The Major Surface Protein of Leishmania Promastigotes Is a Protease

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The major surface protein of Leishmania promastigotes is evolutionarily conserved and is found in isolates of L. donovani, L. major, L. tropica, L. mexicana, and L. braziliensis. The data provided in this communication demonstrate that in L. major this integral membrane protein is a protease, which we now designate promastigote surface protease. The enzyme has an alkaline pH optimum and is active both in its detergent-solubilized form and at the surface of living or fixed promastigotes. A water-soluble form of promastigote surface protease is obtained following digestion with the phospholipase C responsible for the release of the variant surface glycoprotein of Trypanosoma brucei. Possible biological functions of promastigote surface protease during the life cycle of Leishmania parasites are discussed.

In the life cycle of the protozoan parasite Leishmania, the promastigote occurs in the insect vector and during the establishment of the infection of macrophages of the mammalian host. Radioiodination of the proteins exposed at the surface of promastigotes showed that a glycoprotein (M, 62,000-65,000) is predominantly labeled in different isolates of Leishmania species infective for humans (1-6). This protein, provisionally termed p63, is a major amphiphilic constituent of the membrane (500,000 copies per cell) and represents approximately 1% of the total cell protein in L. major (4, 7). A glycoprophospholipid similar to that associated with the variant surface glycoprotein of Trypanosoma brucei anchors p63 to the promastigote pellicular membrane (8).

Peptide maps of iodinated p63 of L. major, L. donovani, and L. tropica (4) and of L. major, L. donovani, and L. braziliensis (5) are practically identical, indicating that the primary surface structures of these proteins have been substantially conserved among the widely separated Leishmania species. Such a conservation suggests that p63 has a biological function necessary for the survival of all Leishmania species in their natural environment. During the purification of p63 (7), we found evidence of a proteolytic activity co-purifying with the surface glycoprotein. The results presented in this communication demonstrate that the major surface protein of Leishmania promastigotes, formerly called p63 (4, 7), is a proteolytic enzyme: the promastigote surface protease.

MATERIALS AND METHODS

Cell Culture, Labeling, and Purification of p63—L. major LEM 513 promastigotes were grown in modified Schaefer's medium (7, 9) and, when needed, surface-radioiodinated with Na125I (Amersham Corp.) and Iodo-Gen (Pierce) (4). Purified hydrophilic and amphiphilic forms of p63 were prepared as described (7). Protein concentrations were determined with the biocinchonic acid protein assay reagent (Pierce) using a bovine serum albumin standard curve.

Detection of the Proteolytic Activity of p63 after SDS-PAGE—SDS-PAGE was performed on 7.5-15% linear gradient gels (10). The proteolytic activity present in the gel after SDS-PAGE was detected according to Belin et al. (11), omitting plasminogen in the underlay gel. The two-dimensional analysis of the protease activity was performed as described in Ref. 12 with the difference that Staphylococcus aureus protease was replaced by a 4-mm layer of 1.5% agarose in stacking gel buffer containing 10 μg/cm of heated and reduced bovine serum albumin as a protease substrate between the first-dimension lane containing p63 and the stacking gel of the second-dimension gel. Electrophoresis was performed overnight at 50 V.

Lipase-mediated Solubilization and Protease Activity of p63—Purified 125I-p63 (amphiphilic p63) (14 μg, 10,000 cpm/μg) in 2.5 ml of 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing 0.07% Triton X-114 and 0.03% Triton X-100 was digested at 30 °C with 8 μl/ml of T. brucei phospholipase which had been affinity-purified on Affi-Gel 501 (Bio-Rad). Aliquots of 250 μl were removed from the digestion at different periods of time, adjusted to 1% Triton X-114, and submitted to phase separation (4, 13). Aqueous and detergent phases were adjusted to 0.5% Triton X-114, 0.1% Triton X-100, 20 mg/ml azocasein (Sigma), and 50 mM PIPES, pH 7.0, in a final volume of 0.5 ml, and incubated for 60 min at 37 °C (14). Reactions were stopped by addition of 0.5 ml of 5% trichloroacetic acid, and the radioactivity present in each tube was determined. Acid-insoluble material was eliminated by centrifugation at 13,000 × g for 3 min, and the absorbance of the supernatant containing the acid-soluble material was measured at 366 nm using the reaction mixture without added enzyme as a blank. One unit of enzyme has an initial rate of reaction producing 1 mg of acid-soluble azocasein peptides/min (14). E260 of azocasein was 36.

pH Optimum, Low Molecular Weight Substrates, and Inhibitors—The pH optimum of promastigote surface protease was determined on azocasein as described above with the exception that PIPES was replaced by a mixture of 50 mM each HEPEs, citrate, borate, and phosphate adjusted to the appropriate pH with NaOH.

The chromogenic low molecular weight substrate N-benzoyl-L-arginine-p-nitroanilide (1 mM) replaced azocasein in the standard assay. Enzyme activity was determined spectrophotometrically at 405 nm. The activity of promastigote surface protease with 1 mM N-benzoyl-L-lysine thiohydroxamate and 1 mM succinyl-L-Ala-Ala-Pro-Phe-thiobenzyl ester was determined according to the procedure described in (23).

Several compounds known to inhibit proteases were tested on promastigote surface protease. The enzyme was preincubated for 20 min at 25 °C with the inhibitor, then assayed on azocasein as described above in the presence of the inhibitor.

Surface Protease Activity of L. major Promastigotes—Washed, living promastigotes or promastigotes which had been fixed for 30 min in 0.1% glutaraldehyde in Hanks' balanced salts solution were resuspended at a concentration of 4 × 106 cells/ml in Hanks' balanced salt. 2

The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPEs, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, 1,4-piperazineethanesulfonic acid.

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solution containing 20 mg/ml azocasein and incubated at 28 °C with constant agitation. After 10 min, half of each reaction was removed and centrifuged for 3 min at 13,000 × g to eliminate the promastigotes, and the supernatants were further incubated at 26 °C. At different times, aliquots of 0.5 ml were removed and mixed with 0.5 ml of 5% trichloroacetic acid on ice. The absorbance of the acid-soluble azocasein was determined at 366 nm (14). Living promastigotes were intact and motile at the end of the 60-min incubation.

RESULTS

Evidence of proteolytic activity was found when fractions containing purified p63 were mixed with proteins used as molecular weight standards for SDS-PAGE. As the activity was not inactivated by SDS, we analyzed the fractions containing purified amphiphilic p63 (Ap63) and hydrophilic p63 (Hp63) by SDS-PAGE with and without prior heating of the samples. In this electrophoretic system, heat-denatured Ap63 and Hp63 have approximately the same rates of migration, whereas unheated Hp63 migrates much more slowly in the gel than Ap63 (7). After electrophoresis, one half of the gel was stained with Coomassie Blue, whereas the duplicate half was processed for detection and localization of the proteolytic activities present in the gel. In this zymogram, a protease activity generates a transparent zone of casein lysis which appears as a black spot with dark field illumination. A comparison of the positions of proteolytic activities in the gel used for the zymogram (Fig. 1, lanes c' and d') with duplicate lanes stained with Coomassie Blue (Fig. 1, lanes c and d) shows immediately that both Ap63 and Hp63 are active proteases after SDS-PAGE. Heating the samples before electrophoresis resulted in an irreversible loss of proteolytic activity with both forms of p63 (Fig. 1, lanes a, b, a', and b'). The proteolytic activity of p63 in the presence of SDS was confirmed in the following experiment. Purified Ap63 was analyzed by SDS-PAGE. After electrophoresis, the lane containing the sample was excised and electrophoresed at a right angle into a second slab gel through a short agarose stacking gel containing bovine albumin. Co-stacking of the protease and albumin resulted in the digestion of the protein at the position of the enzyme. Digestion is revealed in the form of a gap in the otherwise continuous albumin line (Fig. 1e). The protein below the gap is Ap63, the sole radiiodinated protein in the gel revealed by fluorography (Fig. 1f). Low molecular weight degradation products of albumin, not visible in Fig. 1e, were detected under the gap by silver staining of the gel (not shown).

The specific activities of Ap63 and Hp63 were measured in solution using azocasein as substrate. In this assay the specific activity of purified Ap63 was 3.3 units/mg and that of Hp63 was 3.7 units/mg. For comparison, the specific activity of crystalline bovine trypsin (M.,24,000) was 6.6 units/mg. These data show that on a molar basis, p63 is approximately 1.5 times more active than trypsin using azocasein as substrate. The hydrophobic anchor of membrane-bound p63 is a phospholipid (8). The lipase responsible for the release of the variant surface glycoprotein of T. brucei hydrolyzes the hydrophobic anchor of Leishmania p63 and transforms Ap63 into its hydrophilic form, Hp63. The susceptibility of the protease to lipase treatment was tested in the following experiment. Purified 125I-Ap63 was analyzed by SDS-PAGE without prior heating and reduction. The lane containing the protein in the first electrophoresis was excised, and Ap63 was electrophoresed through a short agarose stacking gel containing bovine albumin into a second resolving gel containing SDS. The second-dimension gel was stained with Coomassie Blue, and the position of 125I-p63 was revealed by fluorography (f). Only the relevant part of the gel is shown in e and f.

FIG. 1. Zymogram of p63 and two-dimensional analysis of the proteolytic activity of p63. Samples of purified Hp63 (a, c, a', c') and Ap63 (b, b', b', d') or without (c, d, c, d') prior heating at 95 °C. After electrophoresis, one-half of the gel (a-d) was stained with Coomassie Blue, and the other half, containing duplicate samples, was processed for the detection of proteolytic activities present in the gel on a casein agarose underlay (24 h at 37 °C), and photographed with dark-field illumination (a'-d'). In the second experiment (e and f), purified 125I-Ap63 was analyzed by SDS-PAGE without prior heating and reduction. The lane containing the protein in the first electrophoresis was excised, and Ap63 was electrophoresed through a short agarose stacking gel containing bovine albumin into a second resolving gel containing SDS. The second-dimension gel was stained with Coomassie Blue (e), and the position of 125I-p63 was revealed by fluorography (f). Only the relevant part of the gel is shown in e and f.

µM L-trans-epoxysuccinylleuclamido-(4-guanidino)-butane, 10 mM Nε-EDTA, and 10 mM p-chloromercuri phenylsulfonic acid had no inhibitory effect on the activity of promastigote surface protease. In contrast, 1 mM HgCl2, 1 mM ZnCl2, 1 mM 1,10-phenanthroline, and 10 mM iodoacetamide completely abrogated its protease activity.

The hydrophobic anchor of membrane-bound p63 is a phospholipid (8). The lipase responsible for the release of the variant surface glycoprotein of T. brucei hydrolyzes the hydrophobic anchor of Leishmania p63 and transforms Ap63 into its hydrophilic form, Hp63. The susceptibility of the protease to lipase treatment was tested in the following experiment. Purified 125I-Ap63 was incubated with a small amount of T. brucei lipase. Aliquots were removed at intervals and were submitted to phase separation in Triton X-114 solution. In this procedure, amphiphilic molecules partition into the detergent phase, and hydrophilic macromolecules are recovered in the aqueous phase (13). During lipase digestion,
Ap63 was transformed into a hydrophilic molecule, as shown by the decreasing proportion of $^{125}$I-p63 recovered in the detergent phase (Fig. 2, top, circles). Similarly, the proteolytic activity was measured in the two phases following lipase digestion. The amphiphilic protease was transformed into a hydrophilic enzyme with precisely the same kinetics as $^{125}$I-p63 (Fig. 2, top, triangles). Recoveries of radioactivity and enzymatic activities were constant during the course of the digestion (Fig. 2, bottom). This experiment shows that the amphiphilic character of the protease is due to a covalently attached phospholipid which can be cleaved by the lipase of T. brucei, and confirms indirectly that p63 itself is the protease. In addition, the constancy of the enzymatic activities recovered confirms that Ap63 and Hp63 have approximately the same specific activities.

The present experiments demonstrate that the major surface protein of *Leishmania* promastigotes is a protease. Based on these observations, the descriptive name promastigote surface protease is proposed for the protein formerly named p63.

The proteolytic activity of promastigote surface protease in situ was demonstrated by incubating azocasein with living promastigotes. The generation of acid-soluble azocasein peptides catalyzed by the protease was measured by monitoring the absorbance of the trichloroacetic acid supernatant at 366 nm. The steady increase in absorbance as a function of time demonstrates the proteolytic activity of the promastigote suspension (Fig. 3, solid circles). To rule out the possibility that endocytosis and intracellular azocasein degradation played an important role in this process, an incubation was performed in parallel with the same number of glutaraldehyde-fixed promastigotes (Fig. 3, solid triangles). Both cell suspensions had the same proteolytic activity. Furthermore, to rule out the possibility that proteolysis was due to secreted enzymes or to the release of a substantial amount of soluble protease from damaged promastigotes, cells were removed by centrifugation after 10 min and the incubation continued. Proteolysis of azocasein was completely abrogated when the cells (whether fixed or alive) were separated from the substrate (Fig. 3, open symbols). These data show that membrane-bound promastigote surface protease present at the surface of *Leishmania* promastigotes is enzymatically active on a soluble substrate.

**DISCUSSION**

Protease activities have been found in several *Leishmania* species. Fong and Chang (20) described briefly two major enzymes with molecular masses between 45 and 68 kDa in *L. mexicana amazonensis* which were active on gelatin or fibrinogen after SDS-PAGE. Pupkis and Coombs (14, 15) found a particular protease in *L. mexicana mexicana* promastigotes and also suggested that a soluble 67-kDa protease may be common to both promastigote and amastigote forms of the parasite. The crucial finding presented in this communication is not the identification of a proteolytic activity, but rather the assignment of the activity to the major, and practically unique, protein exposed at the surface of the promastigote (4, 7). The promastigote surface protease described in this communication is an evolutionarily conserved protein found at a high surface density ($5 \times 10^5$/cell in *L. major*) on all species of *Leishmania* examined so far (3–5, 7).

The results obtained with inhibitors and synthetic substrates do not allow the precise characterization of the substrate specificity or the identification of a specific reactive amino acid of promastigote surface protease. However, the absence of inhibition by diisopropyl fluorophosphate (21) and phenylmethylsulfonylfluoride suggest that promastigote surface protease is not a serine esterase.

At the present time the function of promastigote surface protease during the life cycle of the parasite may only be postulated. In the insect gut, promastigote surface protease may degrade proteins from the blood meal to generate small peptides and amino acids which may be transported across the membrane to sustain promastigote growth. As the digestive tract of the insect, like the phagolysosome of the mammalian macrophage, are environments rich in hydrolytic en-

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**Fig. 2.** Lipase digestion and phase separation of p63 in Triton X-114 solution. Purified $^{125}$I-Ap63 was incubated with affinity-purified *T. brucei* lipase. Aliquots taken at different time points were submitted to phase separation in Triton X-114 solution. Aqueous and detergent phases were examined for radioactivity and proteolytic activity. Partitions and recoveries of the activities are shown in the top and bottom panels, respectively.

**Fig. 3.** Surface protease activity of *L. major* promastigotes. Living or glutaraldehyde-fixed promastigotes of *L. major* were incubated at a concentration of $4 \times 10^8$ cells/ml with azocasein. After 10 min, cells were removed from part of the reaction mixtures. Aliquots were collected at the indicated times and the absorbance of the acid-soluble material was determined. Each point is the average of two measures. ◇, ○, living cells; ▲, Δ, fixed cells. Solid symbols, cells present throughout the incubation; open symbols, cells removed after 10 min incubation.

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zymes, an additional protease activity provided by the parasite may seem redundant, since such functions are undoubtedly provided by host enzymes.

In a more challenging hypothesis, the function of promastigote surface protease could be to proteolyze the promastigote from microbial enzymes in the insect or during the establishment of macrophage infection (for a review, see Ref. 16). After phagocytsosis of the promastigote, the local concentration of promastigote surface protease in the phagolysosome will be very high. The protease, which is resistant to proteolysis (16) and is active at the cell surface (see Fig. 3), could then rapidly degrade important host enzymes present in the parasitophorous vacuole, thus inactivating the microbicidal capacity of the macrophage.

Promastigote surface protease is not observed at the surface of amastigotes (1) but is immunogenic during animal and human infection with Leishmania (2, 4–6). This apparent contradiction may be resolved by assuming that the protease is synthesized by both forms of the parasite, but that the glycoprophospholipid that anchors amphiphilic promastigote surface protease to the promastigote membrane is cleaved from the amastigote surface by an enzyme similar to the phospholipase responsible for the release of the soluble form of T. brucei variant surface glycoprotein (17). The surface protease could also be released by a macrophage phosphatidylinositol-specific phospholipase C active in the phagolysosome (18).

Alternatively, biosynthesis of promastigote surface protease in the amastigote may not involve the covalent attachment of a phospholipid membrane anchor, resulting in the secretion of a soluble protease. The experiment presented in Fig. 2 demonstrates that the elimination of the lipid anchor from amphiphilic promastigote surface protease generates a water-soluble, hydrophilic form of the protease, but does not inhibit the enzymatic activity of the enzyme. Regardless of the mechanism by which soluble hydrophilic promastigote surface protease would be produced by the amastigote during the maintenance of infection, it could exert its function at locations distant from the parasitophorous vacuole. For instance, binding of promastigote surface protease to a receptor of the macrophage membrane (19, 22) could concentrate the protease at the surface of the host cell, inducing the degradation of macrophage surface proteins, and possibly interfering with the immunological mechanisms required for the destruction of the intracellular parasites.

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