THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 36, Issue of September 3, pp. 25411–25418, 1999

Printed in U.S.A.

MARCKS-related Protein (MRP) Is a Substrate for the *Leishmania major* Surface Protease Leishmanolysin (gp63)*

(Received for publication, March 30, 1999, and in revised form, June 21, 1999)

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Myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein (MRP; MacMARCKS) are protein kinase C substrates in diverse cell types. Activation of murine macrophages by cytokines increases MRP expression, but infection with *Leishmania* promastigotes during activation results in MRP depletion. We therefore examined the effect of *Leishmania major* LV39 on recombinant MRP. Both live promastigotes and a soluble fraction of LV39 lysates degraded MRP to yield lower molecular weight fragments. Degradation was independent of MRP myristoylation and was inhibited by protein kinase C-dependent phosphorylation of MRP. MRP was similarly degraded by purified leishmanolysin (gp63), a *Leishmania* surface metalloprotease. Degradation was evident at low enzyme/substrate ratios, over a broad pH range, and was inhibited by 1,10-phenanthroline and by a hydroxamate dipeptide inhibitor of leishmanolysin. Using mass spectrometric analysis, cleavage was shown to occur within the effector domain of MRP between Ser^{92} and Phe^{93}, in accordance with the substrate specificity of leishmanolysin. Moreover, an MRP construct in which the effector domain had been deleted was resistant to cleavage. Thus, *Leishmania* infection may result in leishmanolysin-dependent hydrolysis of MRP, a major protein kinase C substrate in macrophages.

Myristoylated alanine-rich C kinase substrate (MARCKS)[1] and MARCKS-related protein (MRP), also known as MacMARCKS, are members of a highly acidic myristoylated family of protein kinase C (PKC) substrates (1, 2). The primary structures of MARCKS and MRP exhibit significant homology, including a highly basic stretch of amino acid residues known as the effector domain (also as the phosphorylation site domain), which contains the serine residues subject to PKC-dependent phosphorylation as well as binding sites for calmodulin and actin. Whereas MARCKS is widely distributed in diverse cell types, MRP is present primarily in brain and reproductive tissue (3, 4) as well as in macrophages, where it was first characterized (5). The biologic functions of MARCKS proteins are unknown. Due to their high effector domain homology, it is also possible that MRP and MARCKS play overlapping roles in some cells. In macrophages, both proteins colocalize in the cytosol in association with components of the actin cytoskeleton (6–9) and consequently are thought to participate in major cellular responses such as phagocytosis, motility, and membrane trafficking.

The expression of MARCKS proteins appears to be highly regulated, and *in vitro* studies have demonstrated up- or down-regulation of MARCKS at both the transcriptional and post-transcriptional levels (5, 10, 11). One mechanism of post-transcriptional regulation involves proteolytic degradation. Spizz and Blackshear (12) recently identified cathepsin B as a cellular MARCKS-cleaving enzyme in fibroblasts. They suggested that cleavage might occur within lysosomes as a result of specific lysosomal targeting sequences identified within the MARCKS primary sequence. At least one cathepsin B-dependent cleavage site was identified within the effector domain, whereas a second site was three amino acids amino-terminal to the effector domain. These results were consistent with the inhibition of MARCKS cleavage observed upon PKC-dependent phosphorylation of its effector domain serines (13).

We recently suggested that MRP levels might also be regulated by proteolysis under certain conditions (14). Activation of murine macrophages by bacterial lipopolysaccharide or cytokines strongly up-regulates MRP mRNA and protein expression (5, 14). However, infection of macrophages with promastigotes of the obligate intracellular parasite *Leishmania* at the time of activation or up to 48 h post-activation results in significant MRP depletion (14). Moreover, experiments with [^{3}H]myristate-labeled macrophages (14) and Western blot analysis of macrophage lysates using an antibody directed against the C terminus of MRP[2] revealed the appearance of lower molecular weight MRP fragments in *Leishmania*-infected cells. These studies suggested that a parasite enzyme might be capable of degrading MRP.

Leishmania promastigotes reside in the midgut of the phlebotomine sand fly and invade host macrophages during a sand fly bite. Promastigotes express a number of proteolytic enzymes that may play various roles in host invasion, evasion of the anti-parasite immune response, or degradation of host proteins (reviewed in Ref. 15). One such enzyme, the zinc metalloprotease leishmanolysin, also known as promastigote surface

* This work was supported by Grant 3100-050667.97 (to J. M.) and Grant 3100-042045.94 (to G. Schwarz, Biozentrum, University of Basel) from the Swiss National Fund for Scientific Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MARCKS, myristoylated alanine-rich C kinase substrate; MRP, MARCKS-related protein; PKC, protein kinase C; OPA, *ortho*-phenanthroline; Cbz, benzyloxycarbonyl; HPLC, high pressure liquid chromatography; PIC, protease inhibitor mixture; PAGE, polyacrylamide gel electrophoresis.

[2] S. Corradin, unpublished observations.

protease or gp63, is expressed at very high density (up to 5×10^5 molecules/cell) at the parasite surface (16), but its physiologic role is still unclear. Leishmanolysin is a glycosylphosphatidylinositol-anchored glycoprotein with neutral metalloproteinase activity for various denatured protein substrates (16), but attempts to identify a physiologically relevant substrate have been unsuccessful. However, a study of synthetic peptide substrates allowed the determination of a consensus cleavage sequence with a hydrophobic residue at the P1′ site and basic amino acid residues at the P2′ and P3′ sites (17). We show here that recombinant MRP is a substrate for leishmanolysin and that a predominant cleavage site ($Ser^{92} \downarrow Phe^{93}$-$Lys^{94}$-$Lys^{95}$) corresponds to the above-mentioned consensus sequence. Furthermore, the presence at this site of one of the two serine residues subject to PKC-dependent phosphorylation suggests that the level of MRP degradation by leishmanolysin in *Leishmania*-infected macrophages may depend on the state of effector domain phosphorylation.

EXPERIMENTAL PROCEDURES

Reagents—Pepstatin A, poly-L-lysine (~2600 Da), fatty acid-free bovine serum albumin, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Sigma. Aprotinin, leupeptin, and phenylmethylsulfonyl fluoride were purchased from Roche Molecular Biochemicals (Rotkreuz, Switzerland), and 1,10-phenanthroline (*ortho*-phenanthroline (OPA)) was from Fluka (Buchs, Switzerland). The hydroxamate-derivatized dipeptide Cbz-Tyr-Leu-NHOH (17) was a gift of Dr. Jacques Bouvier (Novartis, St. Aubin, Switzerland). The pBB131NMT plasmid was a gift from Dr. Jeffrey Gordon (Washington University School of Medicine, St. Louis, MO). Construction of the plasmid pET3dMRPHis has been described elsewhere (18). *Escherichia coli* strain JM109(DE3) and the HisBind resin were obtained from Novagen (Madison, WI). *Pfu* DNA polymerase was from Stratagene (La Jolla, CA). The *Nco*I and *Bam*HI restriction enzymes and T4 DNA ligase were from New England Biolabs Inc. (Beverly, MA). The Qiaquick kit for extraction of DNA from agarose gels was obtained from QIAGEN Inc. (Basel, Switzerland); phenyl-Sepharose was from Amersham Pharmacia Biotech (Uppsala, Sweden); and oligonucleotide primers were from Microsynth (Balgach, Switzerland).

Leishmanolysin—Proteolytically active, glycosylphosphatidylinositol-anchored leishmanolysin from *Leishmania major* LEM513 that migrated as a single 63-kDa band on SDS-polyacrylamide gels was purified as described previously (16).

Mutagenesis—Manipulation of DNA followed standard procedures (19). To delete the effector domain in the MRP gene, the megaprimer method was used (20, 21). Accordingly, three primers were synthesized: the first primer covers the sequences adjacent to the area to be deleted (5′-AGGAATCACCGGGGGCGA′); the "upstream" primer (5′-TTAATACGACTCACTATAGGG-3′) is directed against the T7 promoter region of the pET plasmid, whereas the "downstream" primer (5′GCTAGTTATTGCTCAGCGGT-3′) is complementary to the transcription termination region of the pET plasmid. The deletion mutation was introduced by polymerase chain reaction using *Pfu* DNA polymerase and the pET3dMRPHis plasmid (18) as the template. Guidelines provided by the supplier of the *Pfu* DNA polymerase were followed. In the first round of polymerase chain reaction, the mutagenic primer and the upstream primer were used to generate a megaprimer that was purified by agarose gel electrophoresis, followed by QIAGEN extraction. In the second round of polymerase chain reaction, the megaprimer and the downstream primer were employed. The reaction product was purified as described above; double-digested with *Nco*I/*Bam*HI; and after purification, ligated with the *Nco*I/*Bam*HI-double-digested pET3dMRPHis plasmid using T4 DNA ligase. The ligation mixture was used to transform *E. coli* strain JM109(DE3) by electroporation. The bacteria were plated on LB agar in the presence of 50 μg/ml ampicillin, and transformants harboring the plasmid (named pET3dMRPΔ(82–112)His) were selected based on their ability to express high levels of the unmyristoylated form of MRP-Δ(82–112) (amino acid residues are numbered starting from the N-terminal Gly residue of MRP). Plasmid DNA from several colonies was sequenced (Microsynth) to confirm deletion of the effector domain in the MRP gene.

Recombinant MRPs—For recombinant unmyristoylated MRP and MRP-Δ(82–112), we used MRP constructs containing the thrombin-cleavable His tag at the C terminus (LVPRGSSSGHHHHHH), which were expressed in *E. coli* JM109(DE3) as described above. To obtain myristoylated MRP, the pET3dMRPHis plasmid was electroporated into *E. coli* JM109(DE3)pBB131NMT, a strain harboring a plasmid coding for myristoyl-CoA:protein *N*-myristoyltransferase; and transformants growing in the presence of 50 μg/ml ampicillin and kanamycin each were screened for expression of myristoylated MRP after induction with isopropyl-β-D-thiogalactopyranoside. Large-scale cultures for the production of recombinant proteins were grown, induced, and harvested as described (18, 22). MRPs were purified as described (18) using a modification of a previously published procedure (22). Briefly, cells were lysed, and the supernatant was applied to a HisBind column saturated with Ni^{2+}. Proteins were eluted with an imidazole gradient and applied to a phenyl-Sepharose column. Myristoylated MRP is retained on the column in the presence of 3 M NaCl and eluted by decreasing the NaCl concentration to 1.5 M. The unmyristoylated proteins, on the other hand, are not retained in the presence of 3 M NaCl and elute in the flow-through of the column. Fractions containing the purified proteins were combined, dialyzed in the presence of 2.5 mM imidazole (pH 7.4), concentrated, frozen in aliquots in liquid nitrogen, and stored at −80 °C until used.

MRP is a highly anionic protein despite the presence of a basic cluster within the effector domain and thus exhibits anomalous migration on SDS gels. The calculated molecular mass of native murine MRP (myristoylated, 199 amino acids) is 20,244 Da, but it is recognized as a 42–45-kDa doublet on Western blots (23, 24). The His-tagged MRP constructs used in this study also exhibit anomalous migration (18). Unmyristoylated MRP contains 214 residues with a calculated molecular mass of 21,698 Da and migrates on 15% polyacrylamide gels with an apparent molecular mass of 46 kDa; myristoylated MRP has a calculated molecular mass of 21,908 Da and an apparent molecular mass of 48 kDa. Unmyristoylated effector domain-deficient MRP (MRPΔ(82–112)), in which the 24 amino acid residues of the effector domain and flanking residues 82–85 (KETP) and 110–112 (EGG) are deleted, has 183 residues, a calculated molecular mass of 18,061 Da, and an apparent molecular mass of 39 kDa. Phosphorylation of MRPs was performed with the catalytic subunit of PKC as described previously (25, 26).

Effector Domain Peptides—A 24-amino acid peptide corresponding to the effector domain of murine MRP (KKKKKFSFKKPFKLSGLSFKRNRK) was synthesized using solid-phase Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry in an Applied Biosystems 431A peptide synthesizer (Perkin-Elmer International Inc., Rotkreuz, Switzerland). The crude polypeptide was purified by reversed-phase HPLC on a Vydac C_{18} column (250 × 10 mm) using a 0.1% trifluoroacetic acid-containing H_2O/acetonitrile gradient with a flow rate of 3 ml/min. Purity was >90% as determined by HPLC. The corresponding 25-amino acid bovine MARCKS peptide (KKKKKRFSFKKSFKLSGFSFKKNKK) was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). The molecular masses of both peptides were confirmed by mass spectrometry.

Leishmania—L. major promastigotes (strain MRHO/SU/59/P designated as LV39) were grown at 26 °C in Dulbecco's minimal essential medium on blood agar (27). Stationary phase promastigotes were washed three times in phosphate-buffered saline and resuspended in phosphate-buffered saline at $10^6/\mu l$ for experiments measuring the effect of live parasites on recombinant MRP. For preparation of LV39 lysates, washed parasites were resuspended at $1.2 \times 10^6/\mu l$ in phosphate-buffered saline containing protease inhibitor mixture (PIC; 5 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and sonicated for 10 s. Lysates were then used directly for incubation with MRP or centrifuged at 80,000 rpm for 20 min at 4 °C in a Beckman Airfuge. The supernatant (referred to as LV39 SN) was removed and stored at −20 °C until use. For some experiments, supernatants were heat-inactivated at 95 °C for 5 min, followed by 5 min on ice and centrifugation at 11,000 × *g* for 5 min.

MRP Degradation—For Western blot analysis, 30 ng of myristoylated MRP were incubated with LV39 promastigotes or LV39 lysates in 20 μl of 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl containing 0.4 mg/ml fatty acid-free bovine serum albumin and fresh PIC. Reaction mixtures were then centrifuged for 5 min at 11,000 × *g*, and supernatants were removed and heated for 5 min at 95 °C to obtain heat-stable fractions. After 5 min on ice, samples were centrifuged again for 5 min, and supernatants were collected for analysis of MRP degradation. For analysis by Coomassie Blue staining of SDS-polyacrylamide gels, 2 μg of myristoylated or unmyristoylated MRP were incubated with LV39 SN or purified leishmanolysin in 20 μl of 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl as indicated. Samples were then heated to 95 °C for 5 min, and heat-stable fractions were obtained as described.

SDS-PAGE and Western Blot Analysis—Western blot analysis was

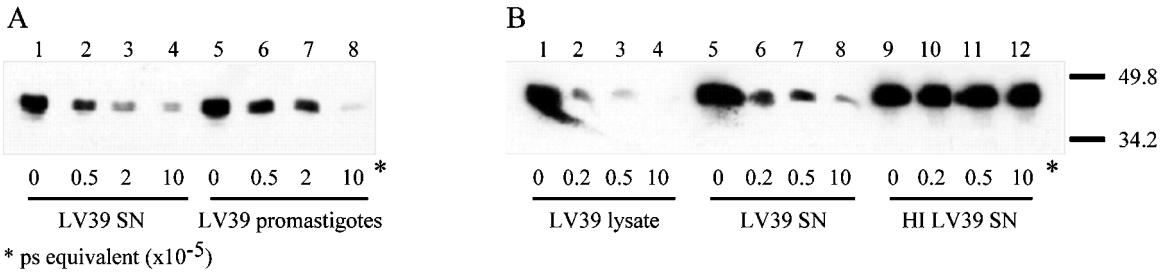

FIG. 1. **Degradation of MRP by *L. major* LV39.** Myristoylated MRP (30 ng) in buffer containing PIC was incubated for 15 min at room temperature with increasing numbers of live LV39 promastigotes (*A*, *lanes 5–8*) or equivalent amounts of LV39 SN (*lanes 1–4*) or with whole LV39 lysate (*B*, *lanes 1–4*), LV39 SN (*lanes 5–8*), or heat-inactivated (*HI*) LV39 SN (*lanes 9–12*). Reaction mixtures were processed as described under "Experimental Procedures," and samples were examined for MRP degradation by Western blot analysis. The *asterisks* indicate parasite (*ps*) equivalent.

performed as described previously (14). The anti-MRP antibody used recognizes both myristoylated and unmyristoylated MRPs as well as MRP phosphorylated *in vitro* by the catalytic subunit of PKC (25).[3] Alternatively, MRP and its degradation products were visualized on SDS-polyacrylamide gels by staining with Coomassie Brilliant Blue R (Serva, Heidelberg, Germany) and destained with 10% isopropyl alcohol and 5% acetic acid in H_2O. SDS-polyacrylamide gels or films exposed to chemiluminescent blots were scanned on a ScanJet 4c/T densitometer (Hewlett-Packard, Geneva, Switzerland) using the Adobe Photoshop software package and NIH Image 1.60 software.

pH Optimum—For determination of pH optimum, MRP was incubated with leishmanolysin in Britton-Robinson universal buffer (28) containing 15 mM each boric acid, sodium citrate, sodium barbital, and NaH_2PO_4 adjusted from pH 4 to 12 with NaOH.

Mass Spectrometry—Unmyristoylated MRP, MRP effector domain peptide, or MARCKS effector domain peptide was incubated with or without leishmanolysin in 10 mM NH_4 acetate buffer (pH 7.0) as indicated. Reactions were terminated by heating to 95 °C for 5 min. One μl of sample was placed into a well of the mass spectrometer sample plate and air-dried at room temperature. One μl of a saturated solution of sinapinic acid (Aldrich, Buchs) in 0.1% trifluoroacetic acid-containing acetonitrile/H_2O (1:2) for MRP samples or α-cyano-4-hydroxycinnamic acid (Sigma, Buchs) in 0.1% trifluoroacetic acid-containing acetonitrile/H_2O (1:1) for effector domain peptide samples was then added to the same well and air-dried at room temperature. Matrix-assisted laser desorption ionization time-of-flight analysis was performed using a time-of-flight mass spectrometer (Voyager-DE, PerSeptive Biosystem, Framingham, MA) equipped with a nitrogen laser (λ = 317 nm) to desorb and ionize the sample; the accelerating voltage was 30 kV. Ion masses were assigned based on an external mass calibration using two points that bracketed the mass range of interest for MRP samples or using bovine insulin (molecular mass $[M + H]^+$ = 5734.59 Da) as an internal standard for effector domain peptide samples. Relative intensities in the matrix-assisted laser desorption ionization spectra are not strictly proportional to relative abundance of the species due to intrinsic variability in the desorption process.

RESULTS

MRP Degradation by Live Leishmania Parasites or Parasite Lysates—To determine whether *Leishmania* parasites are capable of exerting a direct degradative effect on MRP, we first performed Western blot analysis of recombinant myristoylated MRP incubated with intact live LV39 promastigotes. As shown in Fig. 1*A*, MRP levels decreased as a function of parasite number. Moreover, an ultracentrifugation supernatant fraction of the LV39 lysate (referred to as LV39 SN) appeared to be nearly as effective as whole parasites. We then compared whole lysate with LV39 SN or with LV39 SN that had been maintained at 95 °C for 5 min. Fig. 1*B* shows the results of incubating MRP with these parasite fractions in the presence of PIC containing pepstatin, phenylmethylsulfonyl fluoride, leupeptin, and aprotinin. Both whole lysate and LV39 SN dose-dependently decreased MRP levels, whereas heated LV39 SN was no longer effective. These results suggested that an enzyme that is insensitive to the inhibitor mixture might be responsible for the disappearance of immunoreactive MRP.

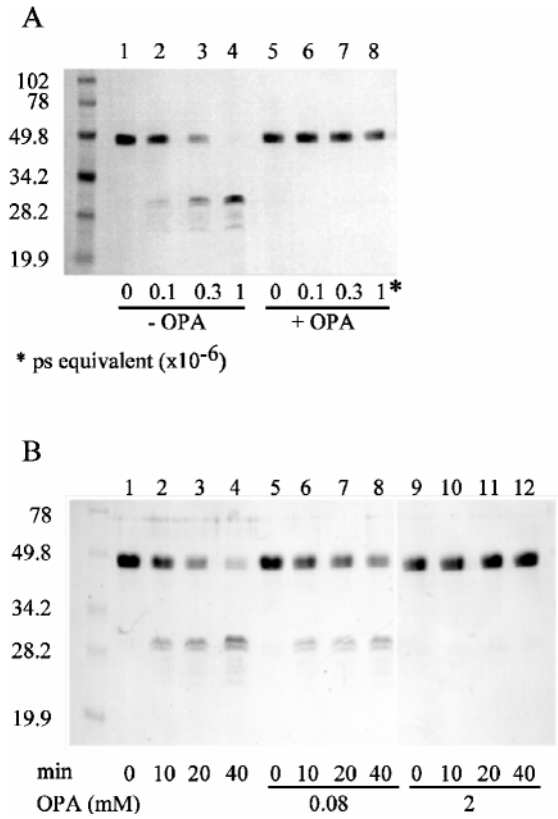

FIG. 2. **Dose and time dependence of MRP degradation by LV39 SN.** Myristoylated MRP (2 μg) in buffer containing PIC was incubated for 15 min at room temperature with increasing concentrations of LV39 SN in the absence (*A*, *lanes 1–4*) or presence (*lanes 5–8*) of 2 mM OPA or for increasing amounts of time with LV39 SN equivalent to 10^6 parasites in the absence (*B*, *lanes 1–4*) or presence (*lanes 5–12*) of OPA. Heat-stable supernatant fractions of the reaction mixtures were assessed for MRP degradation by SDS-PAGE and Coomassie Blue staining. The *asterisk* indicates parasite (*ps*) equivalent.

Inhibition of MRP Degradation by OPA—One *Leishmania* enzyme that is not inhibited by the protease inhibitors listed above is the surface metalloprotease leishmanolysin. We therefore tested the effect of LV39 SN in the presence and absence of OPA, a zinc chelator that is known to inhibit leishmanolysin activity for a variety of protein and peptide substrates when used at millimolar concentrations (17). For the following experiments, we employed an alternative assay that did not rely on antibody recognition of MRP. This method takes advantage of the heat stability of MARCKS proteins (5) to remove the ma-

[3] G. Vergères, unpublished observations.

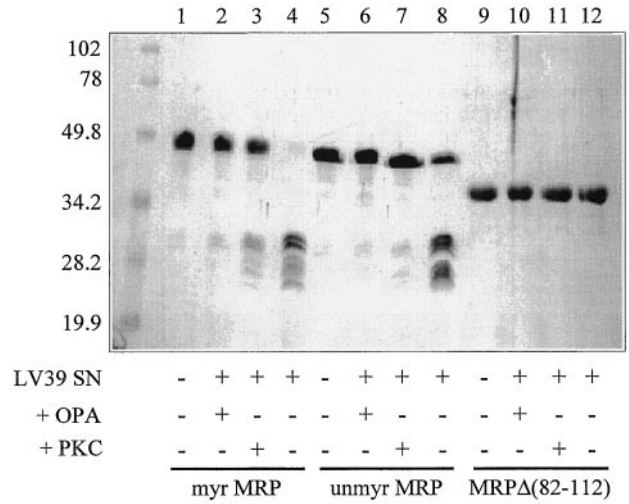

FIG. 3. **Effect of substrate modification on MRP degradation by LV39 SN.** LV39 SN equivalent to 10^6 parasites with or without 2 mM OPA was incubated for 1 h at room temperature with 2 μg (5 μM) of myristoylated (*myr*) MRP (*lanes 1–4*), unmyristoylated (*unmyr*) MRP (*lanes 5–8*), or effector domain-deficient MRP (MRPΔ(82–112)) (*lanes 9–12*). For *lanes 3*, *7*, and *11*, the different MRPs were allowed to undergo PKC-dependent phosphorylation (+ *PKC*) prior to addition of LV39 SN. Heat-stable supernatant fractions of the reaction mixtures were assessed for MRP degradation by SDS-PAGE and Coomassie Blue staining.

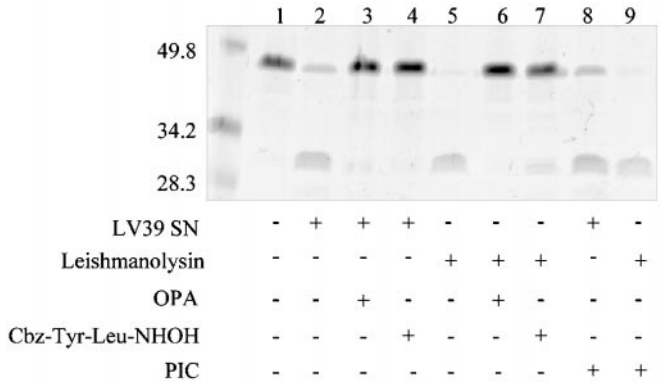

FIG. 4. **Degradation of MRP by leishmanolysin or LV39 SN and effect of inhibitors of leishmanolysin.** MRP (5 μM) was incubated with LV39 SN equivalent to 10^6 parasites (*lanes 2–4* and *8*) or with 40 nM purified leishmanolysin (*lanes 5–7* and *9*) in the absence or presence of 2 mM OPA, 2 mM Cbz-Tyr-Leu-NHOH, or PIC for 1 h at room temperature. Heat-stable supernatant fractions of the reaction mixtures were assessed for MRP degradation by SDS-PAGE and Coomassie Blue staining.

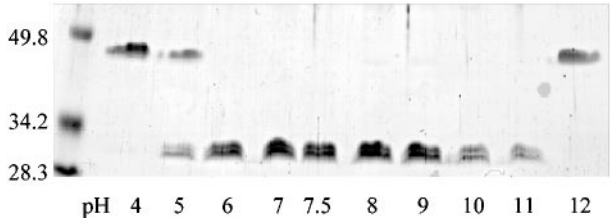

FIG. 5. **pH dependence of MRP degradation by leishmanolysin.** MRP (5 μM) was incubated with 4 nM leishmanolysin in 15 mM Britton-Robinson universal buffer at different pH values for 1 h at 37 °C. Heat-stable supernatant fractions of the reaction mixtures were assessed for MRP degradation by SDS-PAGE and Coomassie Blue staining.

jority of LV39 SN proteins before analysis by SDS-PAGE and Coomassie Blue staining. Indeed, no proteins were revealed in gels containing LV39 SN alone (data not shown). As demonstrated in Fig. 2*A*, LV39 SN decreased MRP levels in a dose-dependent manner, in agreement with Western blot analysis. Moreover, this assay allowed the visualization of lower molecular weight degradation products in addition to intact MRP. Degradation of MRP was completely inhibited by 2 mM OPA. Fig. 2*B* shows a representative time course of MRP disappearance and again the inhibition by OPA.

Degradation of Different Molecular Forms of MRP by LV39 SN—The above experiments were performed with myristoylated MRP. We then examined the effect of LV39 SN on different molecular forms of MRP including myristoylated MRP, unmyristoylated MRP, or unmyristoylated MRP lacking the effector domain (MRPΔ(82–112)). Each of these proteins was also tested after phosphorylation by the catalytic subunit of PKC. Although both myristoylated and unmyristoylated MRPs were degraded by LV39 SN in an OPA-inhibitable manner, MRPΔ(82–112) or the phosphorylated MRPs were largely unaffected (Fig. 3), suggesting that cleavage occurred within the effector domain close to a phosphorylation site.

MRP Degradation by Purified Leishmanolysin—As shown in Fig. 4, the proteolytic activities of purified leishmanolysin and LV39 SN on MRP were indistinguishable, both in their generation of degradation products and in their sensitivity to the metalloprotease inhibitors OPA and Cbz-Tyr-Leu-NHOH, a more specific inhibitor of leishmanolysin (17). Inhibitors affecting other classes of proteases had no effect. Cleavage of MRP by leishmanolysin occurred at enzyme/substrate ratios as low as 1:50,000 (data not shown), which compares favorably with conditions used previously with peptide substrates (17). The activity was dependent on both time and temperature of incubation and occurred over a broad, mostly alkaline range of pH, which is characteristic of leishmanolysin (Fig. 5). As expected, no degradation was observed at pH 12, in agreement with results demonstrating irreversible inactivation of leishmanolysin at pH 11.5 and higher (17).

Inhibition of MRP Degradation by Effector Domain Peptides of MRP or MARCKS—The experiments described above for effector domain-deficient MRP suggested that this domain was necessary for leishmanolysin degradation of MRP. A 10-fold molar excess of peptide covering the effector domain of MRP completely blocked leishmanolysin-dependent MRP degradation (Fig. 6). The homologous MARCKS effector domain peptide was somewhat less efficient. Since these peptides contain a high number of basic lysine residues, we also tested poly-L-lysine, which was inactive under the same conditions (Fig. 6).

Mass Spectrometric Analysis of MRP Degradation Products—As shown in Fig. 7, MRP contains two sites, $Ser^{92} \downarrow Phe^{93}$-$Lys^{94}$-$Lys^{95}$ (site a) and $Ser^{103} \downarrow Phe^{104}$-$Lys^{105}$-$Arg^{106}$ (site c), that closely correspond to the consensus cleavage site of leishmanolysin (17). These sites are both present within the effector domain and include serines 92 and 103, which are subject to PKC-dependent phosphorylation. Fig. 8 shows the mass spectrometric analysis of unmyristoylated MRP and its leishmanolysin-dependent degradation products. The intact molecule gave a major peak at 22,183.0 Da (Fig. 8*A*), considerably larger than its calculated molecular mass of 21,698 Da. The peak at 11,086.2 Da corresponds to the double-charged species. Two additional peaks (22,058.9 and 21,765.7 Da in Fig. 8*A*) were consistently observed as shoulders to the larger peak. Upon incubation with leishmanolysin, a major product of 9166.0 Da was obtained (Fig. 8*B*), which exactly corresponded to the N-terminal peptide expected to result from cleavage at site a (see Fig. 7) (calculated molecular mass $[M + H]^+ = 9165.8$ Da). A minor peak at 13,024.2 Da most probably

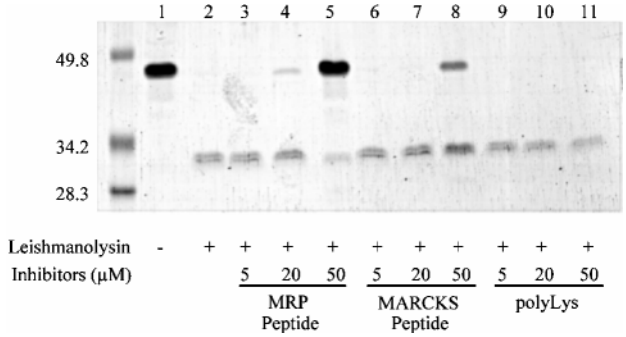

FIG. 6. **Inhibition of leishmanolysin degradation of MRP by effector domain peptides of MRP or MARCKS.** MRP (5 μM) was incubated with 1 nM leishmanolysin for 1 h at 37 °C in the absence (*lane 2*) or presence of 5, 20, or 50 μM MRP effector domain peptide (*lanes 3–5*, respectively); MARCKS effector domain peptide (*lanes 6–8*, respectively); or poly-L-lysine (*polyLys*; *lanes 9–11*, respectively). Heat-stable supernatant fractions of the reaction mixtures were assessed for MRP degradation by SDS-PAGE and Coomassie Blue staining.

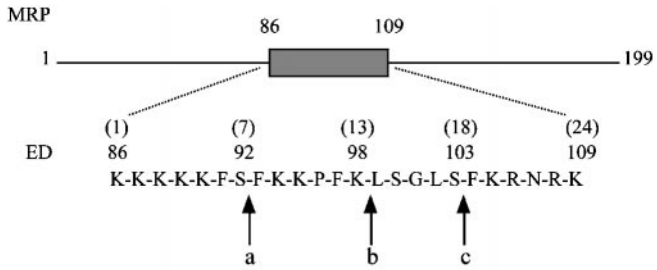

FIG. 7. **Consensus leishmanolysin cleavage sites within the effector domain of MRP.** Shown is the amino acid sequence of the murine MRP effector domain (*ED*). *Arrows a* and *c* represent leishmanolysin sites based on a consensus site where P1′ is a hydrophobic amino acid residue and P2′ and P3′ are basic residues; also shown (*arrow b*) is a cleavage site analogous to a previously described cathepsin B cleavage site in MARCKS (12). Amino acid residues are numbered according to their positions within the intact MRP molecule; the corresponding residue positions in the effector domain peptide are indicated in *parentheses*.

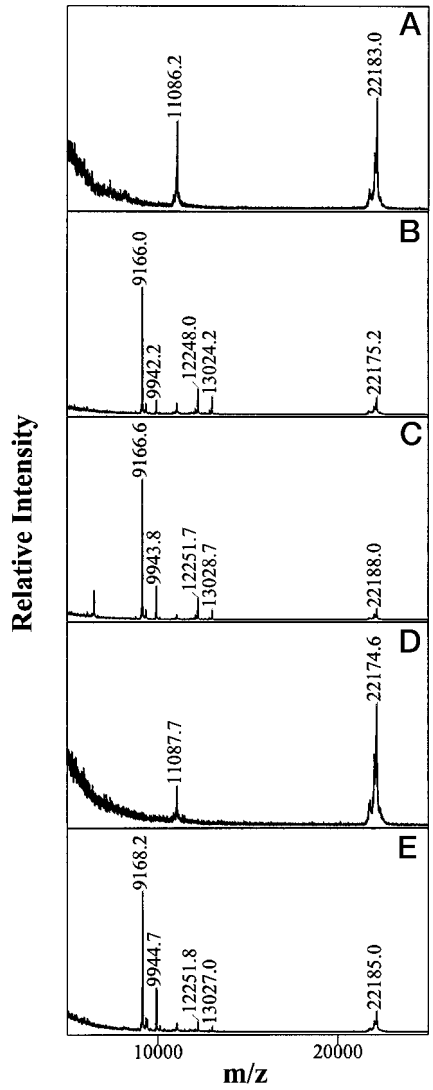

FIG. 8. **Mass spectrometry of MRP and leishmanolysin-generated peptides.** Unmyristoylated MRP (25 μM) was incubated for 1 h at 37 °C alone (*A*) or with 10 nM leishmanolysin (*B*), leishmanolysin plus PIC (*C*), leishmanolysin plus 2 mM OPA (*D*), or LV39 SN equivalent to 10^6 parasites (*E*). Heat-stable products were analyzed by mass spectrometry.

represents the corresponding C-terminal peptide (expected value of 13,028.2 Da based on the intact MRP peak at 22,175.2 Da). Generation of these peptides was unaffected by PIC (Fig. 8*C*), but was completely inhibited by OPA (Fig. 8*D*). Despite the heterogeneity observed for intact MRP, the 9166-Da peptide was always present as a single peak. In addition to this major peptide, minor peaks (9942.2 and 12,248.0 Da in Fig. 8*B*) were consistently observed that could represent the N- and C-terminal peptides resulting from cleavage at site b (calculated molecular mass $[M + H]^+$ of the N-terminal fragment = 9941.8 Da; expected value for the C-terminal fragment = 12,252.0 Da based on the intact MRP peak at 22,175.2 Da). This site is analogous to a site described earlier for cathepsin B cleavage of MARCKS (12). Although some other minor peaks were observed, peptides that might result from cleavage at site c (molecular mass $[M + H]^+$ = 10,399 and 11,803 Da) were not detected. Finally, a similar analysis using LV39 SN in place of purified leishmanolysin resulted in the generation of identical products (Fig. 8*E*), providing strong evidence that leishmanolysin is responsible for the MRP-cleaving activity in LV39 SN.

We then performed a similar mass spectrometric analysis using the effector domain peptide as substrate. As shown in Fig. 9, incubation with leishmanolysin in the presence of PIC generated a 2082.4-Da product corresponding to the C-terminal Phe^8–Lys^{24} peptide (calculated molecular mass $[M + H]^+$ = 2082.5 Da) resulting from cleavage at site a. Although earlier time points were examined in the presence or absence of protease inhibitors (data not shown), the corresponding N-terminal 893-Da peptide was never observed. In addition to the 2082-Da peptide, a second peak at 1306 Da was observed in some experiments. This product most probably represents the C-terminal Leu^{14}–Lys^{24} peptide (calculated molecular mass $[M + H]^+$ = 1305.8 Da) resulting from cleavage at site b. As mentioned above, this site corresponds to an earlier described cathepsin B cleavage site in MARCKS (12).

DISCUSSION

We previously demonstrated that macrophages infected with *Leishmania* promastigotes express very low levels of MRP (14). Depletion of intracellular MRP was demonstrated by several methods, including Western blot analysis, immunofluorescence microscopy, and biosynthetic labeling with myristic acid. In the latter case, the appearance of lower molecular weight labeled products suggested that a proteolytic activity might be responsible for decreasing MRP levels. Such a mechanism appeared particularly interesting in light of recent studies by Spizz and Blackshear (13) demonstrating that cellular concentrations of

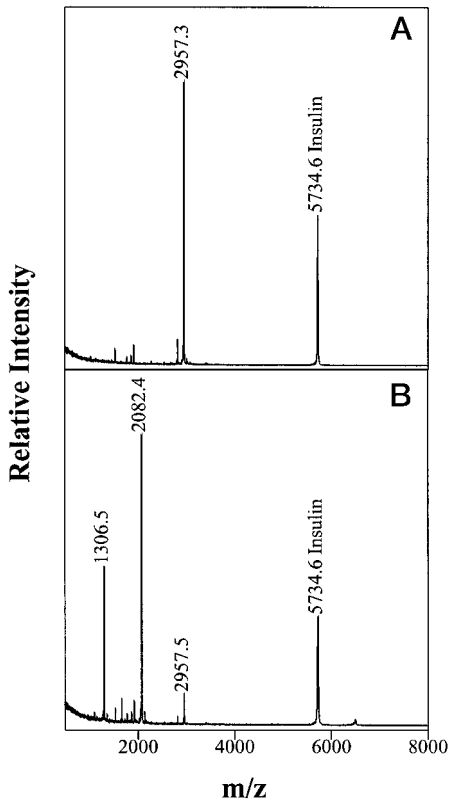

FIG. 9. **Mass spectrometry of MRP effector domain peptide and leishmanolysin-generated fragments.** The MRP effector domain peptide (30 μM) was incubated alone (*A*) or with 10 nM leishmanolysin plus PIC (*B*) for 1 h at 37 °C. Heat-stable reaction products were analyzed by mass spectrometry using bovine insulin as an internal standard.

MARCKS are regulated by proteolysis. A cellular MARCKS-cleaving enzyme was identified as cathepsin B, and the purified enzyme was later shown to cleave MARCKS within its basic effector domain in a cell-free system (12). In the present study, we investigated whether a proteolytic activity associated with *Leishmania* might similarly exhibit MRP-cleaving activity.

MRP and MARCKS contain very homologous effector domains, suggesting that cysteine protease activity could be responsible for MRP depletion in infected macrophages. *Leishmania* parasites are known to express multiple cysteine proteases, some of which display cathepsin B-like activity (29, 30). Although MARCKS degradation in fibroblasts could be inhibited by raising lysosomal pH with NH_4Cl (13), we were unable to inhibit MRP depletion in macrophages treated with NH_4Cl under conditions previously shown in our laboratory to increase intravesicular pH in infected macrophages (31).[2] Moreover, the cysteine protease inhibitor leupeptin was unable to block MRP degradation by *Leishmania* parasites or by a soluble fraction of parasite lysates in a cell-free system. Using direct staining of MRP and its products on SDS-polyacrylamide gels, the appearance of at least two molecular species was observed with apparent molecular masses of ~29–30 and 25–26 kDa. The larger species was generally more strongly stained than the smaller product. However, it was impossible to draw information concerning actual molecular size from such experiments since these smaller fragments apparently exhibited the same anomalous migration on SDS gels as parent MRP. Similar conclusions were made by Spizz and Blackshear (13) for the MARCKS p40 product.

In contrast to the lack of effect of PIC, the zinc chelator OPA completely blocked MRP degradation by LV39 SN or intact LV39 promastigotes, supporting our suspicions that MRP-cleaving activity was due to leishmanolysin, a zinc metalloprotease expressed at relatively high density on the surface of *Leishmania* promastigotes. Indeed, purified leishmanolysin exhibited similar activity as LV39, and both enzyme activities were inhibited by a more specific leishmanolysin inhibitor, Cbz-Tyr-Leu-NHOH. Taken together, our results strongly suggest that the MRP-cleaving activity of LV39 is due to leishmanolysin. Although leishmanolysin is primarily associated with the promastigote surface via a glycosylphosphatidylinositol anchor, a soluble form of the same enzyme has been described (32), which may account for its presence in LV39 SN.

The myristoylation state of MRP did not affect leishmanolysin activity, as both myristoylated and unmyristoylated MRPs were degraded by LV39 SN. However, MRPΔ(82–112) was not degraded, suggesting the presence of a cleavage site for leishmanolysin within the effector domain. In accordance with these results, an excess of MRP effector peptide (and, to a lesser extent, of MARCKS effector peptide; see below) protected MRP from degradation by leishmanolysin. Moreover, MRP subjected to PKC-dependent phosphorylation was resistant to leishmanolysin, strongly suggesting that the unphosphorylated serine residues of the effector domain are part of the leishmanolysin recognition sequence. These data are in line with those of Spizz and Blackshear (12, 13), who found that PKC-phosphorylated MARCKS was a poor substrate for cathepsin B. Similarly, decreased susceptibility of MARCKS to cathepsin L after phosphorylation by PKC has been reported (33), and regulation of proteolysis by substrate phosphorylation was also described for the related GAP-43 protein (34).

The consensus cleavage site of leishmanolysin is characterized by a P1′ hydrophobic residue and P2′ and P3′ basic residues, with P1 often, but not always, a tyrosine residue (17). There are two consensus cleavage sites for leishmanolysin within the MRP effector domain, $Ser^{92} \downarrow Phe^{93}$-$Lys^{94}$-$Lys^{95}$ (site a) and $Ser^{103} \downarrow Phe^{104}$-$Lys^{105}$-$Arg^{106}$ (site c), each of which contains a serine residue subject to phosphorylation (see Fig. 7). The results of mass spectrometric analysis of MRP suggested that the N-terminal peptide resulting from cleavage at site a was indeed a major reaction product. Minor peaks representing the corresponding C-terminal peptide as well as the C- and N-terminal peptides resulting from cleavage at site b were also consistently observed. Interestingly, $Lys^{98} \downarrow Leu^{99}$ corresponds to a major site of MARCKS effector peptide cleavage by purified cathepsin B (12). We can rule out a possible effect of contaminating cysteine protease in our studies because the same peak was observed in samples containing leupeptin, but disappeared in the presence of OPA. Peptides resulting from cleavage at site c were never detected. Results suggesting that leishmanolysin cleaves MRP at site a were further confirmed by the use of the MRP effector peptide as substrate. In this case, the C-terminal peptide product resulting from cleavage between Ser^7 and Phe^8 was detected. The seven-amino acid N-terminal peptide containing five lysine residues was never observed. The short N-terminal peptide resulting from cathepsin B cleavage of the MARCKS effector domain peptide was also undetectable using an alternative method, HPLC (12), suggesting that further degradation may occur within these hydrophilic fragments. As for intact MRP, a second product of the effector domain peptide, most probably resulting from cleavage at site b, was also observed, although not in all experiments (data not shown).

We cannot presently explain the difference in the calculated molecular mass of MRP and our results (21,698 *versus* 22,183 Da) or the finding of at least three peaks in the recombinant preparation. Since sequencing results obtained for our con-

structs are consistent with published data,[3] this could be due to heterogeneity that occurs during synthesis or to modification of the C-terminal portion of the protein (which includes the His tag) during purification or mass spectrometric analysis.

A recent study of the crystal structure of *L. major* leishmanolysin revealed the presence of a large region of negative charge surrounding the active-site cleft (35), which might suggest binding to basic substrates such as the N-terminal portion of the MARCKS or MRP effector domains. Surprisingly, we were unable to demonstrate significant degradation of the MARCKS effector peptide by spectrometric analysis under conditions used to assess cleavage of the MRP peptide (data not shown). The MARCKS effector domain contains two identical Ser-Phe-Lys-Lys sequences and might have been expected to exhibit high sensitivity to leishmanolysin based on our results with MRP. One must, however, consider the possibility that the additional amino acid residue present in the MARCKS effector domain (an arginine at position 7 immediately prior to the putative $Ser^8 \downarrow Phe^9$ cleavage site) might influence enzyme binding and/or activity. In agreement with these mass spectrometry results, we found that the MARCKS effector domain peptide was a somewhat less efficient inhibitor of MRP degradation when compared with the MRP peptide, suggesting that leishmanolysin exhibits some selectivity for MRP as substrate. However, it will be important to examine the effect of leishmanolysin on recombinant MARCKS protein to confirm this conclusion.

The question remains as to how leishmanolysin, which is presumably restricted to the phagosomal/phagolysosomal compartment, might have access to MRP within the macrophage. Spizz and Blackshear (12) addressed a similar question in attempting to explain how fibroblast lysosomal cathepsin B would be expected to exert MARCKS-cleaving activity since MARCKS, like MRP, is generally thought to be confined to the cytosol, where it associates with different membranes and organelles. A putative LAMP1-specific sequence was identified within the MARCKS sequence, which might allow targeting to lysosomes, resulting in eventual uptake and processing by cathepsin B, but this remains to be demonstrated. However, it has been shown that *Leishmania* promastigotes are contained within a LAMP1-negative compartment of host macrophages (36). Moreover, leishmanolysin displayed a pH optimum of 6–9, with little MRP-cleaving activity at pH 4, suggesting that degradation would be less efficient within the phagolysosome than within the neutral cytosolic compartment. In this regard, Rittig *et al.* (37) recently reported that some intracellular promastigotes of *L. major* may be localized in the cytosol of infected macrophages. Alternatively, one might hypothesize that a low level of leishmanolysin is somehow released from the promastigote (perhaps during parasite death) and eventually finds its way into the cytosol. This would not require a high percentage of the total enzyme present on the one to five parasites in each cell. Indeed, we showed that an enzyme/MRP substrate ratio as low as 1:50,000 can result in significant proteolysis in a cell-free system. As the total MRP present in a macrophage varies from ~0.1 to 1 ng/μg of total cell protein depending on the state of cell activation (14), and as *L. major* promastigotes express up to 5×10^5 molecules of leishmanolysin/cell (16), it can be calculated that as little as 0.001–0.01% of the total enzyme might be sufficient to cause detectable MRP depletion in infected macrophages.

We reported that *Leishmania donovani* was considerably less efficient than LV39 in down-regulating MRP expression in infected macrophages (14). Similarly, amastigotes of LV39 were less active than their promastigote counterparts.[2] In this regard, it should be noted that promastigotes of some *L. donovani* strains, including LV636 used in our studies, naturally express lower levels of leishmanolysin than LV39 (38)[2] and that amastigotes of LV39 express 100-fold less enzyme than promastigotes (39), which reinforces the idea that the proteolytic degradation of MRP observed with infected macrophages is in fact due to leishmanolysin. Definitive proof may require the generation of a leishmanolysin knockout parasite in which all of the multiple gene copies encoding leishmanolysin are deleted or the development of specific inhibitors of leishmanolysin that are membrane-permeable and nontoxic for macrophages.

Taken together, our results clearly demonstrate that the PKC substrate MRP is an excellent leishmanolysin substrate. Definition of one major cleavage site within the MRP effector domain may provide important information for further characterization of leishmanolysin enzyme activity as well as for identification of other potential physiologic substrates in the macrophage or within the sand fly vector. Moreover, the MRP cleavage site defined in our studies may provide the basis for developing more potent and specific inhibitors of leishmanolysin and possibly of the parasite itself. Although MRP degradation by leishmanolysin remains to be demonstrated *in vivo*, the possibility that functional alterations observed in *Leishmania*-infected macrophages are related to decreased MRP levels must be considered.

Acknowledgments—We are grateful to Dr. Denis Rivier for the generous gift of purified leishmanolysin used in our preliminary experiments. We thank Dr. Jacques Bouvier for the gift of Cbz-Tyr-Leu-NHOH and for helpful discussion. We also thank Luis Rodrigues for mass spectrometric analyses and Jeannette Holenstein for technical assistance.

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MARCKS-related Protein (MRP) Is a Substrate for the*Leishmania major* Surface Protease Leishmanolysin (gp63)

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J. Biol. Chem. 1999, 274:25411-25418.
doi: 10.1074/jbc.274.36.25411
