Surface Zn-Proteinase as a Molecule for Defense of *Leishmania mexicana amazonensis* Promastigotes against Cytolysis inside Macrophage Phagolysosomes

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The role of the surface membrane Zn-proteinase in protecting the cellular integrity of the macrophage parasite *Leishmania mexicana amazonensis* from intraphagolysosomal cytolysis was studied. These cells lose their infectivity to host macrophages after prolonged cultivation in axenic growth medium. The virulent and attenuated variants of the parasite cells were cloned. Failure of these attenuated parasite cells to survive inside macrophage phagolysosomes is associated with 20- to 50-fold reduction in the expression of surface gp63 protein. In situ inhibition of gp63 proteinase activity inside *Leishmania*-infected macrophage phagolysosomes with targeted delivery of an inhibitor of gp63 proteinase activity, 1,10-phenanthroline, selectively eliminated intracellular *Leishmania* amastigotes, further suggesting the importance of this proteinase in phagolysosomal survival of the parasite. An upstream sequence (US) of the gp63 gene was cloned in front of the bacterial chloramphenicol acetyltransferase (CAT) gene in plasmid pCATbasic. Transfection of *L. mexicana amazonensis* cells with this recombinant plasmid showed that expression of the CAT gene from this US is 15- to 20-fold higher in virulent clones than in avirulent clones of the parasite. Band shift analysis with the cloned US also showed that binding of protein(s) was 15- to 20-fold higher in virulent cell extract than in avirulent cell extract. Coating of attenuated cells or liposomes with proteolytically active gp63 protects them from degradation inside macrophage phagolysosomes. These results suggest a novel mechanism of survival of this phagolysosomal parasite with the help of its surface Zn-proteinase.

The etiological agents for the human parasitic disease leishmaniasis, protozoa of the genus *Leishmania*, are obligate parasites of mammalian macrophages (12). Upon transmission to the human body by the bite of infected sandflies, the parasites invade the macrophage cells by a receptor-mediated process and take shelter inside phagolysosomes (13). Leishmaniae have been shown to be acidophilic organisms (44) and to be resistant to the cytolytic factors of macrophages (2). Without being killed inside macrophage phagolysosome, the parasite cell is transformed into a nonmotile ovoid form called the amastigote and multiplies inside the phagolysosome (13).

The ability of the parasite to survive inside the acidic phagolysosomes of macrophages seems to be the key to the success of leishmanial parasitism. Collective contributions of several parasite surface molecules appear to enable the parasite to establish infection inside the macrophages (12, 36, 44). The Zn-proteinase of ~63 kDa, called gp63 (12, 30, 35), and the lipophosphoglycan (21, 50, 51, 58), two of the abundant parasite surface molecules, seem to be directly involved in this process. gp63 was purified to homogeneity from several species of *Leishmania* (12) and was found to have proteinase activity that requires zinc ion (6, 16). The natural substrate for this proteinase is not known. The optimum pH of gp63 action appears to be dependent on the nature of the substrate used for the in vitro assay (12, 59). An immunoreactive variant of gp63 proteinase isolated from *Leishmania mexicana* amastigotes has been reported (26) to be optimally active at acidic pH. The active site structure of the gp63 proteinase appears to be very similar, if not identical, to those of other well-known

metalloproteinases (11, 32, 35, 37, 56). The strategic location of this glycosylphosphatidylinositol-anchored (23) protein all over the membrane surface of the flagellum and cell body of the parasite (12), its multifaceted potential biological roles (7, 12, 13), its immunogenicity (1, 12, 19, 38, 49, 53), the presence of several differentially expressed copies of its genes (12, 39, 41, 48), and the potential to be regulated at posttranscriptional and posttranslational levels during the life cycle of the parasite (12, 34, 35, 46, 47, 62) make this molecule an excellent candidate for a virulent factor. Evidence presented here points toward the involvement of gp63 in the anticytolytic event that favors survival of virulent *Leishmania* parasites inside macrophage phagolysosomes.

MATERIALS AND METHODS

Cells. *L. mexicana amazonensis* (LV78) was used throughout the study. The parasite cells were cultured in medium 199 plus 10% heat-inactivated (56°C for 30 min) fetal bovine serum (HIFBS) at 25°C (16). J774G8 cells of mouse macrophage origin are the hosts of *L. mexicana amazonensis* (16, 42). These cells were used as cultured macrophages. Monolayers of these cells in 35-mm-diameter plastic petri dishes were incubated at 33°C with *L. mexicana amazonensis* promastigotes in medium A (RPMI 1640 with 20% HIFBS) at a parasite-to-macrophage ratio 10:1. After 4 h of incubation, the infected cells were washed with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered (25 mM, pH 7.4) saline (0.15 M) (HBS) to remove the unabsorbed parasites (16, 42). The test compound was added to the medium. After incubation of the parasite-laden macrophages at 33°C for 20 h, the number of amastigotes per 100 to 200 macrophages was counted microscopically as described previously (16, 42). An avirulent clone of *L. mexicana amazonensis* strains. Also, these sequences are different for other strains of *Leishmania* maintained in this laboratory (*L. major* BT010, *L. major* Lmj144, *L. donovani* DD8, *L. donovani* Jeddah, and *L. chagasi*). Growth kinetics of all clones were studied, and no significant difference was noted. We used late-log-phase cells in all of our studies. *Leishmania* and J774G8

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macrophage cells were originally obtained from K.-P. Chang's laboratory at The Chicago Medical School. Although the infectivities of the strains used were tested in an experimental animal model (BALB/c mice), we have used the term "virulence" to indicate the ability of the parasite cells to establish and maintain infection in cultured macrophage cells. In some experiments, parasite-laden J774G8 cells were lysed by four hand strokes in a Dounce homogenizer with a type A pestle at 4°C. Different components of the cell lysate were separated in a Percoll density gradient (10 to 90% Percoll in phosphate-buffered saline [PBS] containing 2 mM EDTA). The gradients were spun at $18,000 \times g$ for 2 h at 4°C. Fractions (500 μ l) were collected from the bottom of the gradients, and DNA was isolated from each fraction by the standard procedure (3) for PCR analysis (see below).

Cytotoxicity assay in vitro. The cytotoxicity of 1,10-phenanthroline (OP) for cultured macrophages (J774G8 cells) was assayed by the [3 H]thymidine uptake assay as described previously (43). Briefly, 10^5 cells per well were incubated overnight in 1 ml of RPMI 1640–20% HIFBS at 33°C. Each well received the indicated concentrations of OP in free or liposome-encapsulated form, and the cells were incubated for 30 min at 33°C. The cells were then washed thrice in the culture medium and incubated in OP-free medium at 33°C. After 20 h of incubation, [3 H]thymidine (5 μ Ci/ml) was added to each well. After 3 h, the cells were washed three times with 1 ml of medium to remove unincorporated radioactivity. The cells in each well were dissolved in 0.5 ml of 100 mM NaOH, and the radioactivity associated with the cells was determined by scintillation counting.

Cloning of the gp63 gene US. The chromosome-sized DNA carrying gp63 genes was identified by Southern hybridization with a gp63 gene probe of a blot from contour-clamped homogeneous electric field (CHEF) electrophoresis (1% agarose gel; 60- to -90-s pulse for 15 h, 90- to 120-s pulse for 5 h, $0.5 \times$ TBE [45 mM Tris, 45 mM boric acid, 1 mM EDTA], 6°C; Bio-Rad CHEF gel electrophoresis apparatus) of *L. mexicana amazonensis* DNA as described previously (14, 22). This 575-kb chromosomal DNA was purified from the CHEF gels and partially digested with *Rsa*I to a size of 2 to 5 kb. Resulting size-selected DNA fragments were ligated with *Eco*RI adapters and cloned at the *Eco*RI site of the pBluescript KS(+) phagemid (3). The whole ligated mixture was introduced into competent *Escherichia coli* DH5 α cells (GIBCO-BRL) to obtain a chromosome-specific library. Using an antisense primer corresponding to the 5' end of the gp63 gene (10, 35, 41, 46) and T7 primer (Stratagene, La Jolla, Calif.), we isolated a 451-bp upstream regulatory sequence (US) by anchor PCR (3) from the chromosome-specific library in pBluescript KS(+) and cloned it at the *Pst*I site in plasmid pCATbasic. The nucleotide sequence of the cloned US in pBluescript KS(+) was determined by cycle sequencing with 5'- 32 P-labeled T3 and T7 primers, using Promega's fmol cycle sequencing kit according to their protocol. The nucleotide sequence of the US showed identity with several such sequences published for *Leishmania* gp63 (10, 35, 41, 46). This US has nucleotides -1 to -451 of the gp63 gene.

Reverse transcriptase quantitative competitive PCR (RT-QC-PCR). Deoxyoligonucleotide primers were designed from the nucleotide sequences of the *L. major* gp63 gene (10) and the bacterial chloramphenicol acetyltransferase (CAT) gene (sequence provided by Promega), using the MacVector program (IBI-Kodak, New Haven, Conn.). *L. mexicana amazonensis* amastigote DNA was detected by using gp63 gene-specific primers for PCR: GP1 (5'-GCAACACCG ACTTCGTGATGTAC-3') and GP2 (5'-CATGCACTTCTCGTGAGGAAG-3'). This amplification yielded a 566-bp product. CAT gene-specific primers used were CP1 (5'-CCCAATGGCATCGTAAAGAAC-3') and CP2 (5'-TCACAG CCGCATGATGAAC-3'). CP1 and CP2 primers yielded a 550-bp product from pCATbasic plasmid DNA as the template for PCR. CAT PCR-mimic was amplified from the β -galactosidase α -peptide gene in pBluescript KS(+), using chimeric primers CP1-BG1 (5'-CCCAATGGCATCGTAAAGAACCTGTTGG GAAGGGCGATC-3') and CP2-BG2 (5'-TCACAGACGGCATGATGAAC TGTGGAATTGTGAGCGG-3') (3). A gp63 PCR-mimic DNA (420 bp) was made as described above for CAT PCR-mimic, using GP1-BG1 (5'-GCAACA CCGACTTCGTGATGTACCTGTTGGGAAGGGCGATC-3') and CP2-BG2 (5'-CATGCACTTCTCGTGAGGAAGTGTGTTGGAATTGTGAGCGG-3') chimeric primer pairs. Amplified mimic DNA was purified from low-melting-point agarose gels by electroelution (Stratagene) followed by ethanol precipitation (3).

Oligonucleotides needed as primers for PCR or as probes were synthesized by the phosphoramidate method, using an Applied Biosystems model 380A automated DNA synthesizer. Oligonucleotides were purified by electrophoresis through a denaturing polyacrylamide gel, followed by Sep-Pak C₁₈ (Waters, Millipore Corp., Milford, Mass.) reversed-phase column chromatography (3).

gp63 or CAT mRNA levels in *Leishmania* cells were quantitated or compared by QC-RT-PCR (18, 54, 55). Constructed PCR-mimics were used as internal standards (54, 55).

cDNAs were synthesized from total cellular RNA (1 μ g), using Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) and the reverse primer (CP2, 5'-TCACAGACGGCATGATGAAC-3') (3). A Perkin-Elmer (Norwalk, Conn.) RNA PCR kit was used for this purpose. The cDNA synthesis reaction was carried out in a total volume of 20 μ l at 42°C for 15 min, then at 99°C for 5 min, and finally at 5°C for 5 min (3). An MJ Research (Watertown, Mass.) PTC-100 thermocycler was used for PCR with AmpliTaq (Perkin-Elmer) DNA polymerase, using the reagents in the Perkin-Elmer DNA-PCR kit. The

reaction mixture (20 μ l) contained 10 mM Tris-Cl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 10 μ g of gelatin per ml, DNA template, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 100 pmol of each forward and reverse primer, and 25 mU of AmpliTaq per μ l. Mineral oil (Perkin-Elmer) was used to cover the aqueous solution in a 0.5-ml autoclaved tube. The sample tubes were loaded in the prewarmed (95°C) thermocycler and heated for 5 min at 95°C. Thermal cycling was done for 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 to 10 min. After 25 cycles, tubes were incubated at 72°C for 5 min and then chilled at 4°C. The aqueous layer containing the amplified DNA was recovered by extracting the mineral oil with chloroform (20 μ l). The PCR products were analyzed in 2% agarose gel, made and run in $0.5 \times$ TBE (3).

For quantitation, the forward primer used for PCR was 5' end labeled with 32 P, using T4 polynucleotide kinase and [γ - 32 P]ATP (3). The DNAs from the 2% agarose gel were transferred to a Nytran nylon membrane, using the Schleicher & Schuell turbo transfer protocol, and the total radioactivity in the amplified native DNA and the mimic DNA was measured with an AMBIS radioanalytic phosphorimager. The radioactivity (counts per minute) in the native DNA band is expressed as a percentage of that in the corresponding mimic DNA (18, 54, 55).

Transfection of *Leishmania* cells. Late-log-phase (2×10^7 to 3×10^7 cells per ml) *L. mexicana amazonensis* promastigotes were harvested by centrifugation ($2,000 \times g$ for 5 min). Cells were washed twice with ice-cold PBS and resuspended in high-ionic-strength electroporation buffer (20 mM HEPES [pH 7.2], 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose) to a final concentration of 3.3×10^8 cells per ml (5, 28). Cells (0.3 ml) were mixed in a 0.2-cm electroporation cuvette (Bio-Rad) with the plasmid DNA (50 μ g/ 10^8 cells) and exposed to an exponential discharge of 2,250 V/cm from a 500- μ F capacitor (Bio-Rad Gene Pulsar) (5, 28). Electroporated cells were incubated on ice for 10 min, transferred to culture medium (10 ml), and incubated at 25°C for 20 to 24 h before assay of CAT activity.

CAT assay. Electroporated *Leishmania* cells were harvested and washed twice with ice-cold PBS and resuspended in 250 μ l of 0.5% Triton X-100 in 0.25 M Tris-Cl (pH 7.5)–1 mM phenylmethylsulfonyl fluoride as described previously (28). After 5 min of incubation on ice, the lysate was cleared by centrifugation ($16,000 \times g$ for 5 min). The cleared lysate was then incubated at 65°C for 10 min to inactivate transacetylases that can interfere with CAT activity. The lysate was cooled on ice and cleared again by centrifugation in a microfuge for 5 min at full speed ($16,000 \times g$). The cleared and inactivated lysate (200 μ l) was assayed for CAT activity in a mixture of 250 mM Tris-Cl (pH 7.5), 0.4 mM butyryl coenzyme A, and 125 nCi of [14 C]chloramphenicol (50 mCi/mmol; NEN-DuPont) in a final volume of 250 μ l (28). The reaction mixture was incubated at 37°C for 20 h, extracted twice with 250 μ l of ethyl acetate, and dried. The pellet was resuspended in 50 μ l of ethyl acetate and loaded on thin-layer chromatography (TLC) plates. The TLC plates were developed for 2 h at room temperature in chloroform-methanol (95:5 [vol/vol]) and exposed to X-ray film or the AMBIS radioanalytic system for analysis.

Band shift assay. The 0.45-kb *Pst*I fragment from pASCAT-7 containing the gp63 gene US was end labeled with T4 polynucleotide kinase and [γ - 32 P]ATP (3) and was used as a probe for band shift assay according to the instructions and using the reagents provided in the band shift assay kit (Pharmacia Biotech Inc., Piscataway, N.J.). *L. mexicana amazonensis* total protein extracts were prepared exactly as described previously (60). Briefly, 10^6 late-log-phase promastigotes were resuspended in 100 μ l of extraction buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication (checked by phase-contrast microscopy). The lysate was cleared by centrifugation at $10,000 \times g$ for 10 min at 4°C. The cleared extract was stored at -80°C. The binding reaction mixture for the electrophoretic mobility shift assay contained 25,000 cpm of end-labeled probe (~10 ng), 1 μ g of poly(dI-dC) as a nonspecific competitor, and 20 μ g of total protein extract in a total volume of 15 μ l of 10 mM HEPES (pH 7.9)–4% Ficoll 400–5 mM DTT. Binding reaction mixtures were incubated on ice for 90 min. Samples were analyzed in a 4% nondenaturing polyacrylamide gel, using 22 mM Tris-borate (pH 8.3)–0.5 mM EDTA as the electrophoresis buffer (60). The gel was dried following the electrophoresis and autoradiographed for 20 h at -80°C. An unlabeled 0.45-kb *Pst*I fragment (500 ng) from pASCAT-7 was used along with the end-labeled probe for competition experiments to verify the specificity of binding (60).

gp63 purification, proteinase assay, and chemical modification. gp63 was purified from *L. mexicana amazonensis* amastigotes or promastigotes to homogeneity by Triton X-114 detergent extraction followed by anion-exchange liquid chromatography (6). Polyclonal antiserum against purified gp63 was developed in BALB/c mice and purified (16). *Leishmania* cell (10^8) proteins were biosynthetically labeled with [35 S]methionine (100 μ Ci) for 16 h in 10 ml of complete growth medium as described previously (16). Labeled proteins were dissolved in 0.5% Nonidet P-40, and labeled gp63 was immunoprecipitated with polyclonal antibodies and protein A-Sepharose beads as described previously (16). Proteinase activity of purified gp63 was assayed against 125 I-bovine serum albumin (BSA) at 37°C and pH 4.75 for 30 min (15, 16). Surface proteinase activity was assayed against 125 I-BSA at 37°C and pH 4.75 for 60 min, using glutaraldehyde (0.5%, 60 min, 4°C)-fixed promastigotes, as described previously (15). For chemical modifications of its histidine residues (9, 31), gp63 (2 nmol) was treated with 2.5 mM diethyl oxydicarbonate (DOF) for 10 min at 24°C in 20 mM HEPES buffer (pH 7.2) in a total volume of 200 μ l. In some experiments, to reverse the

modifications by DOF (9, 31), the modified gp63 was further treated with neutral hydroxylamine (25 mM) for 30 min at 37°C. gp63 (2 nmol) was also treated with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDO; 2 mM) to modify its carboxylic acid groups (25) at pH 5.8 (50 mM morpholinepropanesulfonic acid) and 30°C for 1 h in a final volume of 200 μ l. Some preparations of gp63 were treated with bacterial phospholipase C (Sigma) for 1 h at 37°C to remove the carboxy-terminal phospholipid (23).

Radioiodination and maleylation of BSA. BSA was radioiodinated with Na¹²⁵I by the ICl catalyzed reaction (15, 17). More than 99% of the radioactivity was trichloroacetic acid precipitable. Specific activity of the radiolabeled protein was 275 to 325 cpm/ng of protein. BSA was maleylated by the reaction of the protein with maleic anhydride at pH 8.0 (17). The change of the electrophoretic mobility of the modified protein was monitored by 0.7% agarose gel electrophoresis (17).

Preparation of liposomes. Dimyristoyl phosphatidylcholine (DMPC) was used to prepare liposomes for encapsulation of OP (16). Briefly, DMPC (10 mg) was made into vesicles with cholesterol (9 μ mol) and dicetyl phosphate (1.2 μ mol) in 1 ml of HBS (sometimes containing 1 mM OP, 10 μ g of plasmid pCATbasic per ml, or 5 mg of ¹²⁵I-BSA per ml). Typically, by this procedure 16 to 20 nmol of OP, 100 to 150 μ g of ¹²⁵I-BSA, or 1.5 to 2 μ g of pCATbasic was trapped per mg of liposomal phospholipids. Amounts of phospholipids in the liposome preparations were assayed by estimating phosphate content (16). Dipalmitoyl phosphatidylethanolamine (100 mg, dissolved in 0.15 M NaCl by ultrasonication; Sigma) was coupled to maleylated BSA (MBSA; 30 mg) by the 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (100 mg)-mediated reaction (17) in a total volume of 3 ml. For coating the liposomes with phospholipid-tailed MBSA, gp63, or gp63 derivatives, 200 μ g of protein was incubated with 1 mg of liposomes in HBS for 20 h at 4°C. Bound protein was separated from unbound molecules by centrifugation at 100,000 $\times$ g for 1 h at 4°C as described previously (16, 17). The efficiency of binding of the proteins to liposomes was approximately 95%, as determined immunochemically (for gp63 or its derivatives) and biochemically as described previously (16). The avirulent promastigotes of *L. mexicana amazonensis* were coated with purified gp63 molecules by incubating the parasite cells (10⁸/ml) with the protein (0 to 250 μ g/ml) in HBS for 2 h at 4°C. The efficiency of binding of the gp63 (or its derivative) to the promastigote cell surface was approximately 90%, as determined immunochemically and biochemically as described previously (16). The control virulent cells were also incubated similarly for 2 h at 4°C in HBS before addition to the macrophage culture.

Assay of uptake and degradation of liposomes. Each petri dish containing a cultured monolayer of J774G8 macrophages received 1 ml of culture medium A (without HIFBS but with 1 mg of BSA per ml) containing different concentrations of ¹²⁵I-BSA-containing liposomes. After incubation for 5 h at 33°C, the amount of labeled material in the cells and the amount of ¹²⁵I-labeled trichloroacetic acid-soluble (noniodide) material in the medium were determined in duplicate dishes as described previously (16, 17). One hundred percent protection of liposome degradation was the value obtained from parallel experiments done in the presence of 75 μ M chloroquine (16, 17).

RESULTS

In situ inhibition of gp63 proteinase activity inside *Leishmania*-infected macrophage phagolysosomes with targeted delivery of OP selectively eliminated intracellular parasites. OP is a strong inhibitor of the proteinase activity of *Leishmania* gp63, particularly at acidic pHs (16). This compound was used to inhibit in situ the proteinase activity of surface gp63 proteinase of the parasite cells inside the infected macrophage phagolysosomes. To avoid potential general toxicity of OP for macrophages, this compound was targeted to phagolysosomes of *L. mexicana amazonensis*-infected (four to five amastigotes per cell) cultured macrophages (J774G8 cells) via the highly efficient scavenger receptor system (4, 8, 27, 42) of these cells. OP, along with ¹²⁵I-BSA as a tracer, was entrapped inside large unilamellar liposomes (16). The liposomes were coated with phospholipid-anchored MBSA, the artificial, highly efficient ligand for the macrophage scavenger receptor (4, 42). When added to the culture medium, J774G8 cells endocytose OP-liposomes via the scavenger receptor (data not shown). The uptake was completely competed out by free MBSA (20-fold-higher concentration) (data not shown). As a steady state was reached (~1 h), liposomes were continuously taken up and degraded to liberate their contents (data not shown), as indicated by the degradation of the tracer ¹²⁵I-BSA, into iodo-tyrosines (detected by TLC [4, 16, 17, 42]). This degradation was inhibited by 75 μ M chloroquine, resulting in continuous

accumulation of the liposomes inside the phagolysosomes, thus suggesting phagolysosomal involvement in this process (4, 16, 17, 42). Degradation of liposomes and their contents, e.g., ¹²⁵I-BSA, by macrophages in the presence of OP also suggests that phagolysosomal cytolytic functions are not inhibited by the presence of OP under the conditions of the experiments.

The growth of leishmanial amastigotes inside macrophage phagolysosomes was inhibited by liposome-encapsulated OP (Fig. 1A). Late-log-phase cells were used for infection, and there was no significant killing in the control cultures. Addition of equimolar zinc chloride with OP during the encapsulation of liposomes eliminated the leishmanicidal effects of OP (Fig. 1A). There was no leishmanicidal effect of the MBSA-coated liposomes (without OP) under the conditions of the study. Further evidence for this targeting is presented in Fig. 1B. Uninfected or *Leishmania*-infected macrophages were incubated with OP-liposomes that also contained pCATbasic plasmid DNA inside the vesicle. The coexistence of this liposomal pCATbasic DNA and *Leishmania* DNA in a subcellular fraction of these cells is shown by PCR using specific oligonucleotide primers (Fig. 1B). Under the conditions of the experiments described (see Materials and Methods), free OP or liposome-encapsulated OP had no effect on the growth of *L. mexicana amazonensis* promastigotes or J774G8 macrophages, as was indicated by incorporation of radiolabeled thymidine into the DNA of these cells (data not shown).

The gp63 gene is poorly expressed in attenuated avirulent cells. Expression of gp63 protein and surface gp63 proteinase activity was depressed in *L. mexicana amazonensis* promastigotes when they were attenuated (12, 30, 34, 35) in vitro in axenic culture medium (Fig. 2). To gain a better understanding of this possible virulence-dependent regulation of gp63 expression, we cloned a 451-bp US of the *L. mexicana amazonensis* gp63 gene and placed it in both sense (pASCAT-7) and antisense (pASCAT-4) orientations next to the CAT reporter gene in pCATbasic (see Materials and Methods). Late-log-phase cells of virulent or avirulent clones of *L. mexicana amazonensis* were transfected with pASCAT-7. CAT enzyme activity was found to be 15- to 20-fold higher in virulent cells than in avirulent cells (Fig. 3). QC-PCR analysis showed equivalent amounts of CAT plasmid constructs in cells transfected with different plasmids (Fig. 4). The CAT mRNA level was also 15- to 20-fold less in avirulent cells than in virulent cells (Fig. 4). The lower net level of CAT enzyme or CAT mRNA in avirulent cell appears to be due to the slower rate of synthesis of the CAT mRNA, possibly due to the presence of a lower concentration of a specific transcription factor(s) which binds to the gp63 US (Fig. 5). The binding buffer contained poly(dI-dC) to compete out any nonspecific DNA-binding proteins of the extract. Moreover, the binding of protein was competed out completely (>99%) by a 50-fold excess of nonlabeled US DNA but not by a 448-bp *Pvu*II fragment of pBluescript KS(+) phagemid (data not shown).

gp63-coated avirulent *Leishmania* cells survived longer inside macrophage phagolysosomes. Clonal cells of avirulent *L. mexicana amazonensis* promastigotes are dramatically deficient in surface gp63 protein (Fig. 2; also see references 30, 34, and 35) and are killed after phagocytosis by J774G8 cells. The reduction of surface proteinase activity (~20-fold) (Fig. 2A) is higher than that of the total gp63 content of the parasite cells (5- to 6-fold) (Fig. 2B). This may be explained by the fact that all gp63 molecules in the parasite cell variants may not be exposed to the outer cell membrane. The survival of these avirulent cells inside the J774G8 cells was found to be increased dose dependently by 1 to 3 days by precoating them

FIG. 1. Selective killing of intracellular *Leishmania* parasites in macrophages by in situ inhibition of gp63 proteinase with targeted delivery of OP. (A) Leishmanicidal effects of free OP or liposome-encapsulated OP and the effect of zinc chloride on the leishmanicidal effect of liposome-encapsulated OP in infected macrophages. More than 90% of macrophages were infected in the control, and there were four to five *Leishmania* amastigotes per macrophage. (B) Distribution of pCATbasic (CAT) in liposome-containing OP and the gp63 gene of *L. mexicana amazonensis* amastigotes before (/N) or after (/IN) incubation of the liposomes with infected macrophages. Radioactivity in the PCR-amplified CAT DNA or gp63 DNA is plotted against the fraction number from which the template DNA for PCR was isolated.

with purified gp63, in vitro, before infection (Fig. 6A). Such induction of parasite survival was absent when cells were coated instead with chemically inactivated gp63 (treated with DOF or EEDQ to modify its essential histidine or carboxylic acid residues, 9, 31) or when incubated with OP-liposomes after infection (Fig. 6B). Simultaneous targeted delivery of OP

to macrophage phagolysosomes via separate liposomes dose dependently inhibited the protection of the gp63-coated avirulent cells from degradation inside J774G8 cells (data not shown). Again, zinc chloride was able to alleviate the action of OP (data not shown), indicating that the metalloproteinase activity of gp63 was important for such protective action.

FIG. 2. Differential expression of gp63 in virulent and avirulent cells. Decreases in surface proteinase activity (A) and gp63 content (B) of cells during axenic culture are shown. In panel B, data from two representative virulent (v) and two representative avirulent (Av) clones are shown.

FIG. 3. Activity of the cloned gp63 gene US in virulent and avirulent cells in driving CAT gene expression as CAT enzyme activity. (A) Autoradiogram of a representative TLC plate showing differential activity of CAT enzyme in extracts (Extr.) obtained from pASCAT-7-transfected virulent and avirulent promastigotes. (B) Compiled scan results of CAT activity in transfected cells from four different experiments. Each value is the average of four experiments $\pm$ standard error.

gp63 coating increased the stability of liposomes inside macrophage phagolysosomes. The surface Zn-proteinase gp63 purified from *L. mexicana amazonensis* promastigotes or amastigotes was reconstituted on the surface of DMPC-liposomes by incubation at 4°C of preformed liposomes with gp63 followed by ultracentrifugation to separate the liposome-bound gp63 from free gp63 molecules (16). More than 95% of gp63 or its proteinase activity becomes associated with the liposomes after incubation. Pretreatment of promastigote gp63 with phospholipase C dramatically decreased (>97%) the binding of gp63 to the liposome surface, indicating that this binding may be mediated by the C-terminal lipid anchor of gp63 (23). In contrast, such treatment of amastigote gp63 marginally ($10\% \pm 2\%$) decreased the binding efficiency. Purified gp63 was modified by specific chemical reagents to destroy its proteinase activity. Modification of gp63 by DOF or EEDQ almost completely (>98%) destroyed the proteinase activity. But these inhibitions were not due to destruction of the protein (as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis) and were completely (94 to 96%) reversed by neutral hydroxylamine (data not shown). Chemical modifications of gp63 with DOF or EEDQ did not affect its binding to DMPC-liposomes. To further substantiate the protective role of gp63 in establishing infection by the leishmanial cells inside macrophages, experiments were done with MBSA-coated liposomes which were also coated with purified gp63. Such liposomes were taken up by the *Leishmania*-infected J774G8 cells by phagocytosis (16) and, in the absence of a gp63 coat, were degraded therein. This was indicated by the degradation of encapsulated 125 I-BSA by phagolysosomal enzymes (16). When those liposomes were coated with purified gp63, they were dose dependently protected from such degradation (Fig. 7A). DOF- or EEDQ-modified proteolytically inactive gp63 failed to protect those liposomes (Fig. 7B), indicating that the proteinase activity of gp63 is essential for such protection. Simultaneous targeted delivery of OP (as described above) to macrophage phagolysosomes via separate MBSA-coated liposomes dose dependently inhibited the protection of the gp63-coated liposomes from degradation inside J774G8 cells (data not shown). Again, zinc chloride was able to alleviate the action of OP (data not shown), establishing that the metallo-proteinase activity of gp63 was important for such protective action.

DISCUSSION

Critical events in establishing intracellular parasitism by a microbe are the molecular interactions at the host-parasite interface. The natural transmission of the mammalian parasite *L. mexicana amazonensis* starts with the delivery of the promastigote stage by the sandfly vector into the susceptible host (12). This follows the establishment of infection in the host macrophage phagolysosome by the parasite. The most intriguing not yet understood aspect of leishmanial parasitism is how the parasite cells overcome a formidable array of hostile activities with which macrophages are equipped to kill ingested microbes. The mechanisms of evasion of macrophage hostility that favor parasitism of some other parasites such as avoidance of phagosome-lysosome fusion, inhibition of acidification of phagolysosomes, or neutralization of phagolysosomal hydrolases directly or indirectly by neutralizing the acidity appeared not to be the mechanisms in leishmanial virulence (12). Evidence presented here further supports the hypothesis (16) that the surface molecules of *Leishmania* cells actively provide a protective shield allowing the parasite cells to function normally inside macrophage phagolysosomes.

We previously (16) have suggested that purified *Leishmania* surface membrane proteinase gp63 may have anticytolytic ac-

FIG. 4. Activity of the cloned gp63 gene US in virulent and avirulent cells in expressing CAT mRNA. (A) Ethidium bromide-stained gel showing products from QC-PCR (lanes 1 to 4) and RT-QC-PCR (lanes 5 to 8) for relative quantitation of CAT DNA and CAT transcript, respectively, from virulent (lanes 1, 3, 5, and 7) or avirulent (lanes 2, 4, 6, and 8) *L. mexicana amazonensis* cells transfected with pCATb (lanes 1, 2, 5, and 6) or pASCAT-7 (lanes 3, 4, 7, and 8). CAT mim represents a 416-bp *E. coli* β -galactosidase fragment having CAT gene-specific primer binding sites at its ends. (B) Summary of the relative CAT mRNA pool size in pASCAT-7-transfected virulent or avirulent cells. Total radioactivity (counts per minute) in the CAT cDNA band is expressed as a percentage of that of the mimic DNA band in each lane (mean $\pm$ standard error, $n = 3$).

FIG. 5. Differential binding of nuclear proteins isolated from virulent (V1 and V2) or avirulent (AV1 and AV2) cells to the cloned gp63 gene promoter in electrophoretic mobility shift assay. C1 and C2 indicate control incubations with boiled extracts from virulent and avirulent cells, respectively.

tivity in protecting coated membrane bodies inside macrophage phagolysosomes. To understand whether the inhibition of gp63 proteinase activity inside macrophage phagolysosomes kills resident *Leishmania* amastigotes, we specifically targeted OP to those phagolysosomes, exploiting the exquisite specificity of the macrophage scavenger receptor (4, 8, 27). We made liposomes encapsulating OP and coated those liposomes with phospholipid-tailed MBSA. MBSA is a potent ligand for the macrophage scavenger receptor (4, 8, 17, 27). This ligand was shown to bind to the macrophage scavenger receptor, to be internalized by absorptive endocytosis, and to be degraded inside the phagolysosomes of these cells (17). When liposomes carrying OP are coated with MBSA, they bind to the cell surface of macrophages with high affinity, resulting in rapid internalization and destruction of these phospholipid vesicles

inside the macrophage phagolysosomes by the cytolytic enzymes therein. Thus, OP is liberated in the immediate vicinity of resident *Leishmania* amastigotes. That inhibition of gp63 proteinase activity is the mode of killing action of targeted OP was further substantiated by the destruction by OP of the gp63-protected avirulent cells or liposomes inside macrophage phagolysosomes. Several lines of evidence presented here strongly favor the hypothesis that the OP, thus targeted, inhibits the surface gp63 proteinase activity of the parasite, resulting in successful cytolysis of the parasite cells by phagolysosomal hydrolases. gp63 proteinase activity thus appears to help the parasite cells as a mechanism for anticytolysis.

OP is particularly effective against gp63 at an acidic pH (16). OP is believed to be unable to permeate the phospholipid membrane (45). Thus, it does not leak through the liposomal vesicle and, once liberated inside the phagolysosomes, probably does not migrate from that vesicle to the cytosol to become toxic to the macrophages. Another Zn^{2+} chelator, *N,N,N',N'*-tetrakis(2-pyridylmethyl)-ethylenediamine, which is membrane soluble (33), showed anti-gp63 activity similar to that of OP but was somewhat toxic to macrophages at the concentration tested (data not shown).

Although several other mechanisms for the antileishmanial effect of OP are possible, OP, being unable to permeate membranes (45), probably does not enter *Leishmania* cells to interact with other vital molecules of the parasite. Arguments in favor of proper targeting of the OP-liposomes to the amastigote-containing phagolysosomes are the followings: (i) OP is carried by liposomes coated with the high-affinity ligand of the lysosomotropic scavenger receptor of macrophages (see above); (ii) the BSA entrapped inside such liposomes is degraded completely into amino acids, indicating lysis of the liposomes in a close compartment (17, 42); and (iii) the degradation of the liposome-entrapped BSA is inhibited by chloroquine, an inhibitor of lysosomal function (17, 42).

FIG. 6. Protection of avirulent parasite cells from cytolysis inside cultured macrophages by in vitro coating with proteolytically active gp63. (A) Induced survival of avirulent promastigotes (AP) in macrophages 5 days after infection. The amounts of gp63 per 10^8 cells were as follows: AP-0, none; AP-10, 10 μ g; AP-30, 30 μ g; AP-50, 50 μ g; and AP-100, 100 μ g. VP, control virulent promastigotes. (B) Kinetics of survival inside macrophages with avirulent promastigotes with no added coat (AP), with a native gp63 (100 μ g/ 10^8 cells) coat (AP-Ngp), with a DOF-treated gp63 (100 μ g/ 10^8 cells) coat (AP-Dgp), and with control virulent promastigotes (VP). Each value is the average of at least three experiments $\pm$ the standard error.

FIG. 7. Protection of gp63-coated liposomes from macrophage phagolysosomal degradation. (A) Effect of gp63 concentration on the surface of liposomes on the protection of encapsulated ^{125}I -BSA from degradation. (B) Kinetics of degradation inside macrophages of liposomes with no coat, a native gp63 coat, or a DOF-treated gp63 (CE-gp63) coat. All coats were 50 μg of protein per mg of liposome phospholipid. Each value is the average of at least three experiments $\pm$ the standard error.

The mechanism of gp63 gene expression in virulent and avirulent cells is not known. The lower level of gp63 in avirulent cells is thought to be due to posttranscriptional or post-translational instability of nascent gp63 molecules (35), and this instability may be due to differential expression of different class of gp63 genes which have unique 3' flanking sequences determining their instability at a certain stage of the cell life cycle (40, 46). Recent evidence regarding *hsp70* gene expression in *Trypanosoma brucei* suggests that the RNA polymerase II initiation site in Kinetoplastida may well exist in the intergenic region of a gene cassette containing tandemly arranged genes (29). The molecular event that initiates the differential expression of gp63 genes in virulent and avirulent cells is still to be determined. While all options remain open, our preliminary data obtained with a cloned segment of the gp63 gene US shows differential orientation-dependent expression of the reporter gene, both at the transcript level and at the enzymatic activity level, in virulent and avirulent cells. Our preliminary gel shift assay data for the nuclear proteins isolated from virulent and avirulent cells also raise the possibility that differential expression of gp63 genes is, at least partly, determined by the pool size of certain nuclear proteins binding to the gp63 gene US. One such protein from *L. major* has been characterized (60). The biological role of this protein in the regulation of gp63 gene expression is not yet clear (61).

We have coated avirulent cells of *L. mexicana amazonensis* with gp63 in vitro and inserted them into cultured macrophages. Increasing the surface abundance of proteolytically active gp63 increased the rate of survival of these otherwise avirulent cells inside the cultured macrophages. Chemically modified, proteolytically inactive gp63 could not offer this protection, indicating the anticytolysis is not due solely to the resistance of this parasite glycoprotein toward the lysosomal hydrolases (12). Targeted delivery of OP to the phagolysosomes inhibited this anticytolysis afforded by the coating of the avirulent cells with proteolytically active gp63. Interestingly, at high concentrations of gp63 (200 $\mu\text{g}/10^8$ cells), the avirulent cells apparently became virulent and survived inside the

J774G8 cells as did the virulent cells. This observation may suggest that promastigote surface gp63 proteinase is important for the early survival of the parasite cells inside macrophage phagolysosomes. Amastigote-specific gp63 (16, 24, 40, 52) may be induced in the parasite cells under the physicochemical pressure therein for the maintenance of infection under the altered environmental conditions (12). Again, cotargeting of the phagolysosomes containing gp63-coated avirulent cells with OP inhibited their survival under such conditions. This inhibition was not due to the nonspecific chemical interaction of OP with some other molecules and probably for its ability to chelate Zn^{2+} , as shown by the ability of Zn^{2+} to reverse the inhibitory effect of OP.

To further verify the anticytolytic activity of gp63 proteinase, we reconstituted purified gp63 on the surface of MBSA-coated liposomes to test whether this gp63 coating can protect the ^{125}I -BSA entrapped inside the liposomes from phagolysosomal degradation. Native, proteolytically active gp63 or chemically modified inactive gp63 spontaneously binds to the surface of liposomes, probably through its C-terminal phospholipid tail. Enzymatic removal of this anchor from gp63 inhibits this binding, thus suggesting that the orientation of this proteinase molecule in this reconstitution may be close to that on the parasite cell surface. The gp63 molecules purified from *L. mexicana amazonensis* amastigotes, as suggested previously (16, 24, 35, 40, 52), appeared not to be glypiated at the C terminus. Thus, pretreatment of the amastigote gp63 with phospholipase C did not prevent its binding to the liposome surface in the reconstitution experiments. As expected, the liposome-entrapped ^{125}I -BSA was dose dependently protected only when coated with native gp63 molecules, not when coated with equivalent amounts of chemically modified, proteolytically inactive gp63. This protection was precluded when the molecules were cotargeted with separate liposomes containing OP, further suggesting that the proteinase activity of gp63 is the determinant factor in the anticytolysis in question.

It is difficult to envision exactly how gp63 proteinase catalyzes anticytolysis inside the phagolysosomes. One possible

model involves continuous degradative inactivation of phagolysosomal hydrolase molecules that interact with the parasite cell body, thus creating a hydrolase-free microenvironment around the parasite cell body and preventing the cytolysis. The ability of gp63 molecules to act as a proteinase against native protein molecules at the phagolysosomal acid pH (16, 59) is very significant in this respect.

The establishment and maintenance of infection by *Leishmania* parasites inside mammalian macrophages is a complex process (12). This study does not implicate gp63 as the only virulent factor of *Leishmania* parasites, nor does it indicate the only biological role of this parasite surface molecule, but it has shown one of the important biological roles of the metalloproteinase activity of this molecule in the establishment of leishmanial parasitism inside macrophage phagolysosomes. The concerted action of other surface molecules of the parasite (12), e.g., lipophosphoglycan, to stabilize the gp63 proteinase activity inside the macrophages is very possible. The presence of multiple copies of the gp63 gene (6 to 10 copies per haploid genome) (10, 40, 41, 48) and aneuploidy in *Leishmania* parasites (20) make mutational studies on the biological function of this surface proteinase a challenging project. Biochemical studies on the possible physicochemical and biochemical interactions of other *Leishmania* surface molecules with gp63 in exerting the protective role of gp63 for *Leishmania* parasites should yield a clearer picture.

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