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The lysosomal gp63-related protein in *Leishmania mexicana* amastigotes is a soluble metalloproteinase with an acidic pH optimum

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Leishmania mexicana amastigotes express a lysosomal protein, which is antigenically related to the promastigote surface metalloproteinase (gp63). It is shown that the purified gp63-related protein from amastigote is also an active metalloproteinase. The pH-optimum of the enzyme is acidic, similar to lysosomal cysteine proteinases, but distinct from the neutral to basic pH-optimum of the promastigote surface proteinase. This study appears to be the first report on a metalloproteinase with a lysosomal localization.

Metalloproteinase; Cysteine proteinase; Lysosome; *Leishmania mexicana*

1. INTRODUCTION

Leishmania are dimorphic parasitic protozoa which live as flagellated promastigotes in the digestive tract of sandflies and as nonflagellated amastigotes in the phagolysosomes of mammalian macrophages. Like many other parasites, *Leishmania* contain large amounts of proteolytic enzymes, which are believed to be important virulence factors [1–3]. In the promastigote stage, the surface expression of an abundant glycosylphosphatidylinositol (GPI)-anchored metalloproteinase, called glycoprotein 63 (gp63) or promastigote surface proteinase, is well established for all *Leishmania* species investigated (see [4] for review). In contrast, expression of related proteins in the amastigote stage appears to be species-specific. In *L. major* amastigotes, expression of the enzyme is repressed to a level < 0.3% of that in promastigotes [5]. In *L. mexicana* amastigotes, expression of gp63-related genes has been demonstrated at both the mRNA [6] and the protein level [7,8]. An early study showed that the majority of gp63-related molecules cannot be labeled by surface iodination and is devoid of a GPI-membrane anchor [7]. More recent experiments suggest that the amastigote protein has hydrophilic properties and is localized in the extended lysosomes of *L. mexicana* [8], which are likewise known to be a rich source of cysteine proteinases (see [2] for review). These observations are consistent with a differential expression of mRNAs derived from a family of gp63 genes [9]. Transcripts found predominantly in promastigotes predict a hydrophobic sequence at the COOH-terminus serving as a GPI-anchor addition signal while the otherwise highly homologous mRNA in amastigotes codes for a COOH-terminal extension lacking this signal. Therefore, the membrane-bound promastigote protein is routed to the cell surface while the soluble amastigote protein is somehow delivered to lysosomes.

In this study the gp63-related protein and lysosomal cysteine proteinases of *L. mexicana* amastigotes were purified and their proteolytic activity was compared to the promastigote surface proteinase. The amastigote gp63-related protein is shown to be a soluble metalloproteinase with a pH-optimum similar to the cysteine proteinases but distinct from that of the promastigote surface metalloproteinase.

Abbreviations GPI, glycosylphosphatidylinositol; gp63 glycoprotein with a molecular mass of 63 kDa; hgp63, promastigote gp63 devoid of the diacylglycerol membrane anchor; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate, HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CRD, cross-reacting determinant.

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2. MATERIALS AND METHODS

2.1. *Parasites*

L. mexicana (MNYC/BZ/62/M379) amastigotes were isolated from dorsal lesions of BALB/c or CBA mice as previously described [8].

2.2. *Antibodies*

An antiserum against the promastigote surface metalloproteinase lacking the diacylglycerol residue of the GPI-anchor (hgp63) was raised as described earlier [8]. Antibodies against the cross-reacting determinant of the *Trypanosoma brucei* variant surface glycoprotein were a kind gift from R. Bülow.

2.3. *Purification of amastigote gp63-related protein and cysteine proteinases*

Amastigotes (1×10^{10}, 13 mg protein) were lysed by sonication in

100 mM Na-acetate, pH 5.5, 20 mM DTT without the addition of proteinase inhibitors and centrifuged for 1 h at 100,000 × g. The procedure was repeated with the pellet The combined supernatants were subjected to prolonged autolysis for 3 h at 37°C. After concentration by ultrafiltration (Centricon 10, Amicon, Witten, Germany) to about 200 ml, the sample was loaded onto a Superose 12-column run in a FPLC-system (Pharmacia, Freiburg, Germany). Chromatography was performed at room temperature using 50 mM Na-acetate, pH 5.0, 200 mM NaCl as elution buffer (0.5 ml/min). Collected fractions were analysed for gp63-related protein by dot-blot-ELISA [10] using anti-hgp63-antiserum, for proteinases by the azocaseinase assay and for protein by SDS-PAGE. Fractions containing anti-hgp63-reactive components were pooled and concentrated by ultrafiltration. Cysteine proteinases were purified as previously described [11] with minor modifications. The cysteine proteinase-containing Superose 12-column fractions were pooled, concentrated by ultrafiltration and loaded onto a Mono-Q column (Pharmacia), equilibrated with 20 mM Tris-Cl, pH 7.5. Bound proteinases were eluted with a salt gradient (0–300 mM NaCl, 20 ml; 300–500 mM NaCl, 5 ml, 1 ml/min). The eluate was analyzed for proteinase activity using azocaseine as a substrate and by SDS-PAGE. Active fractions were pooled and concentrated by ultrafiltration.

2.4. *Determination of proteinase activity*

Samples (2.5–20 μl) were added to 400 μl 100 mM Na-acetate, pH 5.5, 20 mM DTT, 4 mg/ml azocaseine (Sigma, Deisenhofen, Germany) and incubated at 37°C for 1–2 h. Thereafter, azocaseine was precipitated by the addition of 400 μl cold 5% trichloroacetic acid and the samples were incubated on ice for 30 min After centrifugation, the absorbance of the peptide-containing supernatant was measured at 366 nm. For inhibition experiments either 20 μM leupeptin, 10 mM iodoacetamide or 10 mM *ortho*-phenanthroline was added to the incubation mixture and the DTT-concentration was lowered to 1 mM. For assays of metalloproteinase activity, DTT was omitted from the buffers. pH-optima were determined in 50 mM HEPES, 50 mM boric acid, 50 mM citric acid, 50 mM Na_2HPO_4 containing 4 mg/ml azocaseine, titrated to the appropriate pH using NaOH.

3. RESULTS AND DISCUSSION

3.1. *Purification of amastigotes gp63-related protein and cysteine proteinases*

Unless cysteine proteinases were inhibited during preparation of an amastigote lysate, extensive proteolysis of amastigote proteins resulted in the SDS-PAGE pattern shown in Fig. 1 (lane 1, left panel; compare [12]). The prominent band at 24 kDa corresponded most likely to the cysteine proteinase encoded by the *lmcb* gene [13] as shown by immunoblot analysis using antibodies affinity-purified from a rabbit anti-amastigote antiserum on the recombinant *lmcb* gene product expressed in *E. coli* (T. Ilg and M. Wolfram, unpublished results). This proteinase was found in the soluble fraction of the lysate (lanes 2 and 3). In addition (right panel), the lysate (lane 1) and the soluble fraction (lane 2) contained gp63-related proteins. On overloaded gels, small amounts of these proteins were detectable in the washed membrane fraction (not shown).

For purification of the proteinases, the extract was first subjected to prolonged autolysis to degrade contaminating cellular proteins and then chromatographed on a Superose 12 column (Fig. 2). A minor peak of proteinase activity in the 60–70 kDa range (fractions 20 to 28) contained protein reactive with the anti-hgp63 antiserum (panel A). These fractions gave rise to several bands around an apparent molecular weight of 67,000 (panel B). The yield of amastigote gp63-related molecules was 40–50 μg/10^{10} cells (approx. 0.3–0.4% of the cellular protein, corresponding to about 50,000 mole-

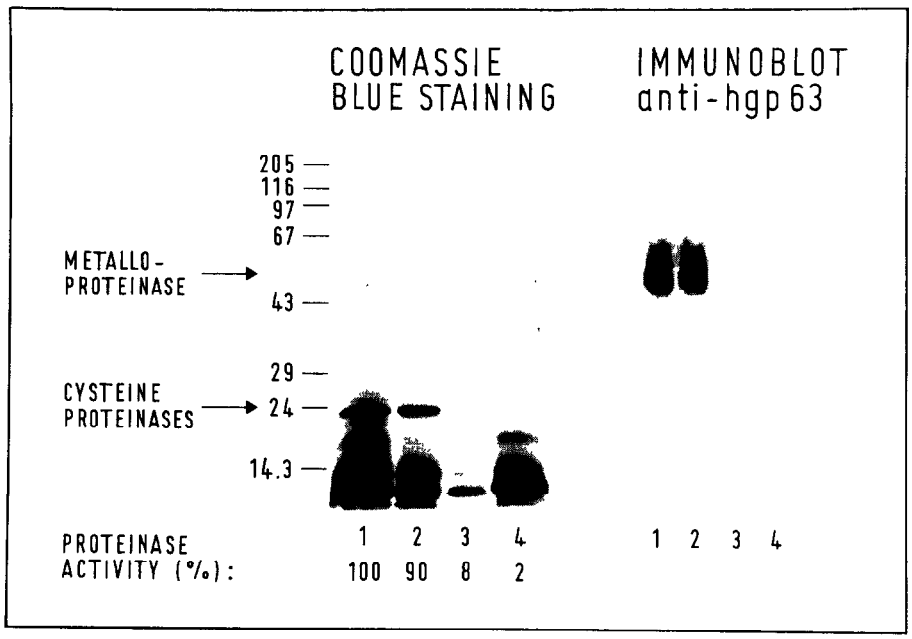

Fig 1. SDS-PAGE analysis of *L. mexicana* amastigote lysate (lanes 1), the 100,000 × *g* supernatant subjected to autolysis (lanes 2), the supernatant of washed membranes (lanes 3) and the membrane fraction (lanes 4). On the left, the gel was stained with Coomassie blue; the right panel shows a blot probed with anti-hgp63 antibodies. Samples equivalent to 4×10^7 amastigotes were applied. The molecular size of standard proteins in kDa, the location of amastigote proteinases and the distribution of total proteinase activity (bottom) are indicated.

cules/cell). This copy number is 3–4 times higher than previous estimates involving metabolic labeling and immunoprecipitation [7,8].

The main peak of proteolytic activity eluting from the Superose 12 column contained the cysteine proteinases which were purified to homogeneity by additional Mono-Q chromatography. The product showed a predominent 24 kDa protein and a smaller amount of a 27 kDa protein (Fig. 3, lane 7). About 450 μg of cysteine proteinase were obtained from 10^{10} amastigotes corresponding to 3.5% of the cellular protein or about 1,200,000 molecules/cell.

3.2. *Comparison of the amastigote gp63-related protein and the promastigote surface metalloproteinase*

Upon SDS-PAGE, gp63-related molecules purified from amastigotes gave rise to about five separate bands between 50 and 60 kDa and a faint band near 190 kDa when the samples were neither heated nor reduced (Fig. 3, lane 2, left panel). Upon heating and reduction, the 190 kDa band disappeared and the bands shifted to a molecular weight range of 60–71 kDa (lane 1). This result agrees with the mobility of the proteins obtained by immunoprecipitation of lysates from metabolically labeled amastigotes with anti-gp63 antibodies [7,8]; in these cases, lysates were prepared in the presence of cysteine proteinase inhibitors. Therefore, gp63-related proteins in amastigotes appear not to be subject to proteolytic modification during purification.

Anti-hgp63 antiserum strongly recognized amastigote gp63-related protein on immunoblots when the samples were not heated and not reduced but only

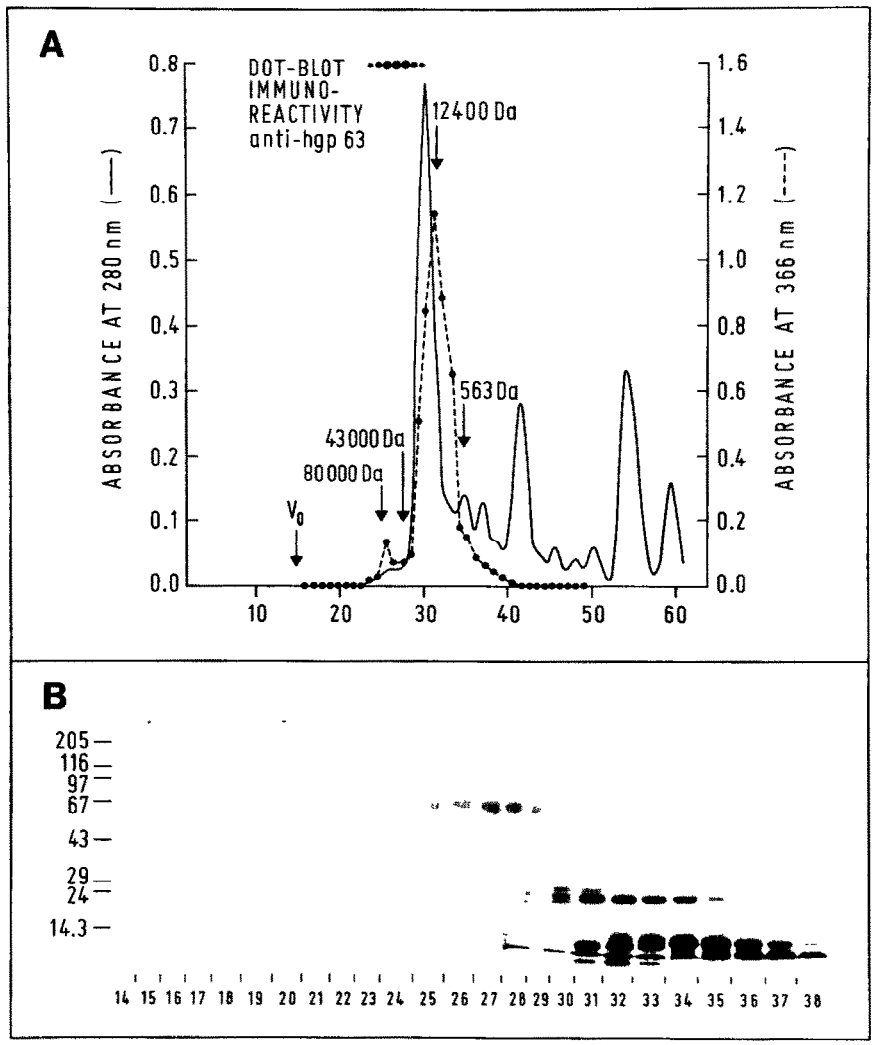

Fig. 2. (A) Superose 12 chromatography of soluble amastigote proteins after autolysis and ultrafiltration. Protein was detected by continuous measurement of the absorbance at 280 nm. Proteinase activity of the fractions was determined by azocaseinase assay (absorbance at 366 nm). Fractions containing gp63-related proteins were identified by dot-blot immunoassay. The column was calibrated using dextrane blue (>2,000,000 Da, void volume V_0), human transferrin (80,000 Da), ovalbumin (43,000 Da), cytochrome *c* (12,400 Da) and CTP (563 Da). (B) Proteinase-containing Superose 12 fractions analyzed by SDS-PAGE and staining with Coomassie blue. The molecular size of standard proteins in kDa are indicated.

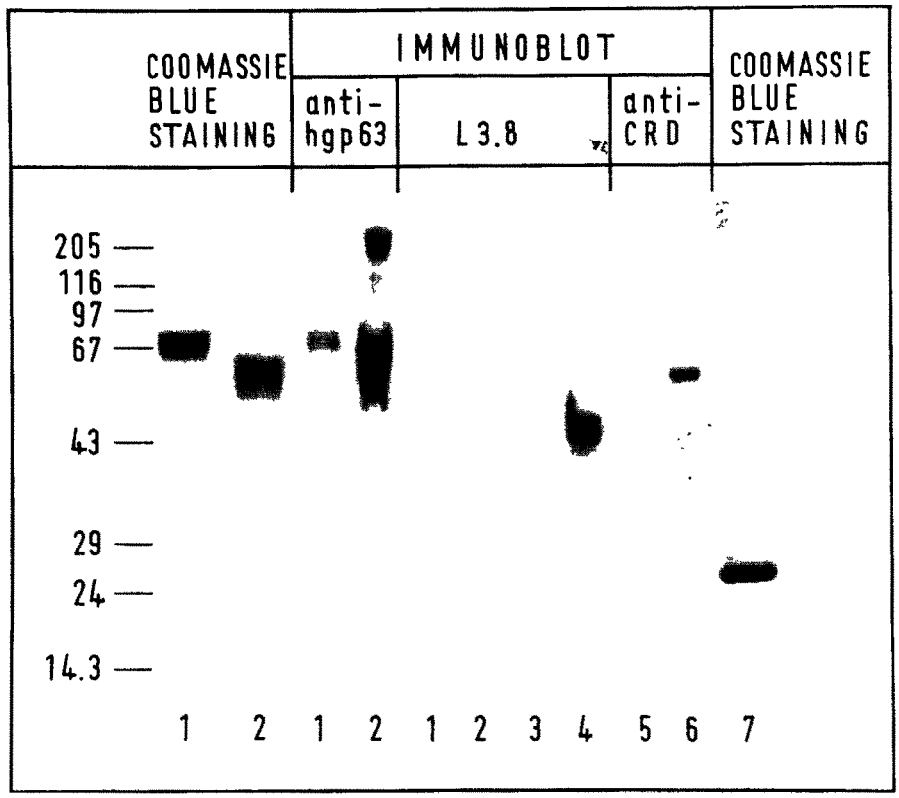

Fig. 3. SDS-PAGE/immunoblot analysis of purified amastigote and promastigote metalloproteinase and cysteine proteinases. (Lanes 1) 1.5 μg amastigote gp63-related protein, heated and reduced; (lanes 2) 1 5 μg amastigote gp63-related protein, not heated and not reduced; (lane 3) 1.5 μg promastigote metalloproteinase lacking the glycosylphosphatidylinositol membrane anchor, heated and reduced, (lane 4) 1 5 μg promastigote metalloproteinase lacking the GPI membrane anchor, not heated and not reduced; (lane 5) 1.5 μg amastigote gp63-related protein, heated and reduced; (lane 6) 1.5 μg promastigote metalloproteinase lacking the GPI membrane anchor, heated and reduced; (lane 7) 2 μg cysteine proteinases, heated and reduced. Gels were either stained with Coomassie blue or derived immunoblots were probed with anti-hgp63 antiserum, monoclonal antibody L3.8 or with antibodies directed against the cross-reacting determinant (CRD) as indicated. The size of standard proteins in kDa is indicated.

weakly after this treatment. Monoclonal antibody L3.8 [7] only recognized unheated and unreduced gp63 from both amastigotes or promastigotes (Fig. 3, center panel). These results indicated that L3.8 as well as the vast majority of the antibodies in the polyclonal antiserum recognized conformational epitopes. Finally, soluble *L. major* promastigote surface metalloproteinase (hgp63) derived from the GPI-anchored membrane form by phospholipase C cleavage was reported to carry the cross-reacting determinant (CRD) [14,15]. *L. mexicana* promastigote hgp63 was likewise recognized by these antibodies, whereas the soluble amastigote gp63-related molecules did not react with this reagent (Fig. 3). Formal proof for the presence of an extended COOH-terminus as predicted from the cDNA sequence [9] rather than a GPI anchor on the amastigote protein requires additional experiments.

The purified amastigote gp63-related protein was proteolytically active against azocaseine as substrate. The proteolytic activity was not inhibited by leupeptin and iodoacetamide, but completely abrogated by *ortho*-phenanthroline, an inhibition pattern identical to that of the promastigote surface metalloproteinase. In contrast, purified cysteine proteinases were not influenced by *ortho*-phenanthroline but strongly inhibited by leupeptin and iodoacetamide, which indicates that the activity of the amastigote metalloproteinase is not due to contaminating cysteine proteinases (Fig. 4A). The promastigote surface metalloproteinase showed a broad activity optimum against azocaseine between pH 7 and 9, whereas the pH-optimum of the lysosomal amastigote metalloproteinase was shifted to the acidic range showing a peak near pH 5.5–6, very similar to the pH-optimum of the cysteine proteinases (Fig. 4B).

4. CONCLUSIONS

L. mexicana amastigotes express a soluble, lysosomal metalloproteinase at 1/10 to 1/3 of the level of the GPI-anchored promastigote surface protease. These observations are consistent with the properties of the proteins predicted to be preferentially expressed in either of the two stages [4]. The sequences share the motives characteristic for Zn^{2+} proteinases but subtle differences must

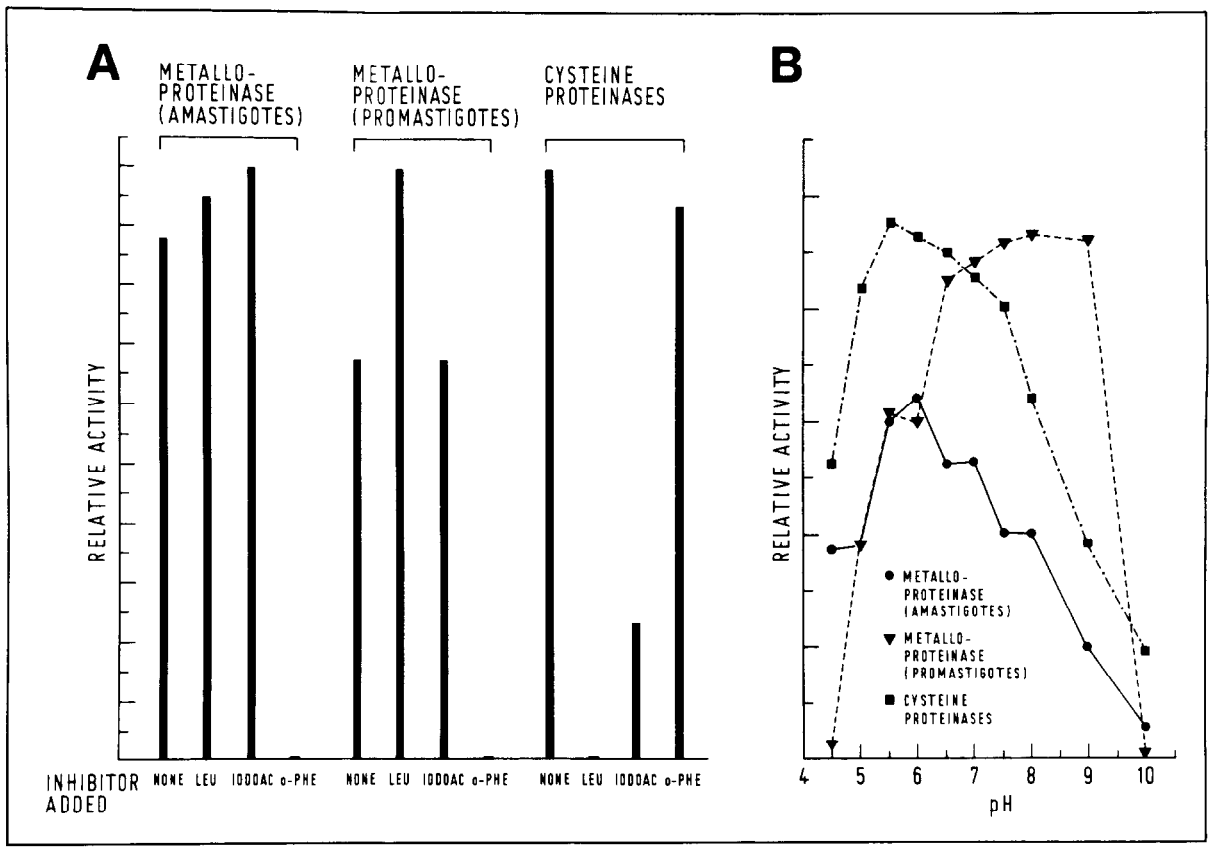

Fig. 4. (A) Sensitivity to proteinase inhibitors: LEU, leupeptin; IODOAC, iodoacetamide; o-PHE, *o*-phenanthroline. (B) pH-dependence of proteinase activity using azocasein as substrate.

modify their pH optimum. While the characteristic COOH-terminus of the promastigote enzyme is clearly instrumental for GPI anchor addition, the importance of the COOH-terminal sequence of the amastigote enzyme for delivery to lysosomes remains to be determined. In spite of extensive work on the structure and properties of the proteinases, their function for the survival of promastigotes in the sandfly's gut and of amastigotes in macrophages remains unknown.

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