Experimental Parasitology 127 (2011) 752–761

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Leishmania mexicana: LACK (*Leishmania* homolog of receptors for activated C-kinase) is a plasminogen binding protein

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ARTICLE INFO

Article history:
Received 3 September 2010
Received in revised form 8 December 2010
Accepted 17 January 2011
Available online 25 January 2011

Keywords:
LACK
Plasminogen
Leishmania mexicana

ABSTRACT

Leishmania mexicana is able to interact with the fibrinolytic system through its component plasminogen, the zymogenic form of the protease plasmin. In this study a new plasminogen binding protein of this parasite was identified: LACK, the *Leishmania* homolog of receptors for activated C-kinase. Plasminogen binds recombinant LACK with a K_d value of 1.6 ± 0.4 μM, and binding is lysine-dependent since it is inhibited by the lysine analog ε-aminocaproic acid. Inhibition studies with specific peptides and plasminogen binding activity of a mutated recombinant LACK have highlighted the internal motif $_{260}$VYDLESKAV$_{268}$, similar to those found in several enolases, as involved in plasminogen binding. Recombinant LACK and secreted proteins, in medium conditioned by parasites, enhance plasminogen activation to plasmin by the tissue plasminogen activator (t-PA). In addition to its localization in the cytosol, in the microsomal fraction and as secreted protein in conditioned medium, LACK was also localized on the external surface of the membrane. The results presented here suggest that LACK might bind and enhance plasminogen activation *in vivo* promoting the formation of plasmin. Plasminogen binding of LACK represents a new function for this protein and might contribute to the invasiveness of the parasite.

1. Introduction

Leishmania parasites are the causal agents of a group of clinical manifestations known collectively as Leishmaniasis. During their life cycle, these parasites alternate between invertebrate host (vector) and mammalian host invading macrophages and reproducing inside phagolysosomes. Infection by *Leishmania* and establishment of the parasite in the mammalian host is known to depend on multiple factors, of which invasive/evasive molecular determinants are key elements (Chang and McGwire, 2002). These invasive/evasive determinants are usually found on the cell surface of the parasite and/or are secreted (Chang and McGwire, 2002; Naderer et al., 2004). Possible molecular determinants in *Leishmania* for invasion and/or establishment in mammalian hosts could be plasminogen binding proteins that allow interaction of the parasite with the fibrinolytic system.

Plasminogen is the zymogenic form of the serine-protease plasmin and is found in plasma and extracellular fluids (Vassalli et al., 1991). In several bacterial pathogens, the acquisition and activation of plasminogen is linked to invasiveness and pathogenecity (Lähteenmäki et al., 2005; Walker et al., 2005; Sun, 2006) and this recruitment of plasminogen is generally due to the presence of several plasminogen binding receptors (Miles et al., 2005). On the surface of the pathogen, plasminogen is transformed into plasmin either by host plasminogen activators or by the pathogen's own activator. This acquired protease can degrade extracellular matrix proteins and fibrin. This latter is part of the host defense against infections (Sun, 2006). Parasites are also among the pathogens that interact with plasminogen (Avilan et al., 2000; Jolodar et al., 2003; Bernal et al., 2004; Almeida et al., 2004; Ramajo-Hernández et al., 2007; Marcilla et al., 2007; Mundodi et al., 2008). In the case of *Leishmania mexicana* this interaction has been previously characterized (Avilan et al., 2000; Calcagno et al., 2002) and contributes to virulence of the parasite (Maldonado et al., 2006). In addition, enolase was identified as plasminogen binding protein on the surface of *L. mexicana* (Vanegas et al., 2007).

In this study we report that LACK (*Leishmania* homolog of receptors for Activated C-kinase) is another plasminogen binding protein in *L. mexicana*. LACK is an analog of the RACK (receptor for activated C-kinase) proteins present in eukaryotes that are members of the family of WD40 repeat proteins (Neer et al., 1994). RACKs are known for their function as stabilizer of the

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0014-4894/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2011.01.008

active form of protein kinase C. In addition these proteins mediate protein–protein interactions serving as adaptors for several multicomplex proteins involved in signaling pathways (Schechtman and Mochly-Rosen, 2001). The molecular function of LACK in *Leishmania* is not clear although the immunological response to this molecule has been well studied (Launois et al., 2007) and used for experimental vaccine studies in the mouse model (Coler and Reed, 2005). It has been clearly demonstrated that LACK is essential for the viability of the parasite and to establish the parasite in the host. LACK mutants of *Leishmania major* with diminished levels of this protein fail to develop lesions in susceptible mice and have reduced capacity to reproduce in macrophages *in vitro* (Kelly et al., 2003). In *Trypanosoma brucei*, the RACK1 homolog is required for cytokinesis (Rothberg et al., 2006). In addition to its cytoplasmic localization (Gonzalez-Aseguinolaza et al., 1999; Taladriz et al., 1999; Kelly et al., 2003), LACK was recently found to also be actively secreted (Silverman et al., 2008, 2010; Cuervo et al., 2009), this secretion occurring via exosomes (Silverman et al., 2010). All these findings suggest that LACK could have several functions, its plasminogen binding capacity representing a novel function of this protein. This function could be important in *Leishmania*–host interaction.

2. Materials and methods

2.1. Parasites and culture conditions

The AZV strain of *L. mexicana* (Pérez et al., 1979) was used in this study. Promastigotes were cultured at 28 °C with gentle shaking in Schneider's medium supplemented with 20% heat-inactivated fetal bovine serum. Promastigotes were harvested by centrifugation (2000*g* for 15 min) and washed in phosphate buffered saline (PBS). In all cases, promastigotes in the logarithmic phase of their growth were used.

2.2. Proteins, peptides and antibodies

Human plasminogen was purified to homogeneity from human blood according to the method of Deutsch and Mertz (1970). Tissue plasminogen activator (t-PA) and urokinase were obtained from Chromogenix (Italy) and Choay Laboratory (France), respectively. The peptides H-VYDLESKAV-NH_2, H-SWDNTIKVW-NH_2, and H-VYALGSLAV-NH_2 were synthesized by a solid-phase approach using the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl (t-Bu) strategy using a Fmoc-Rink-resin (Lloyd-Williams et al., 1997). The purity of the peptides (95%, 93% and 93%, respectively) was established by analytical high-performance liquid chromatography (HPLC). These peptides were dissolved in dimethyl sulfoxide (DMSO). The commercial antibodies used were mouse anti-human α-tubulin (Sigma, USA), goat anti-rabbit IgG conjugated with either alkaline phosphatase (Sigma), peroxidase (Sigma) or Cy3 dye (Amersham Biosciences, Sweden) and goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma). The preparation of rabbit anti-human plasminogen and anti-*L. mexicana* enolase has been described (Vanegas et al., 2007; Quiñones et al., 2007). Mouse anti-*L. mexicana* gp63 was obtained previously by intraperitoneal injection with 50 μg of recombinant gp63 using aluminum (Rehydragel HPA, USA) as adjuvant. The serum was collected after five booster injections. Anti-*T. cruzi* PGK (phosphoglycerate kinase C) was used to detect this protein in *L. mexicana*.

2.3. Recombinant LACK, site-directed mutagenesis and purification

The LACK gene (GenBank accession number AF363976) was amplified by PCR from genomic DNA. The forward primer was 5′_GC**CATATG**AACTACGAGGGTCACCTGAA_3′ (*Nde*I site in bold) and the reverse primer was 5′_CG**GAATTC**TTACTCGGCGTCGGAGATG_3′ (*Eco*RI site in bold). The amplification mixture (50 μl) contained 1 μg of genomic DNA, 1 μM of each primer, 200 μM of each of the four deoxynucleotides, 1.5 mM $MgCl_2$ and 1.5 U of DNA polymerase (Go Taq Flexi DNA polymerasa, Promega, USA) with the corresponding PCR buffer. PCR was performed as follows: an initial incubation at 94 °C for 2 min, 36 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 90 s; the final incubation was at 72 °C for 10 min. The PCR product was ligated into the pGEM-T vector (Promega), cloned and sequenced using the T7 and SP6 primers in an automated sequencer. The gene was then transferred to the pET28a vector (Novagen, Germany) and used to transform the *Escherichia coli* strain BL21(DE3)pLys for the production of the recombinant proteins with an N-terminal 20 residue-long extension containing a poly-His-tag. The strain harboring the expression plasmids was grown at 25 °C for 48 h in ZYM-5052 autoinduction medium (Studier, 2005), supplemented with 33 μg/ml kanamycin and 34 μg/ml chloramphenicol, to $O.D_{600nm}$ values of 10. The bacteria from 50 ml culture were harvested by centrifugation, washed with PBS, resuspended in 20 ml lysis buffer (50 mM potassium phosphate K_2HPO_4:KH_2PO_4, pH 8 and 0.5 M NaCl) in the presence of a cocktail of protease inhibitors (Sigma) and broken by sonication. The lysate was then centrifuged at 12,000*g* for 15 min at 4 °C and the supernatant was applied onto a HisLink resin (Promega) column equilibrated with lysis buffer. After washings, LACK was eluted with 50 mM potassium phosphate buffer pH 6 and 0.5 M NaCl containing 200 mM imidazole. The imidazole was removed from the samples using a PD-10 column (Amersham Biosciences). The purity of LACK was checked by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue R-250 staining. For molecular mass determination of the recombinant protein, gel filtration was performed on a Sephadex G-75 column (76 × 1.4 cm) equilibrated with PBS.

Site-directed mutagenesis of the LACK gene was performed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, USA) following the manufacturer instructions. The gene cloned in the pET28a plasmid was used as template with the individual mutagenic primers. Two recombinant mutated LACK proteins were constructed: LACKseq1 and LACKseq2 using one pair of complementary oligonucleotide primers for each mutant protein. LACKseq1 containing substitutions at positions 262(Asp → Ala) and 266(Lys → Leu) (forward primer: 5′_CTGTCCGTGTACG<u>C</u>CCTCGAGAGC<u>CT</u>GGCCGTGATTGC_3′) and LACKseq2 containing substitutions at positions 173(Asp → Ala) and 177(Lys → Leu) (forward primer: 5′_GCCAGCTGGG<u>C</u>CAACACCATC<u>CT</u>GGTATGGAATGTGAACG_3′). DNA sequence analyses were performed to confirm the mutations introduced using the T7term primer. The mutated recombinant proteins were expressed in *E. coli* strain BL21(DE3)pLys by cell growth in autoinduction medium as specified above.

2.4. Anti-L. mexicana LACK antibodies

Polyclonal antiserum against recombinant LACK was raised in a rabbit by intradermic injection with 200 μg of purified protein in complete Freund's adjuvant for the first inoculation and for four booster injections, at 2-week intervals, using incomplete Freund's adjuvant. The rabbit was bled by cardiac puncture 2 weeks after the last injection. The anti-LACK antibodies were purified using affinity chromatography. Recombinant LACK was coupled to Affigel 10 resin (Bio-Rad, USA) according to manufacturer's instructions and chromatography was carried out using the serum proteins precipitated with ammonium sulfate (50% saturation). After incubation with the resin and washing steps in PBS, the antibodies