP; Pillai et al. 2001). As suggested by the sequence similarity and, as a consequence, the close resemblance between the RP domain of yeast Ddi1 and of the *L. major* Ddi1-like protein, we predict that the *L. major* Ddi1-like protein can accommodate bulkier substrates than those accessible to HIV-1 proteinase. The structure of XMRV proteinase occupies an intermediate situation. Apart from the similarities and differences in the accessibility of large substrates to the active center of the four compared structures (Fig. 4), it is remarkable the presence of an almost identical disposition of the residues in the active center (motif "D[ST]GA") among the three crystallized structures and the modeled one for *L. major* Ddi1-like. This fact

Table 1 Kinetic parameters for hydrolysis of aspartyl proteinase substrates by the *L. major* recombinant Ddi1-like protein

Substrate	Family aspartyl proteinase	$K_{m,}$ µM	pH optime	Inhibition by pepstatin
RE-(EDANS)-SQNYPIVQK-(DABCYL)-R	A_2 specific HIV proteinase	0.57±0.288	5	Yes
Bz-RGFFL-4MβNA	A_1 specific cathepsin D	7.6±2.5	4	Yes
Bz-RGFFP-4MβNA	A_1 specific cathepsin D	1.18±2.5	8	Yes

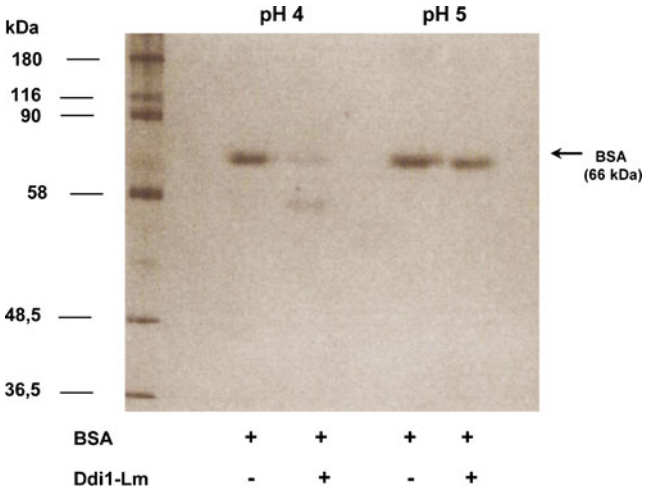

Fig. 3 Electrophoretic analysis of BSA degradation, at different pH values, by the recombinant *L. major* Ddi1-like protein. Samples were separated by 10 % SDS-PAGE under nonreducing conditions. Gel was stained with Coomassie Brilliant Blue

suggests that small proteinase inhibitors would be able to reach the active center in all the structures inhibiting their protease activity. This will explain the susceptibility of *L. major* Ddi1-like protein to common aspartyl proteinase inhibitors. Moreover, this common arrangement can be exploited in comparative studies regarding the effectiveness of HIV proteinase inhibitors on the activity of *L. major* Ddi1-like protein.

Discussion

We identify, clone, express, and characterize the complete *L. major* gene that encodes a protein classified as Ddi1-like. This is the first study to show in a direct fashion that a *Leishmania* protein from this family, which contains within their structure a domain homologous to an RP domain, can perform proteolytic activity. This finding can probably be extrapolated to the other proteins from this family that has an RP domain. Using molecular modeling, this study shows that there is a high degree of structural homology between the RP domains of the *L. major* Ddi1-like protein and that of the orthologous enzyme in *S. cerevisiae* (Sirkis et al. 2006). The RP domain of the parasite protein, just as with that from *S. cerevisiae*, folds to give rise to a homodimer which is characteristic of the A_2 aspartyl proteinases, in which each monomer provides one aspartic acid associated with the catalytic mechanism. Moreover, if we compare the RP domain of the *L. major* Ddi1-like protein with that of the retroviral proteinases, XMRV proteinase, and HIV proteinase, considerable structural homology can also be appreciated. However, the catalytic cavity of the RP domain in the parasite protein is larger than that of XMRV proteinase, which in turn is larger than that of HIV proteinase. All these observations, together with those derived from the model, suggested to us that the retroviral domain of the *L. major* Ddi1-like protein must be functional. Therefore, we expressed the complete parasite Ddi1-like protein in a baculovirus/insect cell eukaryotic expression system to confirm its activity.

The biochemical analysis revealed that the recombinant *L. major* Ddi1-like protein was able of hydrolyze specific aspartyl proteinase peptide substrates: RE-(EDANS)-SQNYPIVQK-(DABCYL)-R, which is selective for HIV proteinase that belong to the A_2 aspartyl proteinase family (Gershkovich and Kholodovych 1996; Sehajpal et al. 1999), and Bz-RGFFL-4MBNA and Bz-RGFFP-4MBNA, which are selective for cathepsin D, an enzyme from the A_1 aspartyl proteinase family (Smith and Van Frank 1975). It should be notice that despite the enzyme being able to hydrolyze the three substrates; it was most selective for the substrate from the A_2 aspartyl proteinase family. As we expected, specific aspartyl proteinase inhibitors blocked the hydrolysis of these substrates. DAN, which esterifies the aspartic acids presents in the catalytic center, completely inhibited the activity of the Ddi1-like protein on the three substrates, while pepstatin inhibited 70 % of the activity at 15 μM and NFV inhibited 60 % of the proteolytic activity at 20 μM. The concentration of NFV needed to inhibit the purified recombinant enzyme was slightly more than that reported using a soluble fraction of *L. mexicana* (Valdivieso et al. 2010) and raw extracts from *L. amazonensis* (Santos et al. 2009), although it was similar to that described using the soluble fraction of promastigotes from *L. infantum* (Valdivieso et al. 2010). To our surprise, with a structure containing part of the *L. major DDI1*-like gene inserted into an *S. cerevisiae* knockout, White et al. (2011a) report an IC_{50} of 0.44 μM for NFV. Substitutions of amino acid residues in the active center of the Ddi1-like proteins from the different species of *Leishmania* could, in part, explain the interspecies differences in sensitivity to antiretroviral drugs (White et al. 2011a). However, in principle, this could not be the reason for the differences observed for the *L. major* Ddi1-like protein. More efficient uptake of these drugs by the cells or an increase in the concentration of the inhibitor within them, could also partially explain the observed phenomenon. Nevertheless, a detailed study using recombinant Ddi1-like protein from the different species of *Leishmania* is necessary to establish the inhibition constants of the antiretroviral drug, and of other inhibitors from this family, in order to determine whether the differences in sensitivity observed depend on the species.

The aspartyl proteinases showed optimum hydrolytic activity between pH2.5 and 5. However, some members of this family, such as renin and the presenilins, show optimum activity at pH values near 7 (Davies 1990; Dunn 2002). In

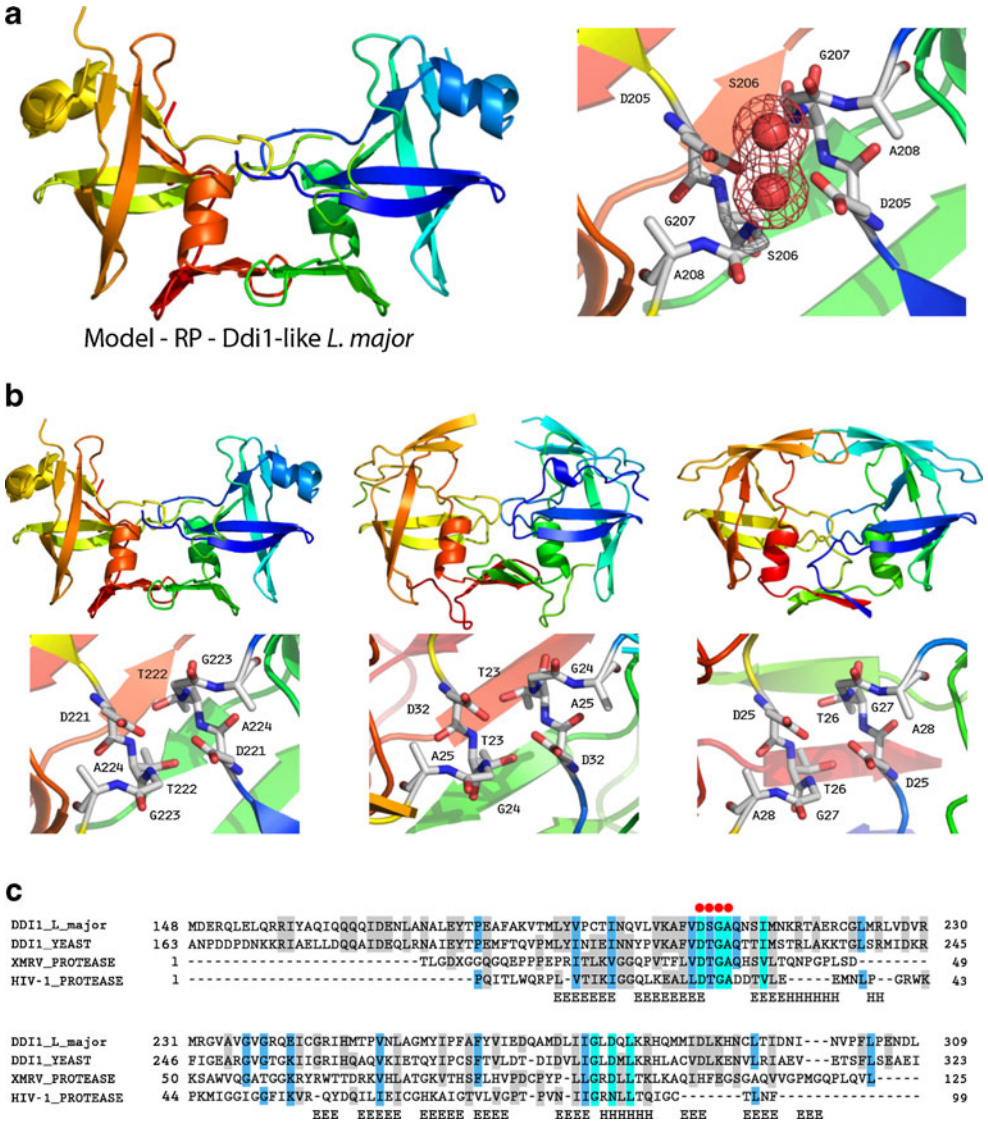

Fig. 4 3D structure model. **a** The dimerized RP domain of the Ddi1-like protein from *L. major*. The ribbon plot is colored *blue* to *red* from the N-terminal end of the RP domain of the first monomer to the C-terminal end of the RP domain of the second monomer. *Right* Detail of the active site of the RP dimer indicating the position of the double Asp-Ser-Gly-Ala amino acid sequence motif. *Red spheres* correspond to water molecules present in the original crystallized template and indicate the putative substrate position. **b** Crystallized RP domains from yeast Ddi1, XMRV proteinase, and HIV-1 proteinase are represented for comparison with the modeled RP domain of the Ddi1-like protein from *L. major*. **c** Multiple sequence alignment of RP domains of Ddi1 proteins from *L. major* (DDI1_L_major; Uniprot ID: E9AC52) and yeast (DDI1_YEAST; Uniprot ID: P40087) and proteinases from Xenotropic murine leukemia virus-related virus (XMRV_PROTEASE; Uniprot ID: P26808) and human immunodeficiency virus (HIV_PROTEASE; Uniprot ID: P20892). Identity percentages of sequences compared to *L_major* Ddi1 are: _YEAST Ddi1 (46.0 %), XMRV_PROTEINASE (14.8 %), HIV_PROTEINASE (18.2 %). Position of residues in the active site "D[ST]GA" is indicated (*red circles*)

the specific case of HIV proteinase, an optimum pH of around 6 has been reported; nevertheless, this could vary slightly depending on the synthetic substrate to be hydrolyzed. It has been reported that 5.5 is the optimum pH for activity with the peptide substrate RE-(EDANS)-SQNY-PIVQK-(DABCYL)-R (Gershkovich and Kholodovych 1996; Sehajpal et al. 1999). This value is very similar to the one we obtained in the present study for the hydrolysis of the same synthetic substrate by the recombinant *L. major* Ddi1-like protein; nevertheless, these range of pHs are not necessarily the same for the target substrates of this parasite enzyme (has not been identified yet).

In the same way, the recombinant *L. major* Ddi1-like protein was able to degrade BSA at pH4; a degradation that was inhibited by the presence of DAN, pepstatin and NFV. At pH5, no degradation of BSA was observed; probably a remnant of BSA degradation could still be present, but the technique sensibility does not allow its detection.

These results demonstrate the capacity of the parasite RP domain not only to act on polypeptides, but also on proteins;

and they are in keeping with the information derived from the model, which suggests that globular proteins of an intermediate size could gain access to or reside in the catalytic cavity of the RP of the *L. major* Ddi1-like protein. This observation could be very important for our understanding of the role that the molecule plays in the biology of the parasite, considering the functions that have been suggested for this family of proteins in other organisms, such as those associated with regulating the cell cycle.

It is important to note the capacity of the RP domain of the parasite Ddi1-like protein to function at different pH values, depending on the substrate to be digested. This plasticity could be very useful when it comes to *Leishmania* carrying out one or more functions, as it is also subjected to a set of natural conditions of stress during its life cycle (Zilberstein and Shapira 1994). Ddi1 shares with the retroviral proteases, in addition to an overall fold and active-site structure, the environment in which it functions. In contrast to most known aspartyl proteases, which cleave substrates in the extracellular milieu (i.e., the insect gut or the bloodstream) or in acidic intracellular compartments (i.e., the lysosome), the retroviral proteases and Ddi1 act in the cell cytoplasm (Diaz-Martinez et al. 2006; Gabriely et al. 2008). Although the biological role of Ddi1 has not been described in *Leishmania*, the possibility is open that this protein could be localized in the cytoplasm as it is homologous and also in other compartment.

The amino acid sequence deduced from the *L. major* Ddi1-like protein includes, in addition to the RP and UBA domains described in the original sequence (GenBank ID: XP_001687454.1), an additional fragment at the N-terminal end, which is homologous to the UBL domain of this family of proteins described in other organisms (Krylov and Koonin 2001), and which had not previously been reported for the *L. major* Ddi1-like protein (Krylov and Koonin 2001; White et al. 2011a). We have obtained the initial ATG using a more complete sequence comparison including a broad representation of related proteins from *L. braziliensis*, *T. cruzi*, *P. falciparum*, yeast, and human, where the position of the initial ATG was located allowing the translation of a common N-terminal domain, shared by all the known Ddi1 proteins. Additionally, as expected the enzymatic activity is detected only when the protein is translated containing the complete sequence, starting from the newly proposed ATG codon.

It is assumed that some of the functions that these molecules perform (which may include marking proteins for their subsequent recognition by proteosomes, control of the cell cycle and suppression or blocking of protein secretion) are mediated by the domains UBL and UBA described (Diaz-Martinez et al. 2006; Gabriely et al. 2008; White et al. 2011b). However, the role that this protein plays in *Leishmania* is still unknown. Previous studies have shown that selective aspartyl proteinase inhibitors inhibited the proliferation of *Leishmania sp.* promastigotes and amastigotes in vitro, possibly in cytokinesis (Valdivieso et al. 2010). These observations would fit in with a possible role that the protein we have now cloned may play in the regulation of the cell cycle of the parasite, either in the marking of proteins with ubiquitin or in the control of the cell cycle, particularly those that occur in the metaphase-to-anaphase transition or those that occur at the end of mitosis. It has been shown that in *S. cerevisiae* both the UBL and the RP domains are involved in these functions (Krylov and Koonin 2001; Diaz-Martinez et al. 2006; Gabriely et al. 2008; White et al. 2011a, b), while the UBA domain seems to be associated with regulation events in the S stage of the cell cycle (Diaz-Martinez et al. 2006).

The presence of aspartyl proteinases in protozoa from the Trypanosomatidae family was only postulated less than a decade ago (Valdivieso et al. 2007; Pinho et al. 2009; Santos et al. 2009), leading to the suggestion that they may play a role in the proliferation of the *Leishmania* parasites (Valdivieso et al. 2007; Santos et al. 2009; Valdivieso et al. 2010; White et al. 2011a). In the present study, we cloned the full *L. major* sequence of a molecule that belongs to a *Leishmania* family of proteins that include an active RP domain, characteristic of the aspartyl proteases from the family A_2. This molecule has two other domains (UBL, described in this work for the first time, and UBA) associated with the regulation of the cell cycle in orthologous proteins from several eukaryotic organisms. These results will allow us to gain more extensive knowledge of the properties of this protein in different species of *Leishmania* and to analyze its function in protozoa, with the possibility of using it as a target for new antileishmanial drugs.

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