Novel peptide inhibitors of *Leishmania* gp63 based on the cleavage site of MARCKS (myristoylated alanine-rich C kinase substrate)-related protein

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The zinc metalloprotease gp63 (leishmanolysin; promastigote surface protease) is expressed at high density at the surface of *Leishmania* promastigotes. Efficient non-toxic inhibitors of gp63 do not exist, and its precise role in parasite physiology remains unknown. MARCKS (myristoylated alanine-rich C kinase substrate) and MARCKS-related protein (MRP; MacMARCKS) are protein kinase C substrates in various cells, including macrophages. We reported previously that MRP is an excellent substrate for gp63. A major cleavage site was identified within the MRP effector domain (ED), a highly basic 24-amino-acid sequence, and the synthetic ED peptide (MRP_{ED}) was shown to inhibit MRP hydrolysis. In the present study, MRP cleavage was used as an assay to measure the capacity of various MRP or MARCKS ED peptides to block gp63 activity. On a molar basis, MRP_{ED} inhibited gp63 to a greater extent than two previously described gp63 inhibitors, *o*-phenanthroline and benzyl-

oxycarbonyl-Tyr-Leu-NHOH. MARCKS_{ED} analogues containing modifications in the gp63 consensus cleavage site showed significant differences in inhibitory capacity. As phosphorylation of ED serine residues prevented gp63-mediated MRP degradation, we synthesized a pseudophosphorylated peptide in which serine residues were substituted by aspartate (3DMRP_{ED}). 3DMRP_{ED} was a highly effective inhibitor of both soluble and parasite-associated gp63. Finally, MRP ED peptides were synthesized together with an N-terminal HIV-1 Tat transduction domain (TD) to obtain cell-permeant peptide constructs. Such peptides retained gp63 inhibitory activity and efficiently entered both macrophages and parasites in a Tat TD-dependent manner. These studies may provide the basis for developing potent cell-permeant inhibitors of gp63.

Key words: metalloprotease, Tat, transduction.

INTRODUCTION

Leishmania parasites are protozoa that can infect humans and several other mammals. During their promastigote developmental stage, the parasites reside in the midgut of the phlebotomine sandfly vectors. Promastigotes are injected into the skin of the host during a sandfly bite, whereafter they invade resident macrophages, transform into amastigotes, and initiate infection. Although little information is available regarding factors that promote infectivity, protozoan parasites are known to express various proteolytic enzymes that are presumably important for host cell invasion, evasion of an immune response or degradation of host proteins (reviewed in [1]). Of particular interest in this context is the zinc metalloprotease gp63 (leishmanolysin; promastigote surface protease) that is expressed at high density as a glycosylphosphatidylinositol (GPI)-anchored protein at the surface of *Leishmania* promastigotes [2]. In addition to the surface-associated enzyme, recent evidence suggests that proteolytically active gp63 is released from promastigotes by autoproteolysis of GPI-linked gp63 and/or secretion of intracellular gp63 [3,4]. Indeed, both amastigotes and promastigotes of some *Leishmania* species have been reported to express a soluble intracellular isoform of gp63 (although at lower levels

than the promastigote surface enzyme) [5–7]. However, the nature of gp63 in the amastigote developmental stage remains an open question.

Despite extensive investigation, the role of gp63 in parasite physiology and infectivity remains uncertain. Seay et al. [8] reported that avirulent clones of *Leishmania amazonensis* had a 20–50-fold reduction in surface gp63, and McGwire and Chang [9] later showed that genetic rescue of such variants increased their infection of macrophages. In related studies, gp63 was shown to protect liposome-encapsulated proteins from phagolysosomal degradation by macrophages [10]. Attempts to produce gp63-deficient parasites have been hampered by the presence of multiple gene copies. Joshi et al. [11] deleted six of the seven genes encoding gp63 in *Leishmania major*. The gp63 gene-1–6-deficient mutants showed increased sensitivity to complement-mediated lysis, particularly in procyclic promastigotes. However, the remaining gene was expressed in metacyclic promastigotes and lesion amastigotes, and mutants were capable of infecting macrophages and differentiating into amastigotes. Recent studies with a GPI:protein transamidase-deficient strain of *L. mexicana*, which lacks surface gp63, showed that the mutant promastigotes were capable of differentiating into replicating amastigotes within macrophages *in vitro* and were infective in mice [12]. Using a

Abbreviations used: Cbz, benzyloxycarbonyl; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ED, effector domain; GPI, glycosylphosphatidylinositol; HBSS, Hanks balanced salt solution; MARCKS, myristoylated alanine-rich C kinase substrate; MARCKS_{ED}, synthetic peptide corresponding to the ED of MARCKS; MRP, MARCKS-related protein; MRP_{ED}, synthetic peptide corresponding to the ED of MRP; 3DMRP_{ED}, pseudophosphorylated peptide in which the three serine residues of MRP_{ED} are replaced by aspartate; rMRP, recombinant MRP; OPA, *o*-phenanthroline; PKC, protein kinase C; TD, transduction domain; TFE, trifluoroethanol.

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different approach, Chen and co-workers [13] transfected promastigotes of *Leishmania amazonensis* with gp63 genes cloned into a *Leishmania*-specific vector in two different orientations. Resulting modulation of sense or antisense gp63 RNA expression suggested that gp63 plays a role in macrophage binding to this parasite and in intramacrophage survival and replication.

Studies of gp63 are also hindered by the absence of effective non-toxic inhibitors. Indeed, the lack of cell-permeant inhibitors precludes investigation of gp63 function within the infected host cell or the role of soluble gp63 within the parasite. The metalloprotease inhibitor *o*-phenanthroline (OPA; 1,10-phenanthroline), which is relatively non-permeant through cell membranes, inhibits gp63-dependent cleavage of synthetic peptide substrates at millimolar concentrations [14]. When added to parasitized macrophages, liposome-encapsulated OPA was reported to decrease the survival of intracellular *L. amazonensis*, pointing to a role for gp63 in parasite maintenance within the cell [8]. The cell-permeant zinc chelator *N,N,N',N'*-tetrakis-(2-pyridylmethyl)ethylenediamine reportedly inhibits gp63 [8], but is of little use in studies involving whole cells, since it is toxic and induces apoptosis in a variety of cell types [15]. Bouvier and co-workers [14] described a hydroxamate-derived dipeptide, benzyloxycarbonyl (Cbz)-Tyr-Leu-NHOH, which inhibited gp63 activity towards synthetic peptide substrates, but this peptide has not been used in studies of gp63 function. Finally, Heumann et al. [16] reported that α_2 -macroglobulin inhibited solubilized gp63, but found no evidence for a specific interaction with the surface of promastigotes or for the inhibition of proteolytic activity on intact cells.

Myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein (MRP; MacMARCKS) are major protein kinase C (PKC) substrates expressed in diverse cell types, including macrophages [17]. Despite extensive investigation, the functions of these proteins are poorly understood. *In vitro* studies have shown that a highly basic 24–25-amino-acid sequence in MRP or MARCKS known as the effector domain (ED) binds with high affinity to actin, calmodulin and acidic phospholipids. The ED also contains the serine residues that are subject to PKC-dependent phosphorylation. We recently reported that MRP is an excellent substrate for purified *L. major* gp63 [18]. A predominant cleavage site between Ser⁹² and Phe⁹³ was identified within the ED. PKC-dependent phosphorylation of ED serine residues prevented MRP degradation by gp63 in a cell-free assay [18]. Using this assay, both OPA and Cbz-Tyr-Leu-NHOH were found to be relatively poor inhibitors of gp63 [18], while *N,N,N',N'*-tetrakis-(2-pyridylmethyl)ethylenediamine was completely inactive (S. Corradin, unpublished work). However, gp63-dependent MRP degradation could be efficiently inhibited by synthetic peptides comprising the MRP or MARCKS ED [18].

Recently, a new technology for introducing macromolecules into diverse cell types was described. Covalent linkage of peptides or full-length proteins to particular membrane-permeant carrier peptides allows the constructs to cross cell membranes by an as yet uncharacterized mechanism (reviewed in [19]). The HIV-1 transactivating protein Tat contains a highly basic amino acid region known as the transduction domain (TD) which has been used extensively for this purpose [19–22]. One application of this technology has been the non-invasive introduction of specific inhibitors of intracellular enzymes as tools to investigate the physiological roles of such enzymes, and also as potential therapeutic agents [19,21–23].

As pointed out above, defining the role of gp63 has been hampered by the lack of appropriate inhibitors. The experiments described in the present paper were aimed at designing peptides

capable of blocking both soluble and membrane-associated gp63 activity, and at producing cell-permeant analogues of promising candidate peptides for further studies of gp63 function within macrophages. To this end, based on the definition of a peptide sequence that is highly sensitive to cleavage by gp63, we have investigated the gp63-inhibitory activity of peptide analogues of the MRP and MARCKS EDs. In addition, MRP ED peptides were linked to the Tat TD in order to obtain potential cell-permeant peptides.

MATERIALS AND METHODS

Materials

Proteolytically active, GPI-anchored gp63 from *L. major* LEM 513 that migrated as a single 63 kDa band in SDS/PAGE gels, purified as previously described [2], was a gift from Pascal Schneider (Epalinges, Switzerland). Hemin was purchased from Sigma (Buchs, Switzerland), and OPA and folic acid from Fluka (Buchs, Switzerland). Preparation of the hydroxamate-derived dipeptide Cbz-Tyr-Leu-NHOH and of the fluorogenic gp63 substrate dansyl-AYLKKWV-NH₂ have been described previously [14,24].

Recombinant MRP and ED peptides

Recombinant His-tagged MRP (rMRP) [18] was a gift from Arndt Schmitz (Cold Spring Harbor, NY, U.S.A.). A 24-amino-acid peptide corresponding to the ED of murine MRP (MRP_{ED}; KKKKKFSFKKPFKLSGLSFKRNRK) and various analogues were synthesized using solid phase Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry in an Applied Biosystems 431 A peptide synthesizer (Perkin-Elmer International Inc., Rotkreuz, Switzerland). MRP_{ED} analogues included the pseudophosphorylated tripartate peptide 3DMRP_{ED} (KKKKKFDKFKPFKL-DGLDFKRNK; the three serine residues of MRP_{ED} are replaced by aspartate), as well as Tat-MRP_{ED} and Tat-3DMRP_{ED}, which contain an additional 13 N-terminal amino acid residues comprising the HIV-1 Tat TD with a glycine residue at either end (GYGRKKRRQRRRG) [20]. For confocal microscopy studies, FITC-conjugated peptides were prepared by coupling FITC to the N-terminal Gly during peptide synthesis. The crude polypeptides were purified by reverse-phase HPLC on a C18 Wydac 250 mm × 10 mm column using a 0.1% trifluoroacetic acid in a water/acetonitrile gradient with a flow rate of 3 ml · min⁻¹. Molecular masses of all peptides were confirmed by MS. The corresponding 25-amino-acid bovine MARCKS peptide (MARCKS_{ED}; KKKKKRFSFKKSFKLSGFSFKKN-KK) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, U.S.A.). Peptide analogues of MARCKS_{ED} [25] were a gift from Felix Althaus (Zurich, Switzerland).

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 [26]. Wild-type *L. mexicana* strain MNYC/BZ/62/M379 or its GPI:protein transamidase-deficient counterpart Δ GPI8, clone W1234 [11], were grown in M199 medium plus hemin folate (12.5 μ g · ml⁻¹ hemin, 25 μ g · ml⁻¹ folic acid) and 10% (v/v) heat-inactivated fetal calf serum. Lesion amastigotes from *L. mexicana*-infected mice were isolated as described previously [27].

MRP degradation assay

rMRP (5 μ M) was incubated with purified gp63 (1–2 nM) in 20 μ l of 10 mM Tris/HCl, pH 7.4, 100 mM NaCl for 15 min at 37 °C, in the presence or absence of various inhibitors as described. Quantification of rMRP remaining was then assessed by SDS/PAGE. To this effect, samples were heated to 95 °C for 5 min, followed by 5 min on ice and centrifugation at 11 000 g for 5 min to obtain heat-stable MRP fractions. Laemmli sample buffer was then added, and samples were placed in a 100 °C heat block for 5 min. MRP and its degradation products were visualized in SDS/12.5% -PAGE gels by staining with Coomassie Brilliant Blue R (Serva, Heidelberg, Germany) and destaining with 10% propan-2-ol/5% acetic acid in water. Gels were scanned on a ScanJet 4c/T densitometer (Hewlett Packard, Geneva, Switzerland) using the Adobe Photoshop software package (Adobe Systems, Inc., Mountain View, CA, U.S.A.) and NIH image 1.60 software (NIH Division of Computer Research and Technology).

Fluorogenic assay of gp63 activity

The activity of purified or parasite-bound gp63 was also monitored using an assay based on hydrolysis of a fluorogenic peptide substrate (see Materials), which measures the increase in tryptophan fluorescence emission at 360 nm using an excitation wavelength of 290 nm, as previously described [24]. For experiments with intact parasites, fluorogenic peptide (3 μ M) was incubated at 26 °C for 30 min with 2×10^6 promastigotes in 1.2 ml of Hanks balanced salt solution (HBSS). After centrifugation for 10 min at 1500 g to remove parasites, hydrolysis of the fluorogenic substrate was determined relative to the HBSS control. In some experiments, promastigotes were first fixed with 0.1% glutaraldehyde (30 min, 26 °C) followed by three washes in HBSS. To control for the possible release of active enzyme and/or molecules that might give a positive fluorescent signal, parasites were incubated in HBSS for 1 h at 26 °C and supernatant collected for assay with or without fluorogenic substrate.

CD spectroscopy

CD spectra of MRP peptides (100 μ g $\cdot$ ml⁻¹ in PBS diluted 1:10 in water) in the presence or absence of trifluoroethanol (TFE) were recorded on a Jobin-Yvon CD Mark VI spectrometer (Longjumeau, France), calibrated with d(+)-10-camphorsulphonic acid, equipped with CDMax software and coupled to a Dell 586 computer. All measurements were performed using a 1 mm path-length cell (Hemmla, Müllheim, Germany). Each complete spectrum was the average of three repeated scans between 190 and 250 nm, with an integration time of 2 s for 0.5 nm steps. Solvent spectra were recorded under the same conditions and the baseline was subtracted. Spectra are presented as mean residue ellipticity (θ) in deg $\cdot$ cm² $\cdot$ dmol⁻¹. Concentrations of peptide solutions for CD analysis were determined by amino acid analysis.

Fluorescence microscopy

Murine bone marrow-derived macrophages, obtained by *in vitro* differentiation of bone marrow precursor cells as previously described [28], were distributed in 24-well cell culture plates (2.5×10^5 macrophages/well), each well containing a round sterile glass coverslip. After 24 h in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, coverslips were washed three times with PBS and then incubated with 5 μ M FITC-Tat-MRP_{ED} or FITC-MRP_{ED} in DMEM for 30 min at

4 °C. Macrophages were washed three times in PBS and fixed with 4% paraformaldehyde. *L. major* LV39 or *L. mexicana* Δ GPI8 promastigotes were incubated with 5 μ M FITC-Tat-3DMRP_{ED} or FITC-3DMRP_{ED} in DMEM for 30 min at room temperature (5×10^6 parasites in 400 μ l). Parasites were washed twice in PBS and then fixed for 10 min with 4% paraformaldehyde followed by two washes in PBS. Fixed parasites were resuspended in 200 μ l of PBS and applied to poly(L-lysine)-coated coverslips. Macrophage or parasite coverslips were mounted with Citifluor (Citifluor Ltd) and examined under a confocal microscope (Zeiss LSM 510) attached to a Zeiss Axiovert 100M microscope equipped with an argon laser. Serial optical sections were recorded at 0.3 μ m intervals with a 63 $\times$ /1.4 Plan Apochromat objective. For co-localization experiments, *L. major* promastigotes were treated with FITC-Tat-3DMRP_{ED} as described, followed by DNA staining with 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 0.5 μ g/ml in PBS. Coverslips were mounted with Citifluor and slides were viewed using an Axioskop microscope fitted with a Coolview CCD camera.

RESULTS

Inhibition of gp63-dependent MRP cleavage by wild-type MRP_{ED} or 3DMRP_{ED}

In a cell-free assay using GPI-anchored gp63 purified from *L. major* promastigotes, we previously showed that MRP_{ED} and MARCKS_{ED} peptides inhibit rMRP degradation by gp63, while polylysine is completely inactive [18]. It was therefore of interest to assess more carefully the peptide inhibitory activity, with the aim of designing an effective, non-toxic gp63 inhibitor. For this purpose, rMRP was first exposed to gp63 in the presence or absence of various inhibitors, followed by SDS/PAGE of heat-stable supernatant fractions and quantification of rMRP remaining by Coomassie Blue staining. Despite the test-to-test variation in absolute values inherent in such a protocol, this assay was found to yield reproducible results when different inhibitors were analysed within the same experiment. Figure 1(A) shows that, on a molar basis, the capacity of MRP_{ED} to inhibit the gp63-dependent cleavage of rMRP was greater than that of two previously described gp63 inhibitors, OPA and Cbz-Tyr-Leu-NHOH. While 400 μ M OPA or Cbz-Tyr-Leu-NHOH resulted in efficient inhibition of rMRP cleavage, 80 μ M was less effective. Under the same conditions, a strong inhibition of rMRP cleavage was obtained with 10 μ M MRP_{ED}.

In order to obtain a cell-permeant analogue of MRP_{ED}, we synthesized Tat-MRP_{ED}, a construct containing an N-terminal HIV Tat transduction sequence. Tat-MRP_{ED} inhibited rMRP degradation at 10 μ M with an efficiency comparable with that of MRP_{ED} (Figure 1A). In as much as phosphorylated MRP is a poor substrate for gp63 [18], we also synthesized a pseudo-phosphorylated analogue of MRP_{ED} termed 3DMRP_{ED}, in which three Ser residues were replaced by Asp. As indicated in Figure 1(B), 3DMRP_{ED} was much more efficient in inhibiting rMRP cleavage than MRP_{ED}.

MARCKS_{ED} analogues as inhibitors of gp63-dependent MRP cleavage

Although MRP_{ED} and its tripartate analogue were capable of blocking gp63 enzymic activity, it was unclear to what extent the gp63 cleavage site previously identified within the MRP ED was either necessary or sufficient for effective inhibition. The MARCKS and MRP ED sequences are very similar (see the Materials and methods section) and both peptides were previously shown to inhibit gp63 activity [18]. We therefore used a

Figure 1 Inhibition of gp63-mediated degradation of MRP

rMRP (5 μ M) was incubated with 2 nM gp63 in the absence or presence of (A) OPA, Cbz-Tyr-Leu-NHOH (Z-Y-L-NHOH), MRP_{ED} or Tat-MRP_{ED}, or (B) MRP_{ED} or its triaspartate analogue 3DMRP_{ED}. Heat-stable supernatant fractions of the reaction mixtures were assessed for rMRP degradation by SDS/PAGE and Coomassie Blue staining. Data from densitometric scans of stained gels are presented as percentage of control rMRP in the absence of gp63.

Figure 2 Inhibition of the gp63-mediated degradation of MRP by MARCKS_{ED} analogues

(A) Peptides contain the alanine substitutions shown in bold and underlined or amino acid deletions. Group 1, some or all hydrophobic residues replaced by alanine; Group 2, N-terminal positively charged residues replaced by alanine or deleted; Group 3, central and/or C-terminal positively charged residues replaced by alanine. Consensus sites for gp63 defined as P1'-P2'-P3' are indicated in italics for wild-type MARCKS_{ED}. The number of unmodified consensus sites is indicated for each peptide. (B) rMRP (5 μ M) was incubated with gp63 in the absence or presence of wild-type MARCKS_{ED} peptide (WT) or analogues shown in (A). Heat-stable supernatant fractions of the reaction mixtures were assessed for rMRP degradation by SDS/PAGE and Coomassie Blue staining.

previously characterized series of MARCKS_{ED} analogues [25] to examine specific requirements for peptide inhibitory activity. Figure 2(A) shows the sequences of three groups of peptides containing alanine substitutions or amino acid deletions as

indicated. In previous studies, synthetic peptide substrates allowed the determination of a consensus gp63 cleavage sequence, with a hydrophobic residue at the P1' site and basic amino acid residues at the P2' and P3' sites [14]. MARCKS_{ED} contains two

Figure 3 Inhibition of gp63-mediated hydrolysis of a fluorogenic peptide substrate

gp63 (2 nM) was preincubated or not with 3DMRP_{ED} or Cbz-Tyr-Leu-NHOH (Z-Y-L-NHOH) at the indicated concentrations (a). Fluorogenic peptide (3 μM) was then added (b) and hydrolysis was monitored by the increase in tryptophan fluorescence at 360 nm (excitation at 290 nm).

such sequences, Phe⁹-Lys¹⁰-Lys¹¹ and Phe²⁰-Lys²¹-Lys²², both of which are preceded by a Ser residue that is subject to PKC-dependent phosphorylation and are identical to the gp63 cleavage site identified in MRP [18]. In earlier studies, synthetic substrates effectively cleaved by gp63 also included a peptide with Leu at the P3' position [14]. Therefore a third potential consensus site (Phe-Lys-Leu), containing Leu rather than Lys at P3', is present in MARCKS_{ED} (Figure 2A). Figure 2(A) indicates the number of unmodified consensus sites, defined as P1'-P2'-P3', for each MARCKS_{ED} analogue.

The capacity of the different analogues and wild-type MARCKS_{ED} to inhibit gp63-dependent MRP degradation was assessed in preliminary experiments, and inhibitory peptides were then retested at concentrations ranging from 3 to 50 μM; inactive analogues were used at 50 μM (Figure 2B). Results from these experiments can be summarized as follows. First, replacement of hydrophobic residues by Ala (Group 1: M-13, M-14, M-16; but also Group 2: M-10, M-12, M-15; Figure 2A) led to considerable loss of inhibitory activity, suggesting that the consensus site P1' hydrophobic residues may be critical to the inhibitory effect. Secondly, deletion or replacement by Ala of N-terminal positively charged residues either failed to decrease activity (M-8) or had little effect (M-1, M-9, M-11). The decreased inhibitory activity of the Group 2 peptides M-10, M-12 and M-15 is likely to be due to additional modifications such as replacement of hydrophobic residues (see above). Thirdly, modification of the P2' and P3' basic residues (M-2, M-3, M-4) was also associated with decreased inhibition of gp63 activity relative to the wild-type peptide. Taken together, these results indicate that peptide inhibitory activity largely correlated with the number of unmodified consensus sites. For example, Figure 2(B), panel (IV) includes results of MRP degradation by gp63 obtained in the presence of peptides containing between zero and three intact consensus sites. Inhibition was clearly less efficient with M-2 (one site) and M-3 (two sites) than with M-8 or wild-type MARCKS_{ED}, which contain three unmodified sites; M-4 (no sites) was completely inactive at the highest concentration tested.

Peptide inhibition of fluorogenic substrate hydrolysis by purified and membrane-associated gp63

Having demonstrated that the MRP_{ED} peptide and its analogues effectively inhibited gp63-dependent MRP degradation, it was of interest to perform similar experiments with a gp63 substrate containing a different cleavage site. Moreover, in as much as most gp63 is anchored to the surface of the *Leishmania* parasite, it was important to demonstrate that inhibition was effective

Table 1 Hydrolysis of fluorogenic substrate by intact *Leishmania* parasites and inhibition by 3DMRP_{ED}

(A) Data represent increases in tryptophan fluorescence [mean ± S.D. (n)] obtained with 2×10^6 *L. mexicana* wild-type (WT) or surface-gp63-deficient (Δ GPI8) promastigotes, or with wild-type amastigotes. (B) Data were obtained using *L. major* promastigotes, and are presented as percentage fluorescence increase [mean ± S.D. (n)] relative to that obtained with 2×10^6 (glutaraldehyde-fixed) LV39 promastigotes alone.

(A)	
Parasite	ΔFluorescence 290/360 nm (units)
Promastigotes	
WT	75.4 ± 11.9 (4)
ΔGPI8	0.0 ± 0.0 (6)
Amastigotes	
WT	2.5 ± 2.6 (3)
(B)	
3DMRP _{ED} concn (μM)	ΔFluorescence 290/360 nm (% of control)
3	38.9 ± 11.2 (3)
10	20.0 ± 5.6 (9)
30	8.0 ± 4.7 (3)

using whole parasites as well as purified enzyme. For these and subsequent studies, we focused on the 3DMRP_{ED} peptide, which had shown strong inhibition of rMRP degradation by gp63. Figure 3 shows the capacity of 3DMRP_{ED} to inhibit gp63-mediated cleavage of a previously described fluorogenic substrate unrelated to MRP [24]. The synthetic peptide dansyl-AYLKKWV-NH₂ is cleaved between the tyrosine and leucine residues by gp63, resulting in a time-dependent increase in tryptophan fluorescence. 3DMRP_{ED} at 2 μM completely inhibited cleavage of the fluorogenic peptide, whereas inhibition by 40 μM Cbz-Tyr-Leu-NHOH was incomplete. Subsequent experiments (results not shown) verified that addition of the Tat TD moiety to 3DMRP_{ED} did not decrease its inhibitory activity measured with the fluorescent substrate and purified gp63.

Cleavage of the fluorogenic substrate was also used to assess the inhibitory activity of 3DMRP_{ED} on membrane-associated gp63 of whole *Leishmania* parasites [24]. Table 1(A) presents the results of experiments comparing wild-type *L. mexicana* and a mutant line (Δ GPI8) lacking the GPI-anchored form of gp63, due to loss of GPI:protein transamidase [12]. No cleavage of the fluorogenic peptide was observed with intact Δ GPI8 promastigotes, while substantial cleavage was observed with

Figure 4 Effect of tripartate substitutions on the secondary structure of MRP_{ED} peptides

CD spectra of (A) MRP_{ED} and 3DMRP_{ED} and (B) Tat-MRP_{ED} and Tat-3DMRP_{ED} in the presence and absence of 80% TFE.

promastigotes from wild-type *L. mexicana*. While other GPI-linked proteins besides gp63 are absent from the mutant parasites [12], the complete lack of substrate cleavage by Δ GPI8 strongly suggests that most of the enzymic activity of wild-type promastigotes was due to membrane-associated gp63. Based on standard curves obtained using purified gp63, fluorescence values for 2×10^6 wild-type *L. mexicana* parasites in 1.2 ml corresponded to approx. 0.5 nM gp63 (results not shown). Consistent with these findings, lesion amastigotes of wild-type *L. mexicana*, which express less surface gp63 than promastigotes [29], gave only a low level of substrate cleavage (Table 1A). We then examined the capacity of 3DMRP_{ED} to inhibit parasite-associated gp63 using *L. major* promastigotes lightly fixed with glutaraldehyde. Such treatment preserves proteolytically active gp63 on the parasite surface [3,24] and further ensured that enzyme known to be present at low levels intracellularly was not released during the assay [24]. Indeed, control supernatants prepared by incubating the fixed parasites in HBSS for 1 h at 37 °C were consistently negative when tested in parallel for substrate-cleaving activity. As shown in Table 1(B), 3DMRP_{ED} efficiently inhibited the cleavage of fluorogenic peptide by *L. major* LV39 promastigotes. Under the conditions used in these experiments, the ID₅₀ of 3DMRP_{ED} was approx. 1 μ M. 3DMRP_{ED} also inhibited substrate cleavage by unfixed *L. major* or wild-type *L. mexicana* promastigotes (results not shown).

CD spectroscopy

Taken together, these results demonstrate that peptide analogues of the MRP or MARCKS ED can be used as inhibitors of purified or parasite-associated gp63. A remaining question was how the substitution of Ser residues by Asp to mimic phosphorylation [30] enhanced inhibitory activity in our assays. Bubb et al. [31] reported that MARCKS_{ED}, which normally exhibits no secondary structure, showed significant α -helical structure if phosphorylated by PKC. We therefore performed CD spectroscopy with MRP_{ED} and 3DMRP_{ED}. In addition, Tat TD constructs of both peptides were examined to determine if the Tat moiety had a significant effect on peptide structure. As shown in Figure 4, MRP_{ED} and 3DMRP_{ED} with (Figure 4B) or

without (Figure 4A) the Tat TD showed random coil conformation in aqueous solution. In the presence of TFE, both MRP_{ED} and 3DMRP_{ED} exhibited a low degree of α -helical structure, while Tat-MRP_{ED} and Tat-3DMRP_{ED} showed an increased propensity to form α -helices. Although α -helix formation in the presence of TFE implies that certain molecules could theoretically adopt such a conformation upon interaction with other proteins or membranes, studies with the intact MRP protein strongly suggest that the ED does not adopt an α -helical conformation upon binding to membranes or calmodulin [32,33].

Transduction of Tat TD-conjugated peptides into macrophages and *Leishmania*

In order to verify that the Tat-conjugated peptides were capable of penetrating cells, murine bone marrow-derived macrophages or *Leishmania* promastigotes were examined by confocal microscopy after treatment with FITC-Tat-3DMRP_{ED} or FITC-3DMRP_{ED} at room temperature. For experiments with parasites, promastigotes of both *L. major* LV39 and *L. mexicana* Δ GPI8, which lacks surface gp63, were used. Under these conditions, macrophages showed significant uptake of both the non-Tat-conjugated FITC-3DMRP_{ED} peptide and FITC-Tat-3DMRP_{ED}, possibly due to the strong endocytotic activity of these cells (results not shown). Since Tat TD constructs are known to enter cells even at low temperatures, at which endocytosis is blocked, we performed subsequent experiments with macrophages at 4 °C. As shown in Figure 5(A), macrophages treated with FITC-Tat-3DMRP_{ED}, but not FITC-3DMRP_{ED}, exhibited diffuse fluorescence, indicative of peptide entry. In the case of *Leishmania*, a diffuse cytoplasmic staining was also observed, which was strictly Tat TD-dependent; no differences could be noted between the two parasite strains (Figure 5A). In addition, there was a pronounced accumulation of peptide at two intracellular locations. We therefore performed double staining experiments with FITC-Tat-3DMRP_{ED} and DAPI in *L. major* promastigotes. As shown in Figure 5(B), FITC and DAPI staining co-localized in the nucleus and kinetoplast. In contrast, macrophages did not show marked accumulation of FITC-

Figure 5 Transduction of Tat-3DMRP_{ED} into macrophages and *Leishmania* promastigotes

(A) Confocal microscopic analysis of bone marrow-derived macrophages (BMMφ) or *Leishmania* promastigotes (*L. major* LV39 or *L. mexicana* ΔGPI8) treated with 5 μM FITC-Tat-3DMRP_{ED} (a) or FITC-3DMRP_{ED} (b). Nomarski images of cells are shown in left-hand panels and accumulation of FITC-conjugated peptide is shown in right-hand panels. (B) Fluorescent images of *L. major* LV39 promastigotes treated with 5 μM FITC-Tat-3DMRP_{ED} followed by DNA staining with DAPI. DAPI fluorescence was consistently brighter for the kinetoplast (k) when compared with the nucleus (n). FITC co-localizes with nuclear and kinetoplast DAPI staining, but also occurs in a diffuse pattern throughout the parasite.

conjugated peptide in the nucleus, even at higher temperatures (results not shown).

DISCUSSION

We reported previously that infection of murine macrophages with *Leishmania* promastigotes markedly decreases MRP levels in cell lysates, apparently as a result of proteolysis [28]. Subsequently, we demonstrated that rMRP or a 24-amino-acid peptide comprising the MRP ED are excellent substrates for the promastigote surface metalloprotease gp63 [18]. While there is some evidence that gp63-dependent proteolysis may occur within infected macrophages, definitive proof awaits the development of specific cell-permeant inhibitors of gp63. Although gp63 is expressed at high levels during the promastigote stage of the *Leishmania* cell cycle and is present as a soluble intracellular form during the amastigote stage, attempts to identify its normal substrate(s), and thus to understand its role in parasite physiology, are similarly hindered by the lack of suitable inhibitors.

In the present study, we investigated the capacity of analogues of MRP_{ED} to inhibit gp63. Cell-permeant constructs were then obtained by addition of the HIV-1 Tat TD, with the goal of using these peptides to interfere with gp63 activity on parasites within

their host cells. Peptides based on the MRP ED were more efficient than the previously described inhibitors OPA and Cbz-Tyr-Leu-NHOH in blocking gp63-dependent cleavage of rMRP or a synthetic fluorescent peptide substrate. While the latter assay was generally more quantitative, the MRP degradation assay presents a distinct advantage of measuring gp63 proteolytic activity towards a potential physiological protein substrate [18].

Interestingly, the triaspartate analogue 3DMRP_{ED} was considerably more effective than MRP_{ED}. MS analysis suggests that 3DMRP_{ED} is a poor substrate for gp63 when compared with MRP_{ED} (S. Corradin, unpublished work). It can be postulated that an inhibitor that is cleaved by gp63 would then leave the active-site pocket, while an uncleaved peptide would remain bound for longer periods. More gp63 molecules per unit time would thus be occupied by the uncleaved inhibitor molecules. Why 3DMRP_{ED} should exhibit reduced sensitivity to proteolysis is not clear. We reported previously that prior PKC-dependent phosphorylation of rMRP inhibited its degradation by gp63 [18]. Both phosphorylated MRP and 3DMRP_{ED} contain modified P1 residues (phosphoserine or aspartate respectively) preceding the gp63 consensus sites described [18]. Unlike its non-phosphorylated counterpart, phosphorylated MARCKS_{ED} is reported to exhibit significant α-helical structure [31], which

might result in altered interaction with gp63 and reduced proteolysis. However, CD spectroscopic analysis of the wild-type and tripartate peptides revealed no major differences in secondary structure. In particular, no significant α -helical structure was observed with the tripartate-substituted peptide. Indeed, modest differences in the CD spectra at low wavelengths might suggest a greater tendency for random coil conformation in 3DMRP_{ED}.

The active-site cleft of gp63 from *L. major* has been reported to contain a large region of negative charge [34]. Thus the additional negative charges resulting from the Ser → Asp substitutions would be likely to decrease rather than increase the peptide's binding affinity for gp63. One possibility that might explain the enhanced inhibitory activity of 3DMRP_{ED} is that the additional carboxylic group of the Asp residue at P1 of the cleavage site is able to chelate the active-site zinc of gp63. Most, if not all, inhibitors of metalloproteases contain a functional group that is able to interact with the zinc atom of the active site. Studies of matrix metalloprotease inhibitors emphasized the critical choice of the inhibitor–zinc ligand [35]. A carboxylate inhibitor of matrilysin was considerably less effective than the analogous hydroxamate compound; thus it may be possible to increase 3DMRP_{ED} inhibitory capacity by using another zinc ligand.

Using a series of MARCKS_{ED} analogues in which amino acid residues were deleted or replaced by alanines, we demonstrated that inhibition of rMRP cleavage correlated with the number of unaltered consensus cleavage sites in the different peptides. Both the P1' hydrophobic residue and the P2' basic residue appeared to be critical to maintain inhibitory activity. While no attempt was made to examine whether the MARCKS_{ED} peptide analogues are cleaved by gp63, inhibition of MRP cleavage is a useful indicator of their capacity to bind to gp63. Further studies are necessary to determine if the peptides are rapidly cleaved and, if so, to attempt to modify amino acid composition to obtain stable inhibitors.

Importantly, 3DMRP_{ED} was also an effective inhibitor of parasite-associated gp63. In preliminary studies, we have not observed any effects of 3DMRP_{ED} on the viability of extracellular parasites (results not shown). While these peptides can now be used to examine the role of surface gp63 in the interaction between macrophages and extracellular promastigotes, cell-permeant analogues may be useful for investigating the role of gp63 in maintaining parasite viability or inhibiting host cell signal transduction and defence mechanisms within *Leishmania*-infected macrophages. Moreover, cell-permeant peptides might be interesting for studies of the intracellular amastigote gp63 isoform. We therefore synthesized analogues of MRP_{ED} and 3DMRP_{ED} containing an N-terminal Tat TD. Peptides or proteins synthesized or expressed in tandem with the Tat TD have been shown to enter cells and exert biological activity in multiple systems, both *in vitro* and *in vivo*, although prior denaturation is required if the constructs exhibit significant secondary structure [19,20,36]. CD analysis of the Tat constructs used in our experiments revealed little or no structure in physiological buffer. Tat–3DMRP_{ED} retained gp63 inhibitory capacity and was found to enter both macrophages and *Leishmania* promastigotes in a Tat TD-dependent manner. To our knowledge, this is the first report of the capacity of the Tat TD to mediate the transduction of a peptide into *Leishmania* parasites. Similar results were obtained with Tat–MRP_{ED}, and both Tat peptides were able to enter other cell types in addition to macrophages, including fibroblasts, mast cells and B cells (results not shown). Apparent differences in the intracellular targeting of Tat–3DMRP_{ED} in macrophages and *Leishmania*

require further investigation. In *Leishmania*, preliminary time course experiments suggested that staining is first evident at the plasma membrane, followed by entry and eventual accumulation in the DNA-containing organelles (S. Corradin, unpublished work). Given the basic nature of Tat–3DMRP_{ED}, as well as the potential nuclear targeting sequence present in the Tat TD [37], it is not surprising that the peptide eventually concentrates in the nucleus or kinetoplast. This does not mean, however, that significant amounts of the protein are not present at other locations; indeed, cytoplasmic staining was also evident (Figure 5). Still, differential targeting of the ED peptides in various cell types might occur due to the presence or absence of proteins or lipids which are known to bind with high affinity to MRP [17]. The expression or not of a MARCKS-like protein in these cells might also affect eventual peptide localization.

Taken together, these results suggest that the Tat constructs or similar cell-permeant molecules may provide interesting and novel tools for investigating *Leishmania* physiology. As the *Leishmania* genome is sequenced and proteomics studies expanded, it will be important to understand the function of many other parasite proteins in addition to gp63. Transduction of specific inhibitors or of the various proteins themselves provides an alternative to DNA transfection that is rapid, non-invasive and applicable to almost all cell types, including primitive eukaryotes as shown here.

Experiments are now in progress to assess the effects of Tat–3DMRP_{ED} on various cell functions. Preliminary experiments designed to investigate a putative effect on macrophage infection by *L. major* promastigotes showed no effects (S. Corradin, unpublished work). When tested at 20 μ M, Tat–3DMRP_{ED} was not toxic, and failed to decrease either the number of intracellular parasites or the percentage of infected macrophages. Intracellular killing induced by interferon- γ plus tumour necrosis factor- α occurred normally, further demonstrating a lack of peptide toxicity for host macrophages. These first experiments might suggest that gp63 is not involved in intracellular infection of macrophages *in vitro*. However, interpretation of negative results is complicated, for several reasons. Possibly our *in vitro* model of macrophage infection is not suitable for detecting the consequences of gp63 inhibition on parasite virulence. Moreover, the high expression of gp63 and its significant role in pathogenesis do not necessarily imply that it is essential for virulence, as recently pointed out by Joshi and colleagues [38]. On the other hand, it is also possible that the Tat constructs are unable to inhibit intracellular gp63 to a sufficient degree. This could be due to peptide degradation, entry into the wrong intracellular compartment(s) and/or sequestration of the peptide by other molecules. Despite the lack of an effect of these peptides in our early experiments, the rationale and results described here form the basis for developing more effective gp63 inhibitors than are currently available. Moreover, Tat TD analogues with increased activity and stability have recently been described [39]. The Tat–MRP constructs should now be tested in other *in vitro* models (with either promastigotes or amastigotes), and possibly *in vivo* in either the sandfly vector or the mammalian host.

While this paper was under review, a paper by Joshi and colleagues [38] appeared describing a total gp63 knockout in *L. major*. Mutant promastigotes showed an increased sensitivity to complement-mediated lysis and delayed lesion formation in mice, further supporting the role of gp63 as a virulence factor in the mammalian host. Whether or not phenotypic differences would also be manifest during macrophage infection by gp63-deficient promastigotes *in vitro* is unclear. However, these parasites provide an additional tool for investigation of gp63 in *Leishmania* biology.

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