Analysis of the roles of cysteine proteinases of *Leishmania mexicana* in the host–parasite interaction

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SUMMARY

Promastigotes of *Leishmania mexicana* mutants lacking the multicopy *CPB* cysteine proteinase genes (Δ*CPB*) are markedly less able than wild-type parasites to infect macrophages *in vitro*. Δ*CPB* promastigotes invade macrophages in large numbers but are unable to survive in the majority of the cells. In contrast, Δ*CPB* amastigotes invade and survive within macrophages *in vitro*. This extreme *in vitro* stage-specific difference was not mimicked *in vivo*; both promastigotes and amastigotes of Δ*CPB* produced lesions in BALB/c mice, but in each case the lesions grew considerably more slowly than those caused by wild-type parasites and only small lesions resulted. Inhibition of CPB *in situ* using cell-permeant peptidyl-diazomethylketones had no measurable effect on parasite growth or differentiation axenically *in vitro*. In contrast, *N*-benzoyloxycarbonyl-phe-ala-diazomethylketone reduced the infectivity of wild-type parasites to macrophages by 80%. Time-course experiments demonstrated that application of the inhibitor caused effects not seen with Δ*CPB*, suggesting that CPB may not be the prime target of this inhibitor. The data show that the *CPB* genes of *L. mexicana* encode enzymes that have important roles in intracellular survival of the parasite and more generally in its interaction with its mammalian host.

Key words: cysteine proteinase, virulence, cosmid, *Leishmania mexicana*.

INTRODUCTION

Parasite proteinases, including those of trypanosomatids, have been the subject of extensive research in recent years (reviewed McKerrow *et al*. 1993; Robertson *et al*. 1996; Coombs & Mottram, 1997*a*, *b*; Mottram, Brooks & Coombs, 1998; Rosenthal, 1999). The enzymes have been implicated with differentiation, nutrition, host cell invasion, and evasion of the host's immune response. Many proteinases exhibit marked stage-specificity, as appropriate for their role. The cathepsin L-like cysteine proteinases (CPs) of parasitic protozoa have attracted particular attention because of their apparent importance in various vital parasite activities such as the destruction of host proteins (Stanley *et al*. 1995), nutrition (Rosenthal, 1999) and neutralization of the host immune response (Alexander, Coombs & Mottram, 1998). The interest in these enzymes has been enhanced further in recent years because several appear to be promising targets for the development of new anti-parasite drugs (Coombs & Mottram, 1997*b*; Selzer *et al*. 1997, 1999; Engel *et al*. 1998*a*, *b*; Rosenthal, 1999).

Leishmania are parasites of mammals in which they reside within vacuoles of macrophages as small forms known as amastigotes. They are transmitted from mammal to mammal via sandflies, in which they exist as several extracellular, motile forms all known as promastigotes. Both of these main developmental forms can be cultured axenically, although the so-called axenic amastigotes are believed to differ in some ways from those occurring in lesions in mammals. The mechanisms whereby *Leishmania* survive and proliferate in the hostile environment of the macrophage phagolysosome are still largely unknown (reviewed by Garcia-del Portillo & Finlay, 1995; Russell, 1995; Mauël, 1996; Antoine *et al*. 1998; Mottram *et al*. 1998; Bogdan & Röllinghoff, 1999). The collective contribution of several of its surface molecules has been thought to aid the parasite in establishing infection inside macrophages (reviewed by Chang & Chaudhuri, 1990; Mauël, 1996; Ferguson, 1999). Two abundant surface molecules of promastigotes, gp63, a zinc metalloproteinase and lipophosphoglycan (Descoteaux & Turco, 1993; Desjardins & Descoteaux, 1997), have received most notoriety in this respect, although mutants lacking gp63 are capable of macrophage invasion and survival (Joshi *et al*. 1998). Stage-specific CP activity has been implicated in intracellular survival of the *L. mexicana* complex (North & Coombs, 1981; Leao *et al*. 1995, Mottram *et al*. 1996, 1998; Coombs & Mottram, 1997*a*, *b*). Three types of CP genes (*CPA*, *CPB* and

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CPC) have been identified in *L. mexicana* (reviewed by Coombs & Mottram, 1997*a*, *b*), and homologues have been isolated in other *Leishmania* species (Traub-Cseko *et al*. 1993; Sakanari *et al*. 1997, Omara-Opyene & Gedamu, 1998). In *L. mexicana*, 19 non-identical copies of the *CPB* genes are arranged in a tandem array (Mottram *et al*. 1997), these have been designated Type I CPs (Coombs & Mottram, 1997*a*). Generation of mutants deficient in each of the *L. mexicana* CP genes revealed differences in their roles in the parasite. *CPA*-deficient mutants (Δ*CPA*) had little or no phenotypic difference from wild type parasites (Souza *et al*. 1994), whereas *CPC*-deficient mutants (Δ*CPC*) showed a reduced ability to infect macrophages but formed lesions in mice to a similar extent as wild-type parasites. In contrast, analysis of Δ*CPB* mutants showed that these enzymes are important virulence factors (Mottram *et al*. 1996) and studies on *CPB*-deficient mutants re-expressing different copies of the *CPB* array revealed that individual isoenzymes apparently differ in their substrate preferences and ability to complement the loss of virulence associated with the *CPB* null mutant (Mottram *et al*. 1996, 1997). These data suggest that individual isoenzymes have distinct roles in the parasite's interaction with its host.

The study reported here aimed at elucidating the role of CPB enzymes in the life-cycle of *L. mexicana*, and hence the suitability of CPB as a drug target, by analysing in detail how CP-deficient mutants differed phenotypically from the parent line. Features compared include growth and differentiation axenically *in vitro* and the kinetics of infectivity to peritoneal macrophages and mice. A complementary approach was the utilization of irreversible CP inhibitors, that permeate into living parasites, to inhibit the parasite CPs *in situ* and so provide data on the roles the enzymes play at different stages in the parasite's life-cycle.

MATERIALS AND METHODS

Transfected cell lines of L. mexicana

The lines studied in this work were derived from *L. mexicana* (MNYC/BZ/62/M379) as described elsewhere (Souza *et al*. 1994; Mottram *et al*. 1996, 1997). Δ*CPB* is a *CPB* null mutant, Δ*CPA* is a *CPA* null mutant and Δ*CPA*/*CPB* is a *CPA* and *CPB* double null mutant (Mottram *et al*. 1996). Δ*CPB2.8* is the Δ*CPB* null mutant expressing the *CPB* gene 2.8 (a gene internal to the array) from the pTEX episome (Mottram *et al*. 1996, 1997).

Cell culture

The 3 major developmental stages of *L. mexicana* were cultured and harvested as described previously (Bates *et al*. 1992). Promastigotes were grown axenically *in vitro* at 25 °C in HOMEM medium, pH 7·5, supplemented with 10% (v/v) heat-inactivated foetal calf serum (Labtech) and 25 μg/ml gentamycin (Sigma). Where appropriate, the following antibiotics were included, singly or in combination, for selection of transfectants: hygromycin B (Sigma) at 50 μg/ml; phleomycin (Cayla, France) at 10 μg/ml; nourseothricin hydrosulphate (Hans-Knoll Institute, Thüringen, Germany) at 25 μg/ml; puromycin (Sigma) at 10 μg/ml; G418 (Geneticin, BRL) at 25 μg/ml (initially) or 500 μg/ml (to increase copy number of the episomal vector expressing *CPB2.8*). Axenic amastigote cultures were initiated at a starting density of 10^6 cells/ml from stationary phase promastigote cultures, were at 32 °C under 5% CO_2/95% air, and were subcultured every 7 days to the same starting density. The growth medium consisted of Schneider's Drosophila Medium (SDM, GIBCO), supplemented with 20% (v/v) heat-inactivated foetal calf serum (Labtech) and 25 μg/ml gentamycin. The pH was adjusted to 5·5 using 1 M HCl. For the inhibitor studies, *N*-benzoyloxycarbonyl-phe-ala-diazomethylketone (Z-FA-DMK, Sigma) and Z-leu-val-gly-DMK (Z-LVG-DMK, Sigma) were made up as 10 mg/ml stock solutions in dimethylsulphoxide (Me_2SO), and included in the appropriate promastigote or axenic amastigote culture medium to a final concentration of 10 μg/ml inhibitor/0·1% Me_2SO (v/v) (approximately 25 μM inhibitor).

Gelatin–SDS–PAGE

Parasite proteinases were analysed by substrate–SDS–PAGE, using Coomassie Blue stain to visualize the hydrolysis of 0·2% (w/v) gelatin co-polymerized in the 12% (w/v) separating gel, essentially as described by Robertson & Coombs (1990). DMK proteinase inhibitors were added (to 10 μg/ml) to the appropriate parasite lysates prior to electrophoresis. For analyses of cells grown in the presence of proteinase inhibitors, the cells were thoroughly washed so that they were free of unbound inhibitors before lysis.

Western blotting

Preparation of parasite protein extracts and electroblotting were carried out as described previously (Mottram *et al*. 1996). Proteinase detection was achieved using a 1:500 dilution of polyclonal antiserum to CPB (Robertson & Coombs, 1994), followed by a 1:1000 dilution of horseradish peroxidase-coupled secondary antibodies. Five% (w/v) dried milk and 10% (v/v) horse serum in TBS-Tween was used as a blocking agent and blots were developed using the Amersham ECL system.

Infection of peritoneal exudate cells

The methods used were modified from those described previously (Mallinson & Coombs, 1989). Peritoneal exudate cells (PECs) were obtained by peritoneal lavage of female BALB/c mice and stationary phase promastigotes or lesion amastigotes were used to infect the PECs in chamber slides (NUNC) using a parasite:PEC ratio of 1:1 and either a 4 h (promastigotes) or a 1 h (amastigotes) incubation. Subsequently, free parasites were removed by repeated washing with medium. The medium used for these studies was RPMI 1640 (Labtech), pH 7·2, supplemented with 10% (v/v) heat-inactivated FCS (Seralab), 25 μg/ml gentamycin (Sigma) and 2 mM L-glutamine (Gibco) (designated complete RPMI). After appropriate incubation (routinely 7 days), cells were fixed, stained with Giemsa's stain and the percentage of infected PECs determined by counting 200 cells. Where time-course experiments were undertaken, PECs were allowed to adhere overnight as usual and then incubated in medium axenically for a further 2 days before initiation of infection; this allowed intracellular parasites to be more easily observed at the early time-points of 2, 4 and 8 h as the macrophages had spread sufficiently during the 2 days prior to infection.

For inhibitor studies, parasites were resuspended to the required density in complete RPMI medium containing 10 μg/ml inhibitor and 0·1% Me_2SO and incubated for 15 min at 25 °C (promastigotes) or 32 °C (amastigotes). Parasites were then added to the PECs in chamber slides and incubated as appropriate, the slides were washed 3 times in complete RPMI to remove free parasites, incubated usually for either 72 h or 7 days in complete RPMI medium containing 10 μg/ml inhibitor, and then the cells were fixed and stained as before.

Infection of BALB/c mice

The inoculation of parasites into BALB/c mice and the monitoring of resulting lesions were carried out as described previously (Bates *et al*. 1992). Each mouse was inoculated subcutaneously in the shaven rump with either 5×10^6 promastigotes or axenic amastigotes or 5×10^5 lesion amastigotes. Amastigotes were purified from lesions in mice as described previously (Bates *et al*. 1992).

RESULTS

Kinetics of infectivity of CP-deficient mutants to peritoneal exudate cells and BALB/c mice

We reported previously (Mottram *et al*. 1996) that there was a great difference in infectivity to PECs between stationary phase promastigotes of wild-type *L. mexicana* and Δ*CPB*, with Δ*CPB* being present in very few macrophages after 7 days and having a lower number of amastigotes per infected PEC. To address the question as to whether the difference was due to lower uptake or poorer intracellular survival, time-course experiments were carried out (Fig. 1). It was found that similar numbers of PECs were initially infected by both wild-type and Δ*CPB* stationary phase promastigotes, but between 4 and 24 h there was a rapid decline in the number of PECs infected with Δ*CPB* such that by 24 h the percentage infected was similar to that at 3 days (Fig. 1 A). The number of amastigotes per infected macrophage was similar with both lines up to 24 h, whereupon wild-type parasites multiplied whereas the numbers of the mutants remained roughly constant (Fig. 1 B). Δ*CPB* amastigotes were found to reside in large parasitophorous vacuoles showing that the CPB enzymes do not contribute to the characteristically large parasitophorous vacuoles associated with macrophage infections with members of the *L. mexicana* complex.

The infectivity of the lines to BALB/c mice was investigated. Inoculation of stationary phase promastigotes of wild-type parasites produced large lesions by 20–25 weeks (not shown), and the inoculation of axenic amastigotes resulted in somewhat bigger lesions more quickly (Fig. 2). Inoculation of axenic amastigotes of Δ*CPB* resulted in the formation of lesions, but they took between 10 and 16 weeks longer to appear than those caused by wild-type parasites and grew very poorly in comparison (Fig. 2). Δ*CPB2.8*, the line re-expressing a single copy of the *CPB* array (gene 2.8), was somewhat more virulent than Δ*CPB*. Axenic amastigotes of Δ*CPB2.8* produced lesions only 3 weeks later than wild-type parasites (by week 14) and 7–13 weeks before the null mutant (Fig. 2) – suggesting that some complementation of the phenotype was occurring. However, the resulting lesions increased in size only very slowly. The double null mutant (Δ*CPA*/*CPB*) did not produce lesions by 9 months, when the experiments were terminated, but viable parasites could still be isolated from the site of inoculation at this time.

It was confirmed by gelatin–SDS–PAGE analysis that parasites obtained from the lesions in mice resulting from inoculation of Δ*CPB* indeed lacked active CPB (Fig. 3, lane 3). Southern and Western blotting also confirmed the lack of *CPB* genes and CPB protein (not shown). Parasites isolated from animals infected with Δ*CPB2.8* possessed CPB activity, but at an extremely low level that was not detectable under standard conditions of gelatin–SDS–PAGE analysis (Fig. 3, lane 1). CPB activity was increased by culturing Δ*CPB2.8* promastigotes in 500 μg/ml G418 (Fig. 3, lane 2). Mutant parasites passaged through mice, re-isolated, transformed to promastigotes and used to infect PECs *in vitro* gave

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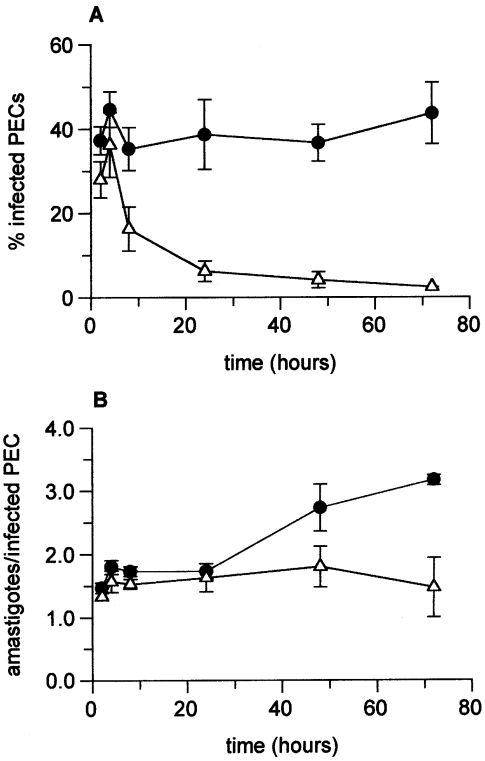

Fig. 1. The fate of wild-type (-●-) and Δ*CPB* (-△-) stationary phase promastigotes after entry into PECs *in vitro*. PECs were infected with stationary phase promastigotes at a ratio of 1:1 and the parasite load determined by counting the number of infected PECs (A) and number of parasites per infected cell (B). The data are means ± S.D. from 3 independent experiments.

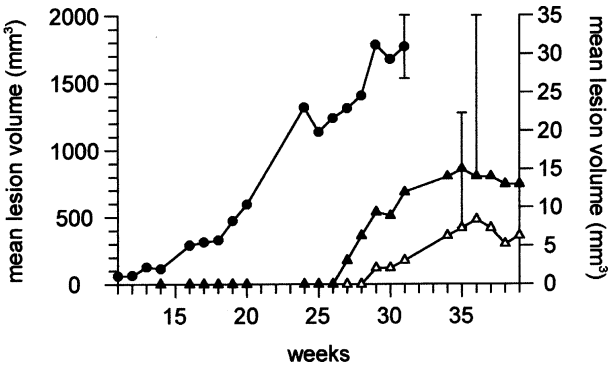

Fig. 2. Infectivity of axenic amastigotes of *Leishmania mexicana* to BALB/c mice. Subcutaneous lesions in the rumps of female BALB/c mice resulting from an inoculum of 5×10^6 axenic amastigotes were measured weekly using a micrometer, and the mean lesion volume ± S.D. per group (5 mice) calculated using the equation for the volume of a hemisphere. (-●-) Wild-type (left hand Y axis); (-△-) Δ*CPB* and (-▲-, △)*CPB2.8* (right hand Y axis); note the 100-fold difference between the two Y axes. Lesions appeared in some mice inoculated with Δ*CPB2.8* from week 14 but they were extremely small and only started to grow significantly from week 27. The majority of error bars have been omitted for clarity.

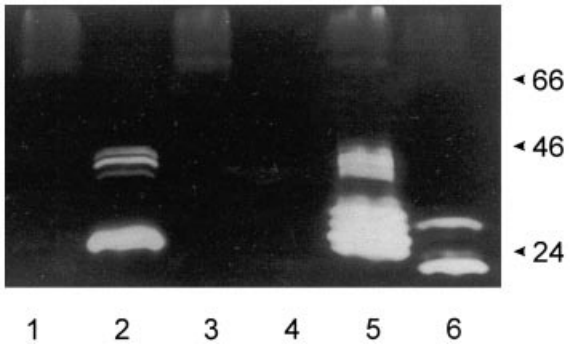

Fig. 3. Gelatin–SDS–PAGE analyses of wild-type and mutant parasites. Samples containing lysates (equivalent to 10^7 cells) were analysed by gelatin–SDS–PAGE: Δ*CPB2.8* amastigotes (lane 1) and promastigotes (lane 2), Δ*CPB* amastigotes (lane 3) and promastigotes (lane 4), wild-type amastigotes (lane 5) and promastigotes (lane 6). The positions of molecular mass markers (kDa) are indicated. The multiple CPBs of wild-type *Leishmania mexicana* result in several activity bands in the 24–30 kDa region of the gel. The stage specificity of the CPB isoenzymes is clearly demonstrated in lanes 5 and 6. The activities at around 35 kDa are thought to be due to CP precursors. Neither CPA nor CPC can be detected by gelatin–SDS–PAGE, whereas the activities > 60 kDa are due to proteinases other than CPs. For further details see Coombs & Mottram (1997*a*, *b*).

the results presented in Fig. 1 and were the cells used throughout the remainder of this study.

The lesion-derived amastigotes of Δ*CPB* were as infective as wild-type amastigotes to PECs, and the multiplication of amastigotes within the macrophages was similar with the two lines (Fig. 4). Δ*CPB* and Δ*CPB2.8* lesion amastigotes were also used to infect BALB/c mice (Fig. 5). As found with Δ*CPB* promastigotes, Δ*CPB* lesion amastigotes produced lesions in animals much more slowly than did the wild-type line – indicating that the situation *in vivo* is more complex than that *in vitro*. Δ*CPB2.8* lesion amastigotes produced lesions only 5 weeks later than the wild-type amastigotes; however, these lesions grew poorly. In contrast, Δ*CPA* was as infective as wild-type parasites.

Effects of peptidyl-diazomethylketones on parasite growth and proteinase activity

Promastigotes and axenic amastigotes of both wild-type and mutant lines cultured in the presence of 10 μg/ml Z-LVG-DMK or 10 μg/ml Z-FA-DMK grew similarly to control cultures (data not shown). Furthermore, neither inhibitor appeared to affect transformation of multiplicative promastigotes to metacyclics or transformation of stationary phase promastigotes to axenic amastigotes (as judged from morphological observations and the appearance of stage-specific CP activities in both cases).

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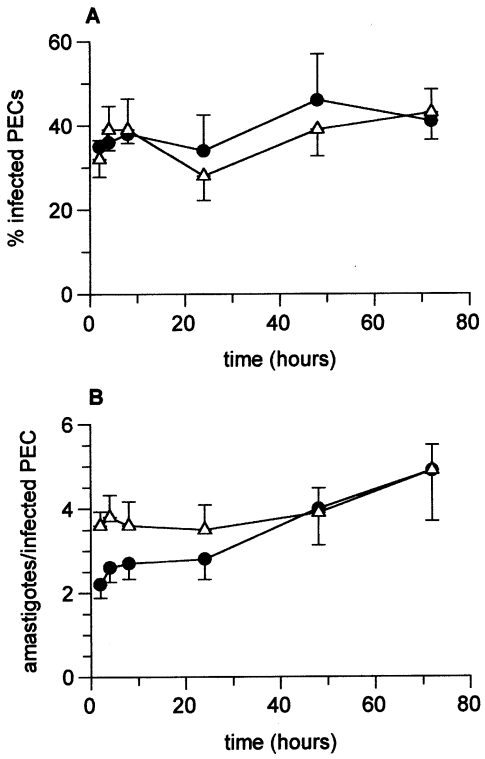

Fig. 4. The fate of wild-type (-●-) and Δ*CPB* (-△-) lesion amastigotes after entry into PECs *in vitro*. PECs were infected with lesion amastigotes of the *Leishmania mexicana* lines at a ratio of 1:1 and the parasite load determined by counting the number of infected PECs (A) and number of parasites per infected cell (B). The data are means ± S.D. from 3 independent experiments.

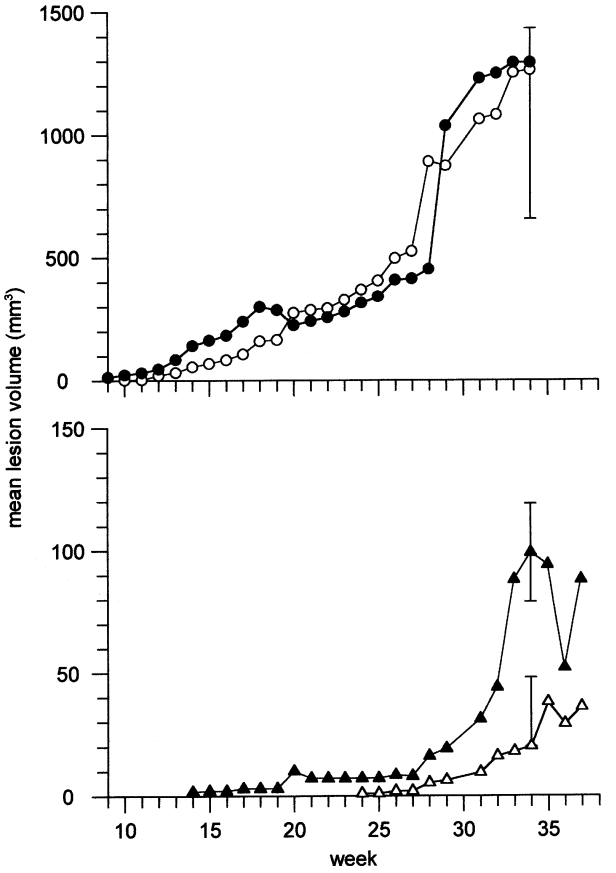

Fig. 5. Infectivity of lesion amastigotes of *Leishmania mexicana* lines to BALB/c mice. Subcutaneous lesions in the rumps of female BALB/c mice resulting from an inoculum of 5×10^5 lesion amastigotes were measured weekly using a micrometer, and the mean lesion volume ± S.D. per group (5 mice) calculated using the formula for a hemisphere. (-●-) Wild-type; (-△-) Δ*CPB*; (-▲-) Δ*CPB2.8*; (-○-) Δ*CPA*. Note the difference between the scales of the Y axes. The majority of error bars have been omitted for clarity.

Gelatin–SDS–PAGE analyses of stationary phase promastigotes of wild-type parasites grown in the presence of inhibitors revealed that they contained very little of the high mobility CP activities of apparent molecular masses between 25 and 20 kDa, which are characteristically present in untreated wild-type parasites (Fig. 6, compare lanes 1–3) and are encoded by genes from the *CPB* array (Robertson & Coombs, 1994; Mottram *et al.* 1996). However, the slightly slower mobility CP activities of apparently 35 to 40 kDa, which are likely to be precursor molecules activated *in situ* in the gel and normally are at very low activity in wild-type promastigotes, were at relatively high activity. The low mobility activities of > 60 kDa (due to proteinases other than CPs, such as the metalloproteinase gp63) also appeared to be up-regulated in cells grown in the presence of the inhibitors. Cell extracts from parasites grown in the absence of inhibitor incubated with 10 μg/ml Z-FA-DMK (not shown) or Z-LVG-DMK (Fig. 6, lane 4) lacked both the high (20–25 kDa) and lower mobility (35–40 kDa) gelatinase activities. This confirmed that the 35–40 kDa activity bands are able to be inhibited by the DMKs, although this did not apparently occur in the living cell. Similar experiments with axenic amastigotes showed that those grown in the presence

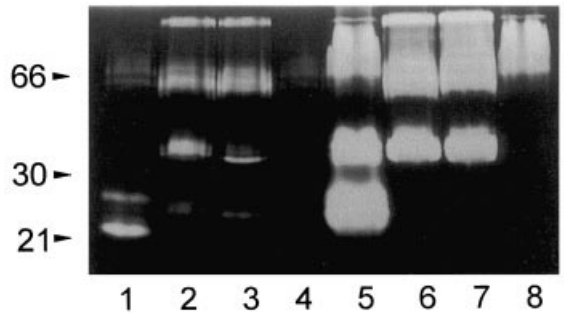

Fig. 6. Gelatin–SDS–PAGE analyses of cell extracts from *Leishmania mexicana* grown in the presence of CP inhibitors. Cells grown for 7 days with inhibitors were washed thoroughly, lysed and subjected to gelatin–SDS–PAGE. Lane 1, untreated stationary phase promastigotes; lanes 2 and 3, promastigotes grown with Z-LVG-DMK and Z-FA-DMK, respectively; lane 4, promastigotes lysed with Z-LVG-DMK. Lanes 5–8: as lanes 1–4 except axenic amastigotes. The positions of molecular mass markers (kDa) are indicated.

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Table 1. Effect of proteinase inhibitors on the infectivity of stationary phase promastigotes of *Leishmania mexicana* to peritoneal exudate cells (PECs)

(The parasites were pre-incubated with the inhibitors for 15 min and the inhibitors were present throughout the 7-day duration of the experiment. The cells were then fixed, stained with Giemsa's stain, and parasite load determined. The values given are the means ± S.D. from 3 independent experiments. The percentage infected PECs for untreated wild-type parasites, and those exposed to Me_2SO or Z-LVG-DMK, were significantly different from both Z-FA-DMK-treated wild-type parasites and Δ*CPB* (P values of $< 0{\cdot}01$, 0·01 and 0·1, respectively) but not from each other; infection rates for Z-FA-DMK-treated wild-type parasites and Δ*CPB* were not significantly different. Values of amastigotes/infected PEC for wild-type parasites treated with Me_2SO, Z-LVG-DMK and Z-FA-DMK were all significantly different from that for wild-type parasites alone, as was the figure for Δ*CPB*, (P values of at least $< 0{\cdot}1$) but they were not significantly different from each other.)

Cell line, Inhibitor	Infected PECs (%)	Amastigotes/Infected PEC
Wild-type	53 ± 6·1	4·8 ± 0·9
Wild-type + Me_2SO	45 ± 11	3·4 ± 0·7
Wild-type + Z-LVG-DMK	42 ± 24	3·0 ± 1·5
Wild-type + Z-FA-DMK	7·0 ± 8·7	1·9 ± 1·3
Δ*CPB*	5·3 ± 5·5	3·6 ± 4·1

of Z-LVG-DMK (Fig. 6, lane 6) or Z-FA-DMK (Fig. 6, lane 7) lacked the typical high mobility CP activity present in parasites grown in the absence of inhibitor (Fig. 6, lane 5) but that the characteristic lower mobility activities (approximately 35–40 kDa) of axenic amastigotes (Bates *et al*. 1992) were active. These lower mobility activities were inhibited by Z-LVG-DMK (Fig. 6, lane 8). This confirmed that the activities were due to CPs. Addition of spent medium from day 7 of these growth experiments to the cell lysates also resulted in inhibition of the CP activities, which confirmed that the inhibitors remained active throughout the course of the 7-day incubation (data not shown). As with promastigotes, there was increased activity of enzymes of around 60 kDa in the amastigotes grown with inhibitors. Apparently these are predominantly metalloproteinases, since they were fully inhibited by 1 mM *o*-phenanthroline added during substrate digestion (not shown). The lack of CP activity in the inhibitor-treated cells was not due to inhibition upon cell lysis by inhibitor bound to the cell surface as analysis by gelatin–SDS–PAGE of lysates of axenic amastigotes grown in the presence of DMK mixed with lysates of control axenic amastigotes showed the CP activity of the control cells was not inhibited (data not shown).

Effects of inhibitors on parasite infection of peritoneal exudate cells

Z-FA-DMK greatly reduced the infectivity of wild-type promastigotes to PECs, to a level similar to that of Δ*CPB* in the absence of CP inhibitors (Table 1). There was also a lower number of amastigotes per infected cell when Z-FA-DMK was present compared with when the drug was absent. However, Z-LVG-DMK, which also inhibited the CPs in axenic parasites (Fig. 6, lane 6), surprisingly did not reduce the infectivity of promastigotes to PECs (Table 1).

To determine the stage of the infection process most susceptible to Z-FA-DMK, the inhibitor was applied for different periods. Similar results were obtained irrespective of whether the promastigotes were pre-incubated with Z-FA-DMK for 15 min, and the inhibitor subsequently included throughout the incubation, or if the inhibitor was added 4 h or 24 h after infection of the PECS with the parasites (Table 2). However, the length of the incubation was found to be important. The inhibitor had much greater anti-parasite effect over 7 days, after which the number of parasite-infected cells was found to be less than 10% of the control, than when the experiment was stopped after 3 days. The mean number of parasites per infected macrophage was not affected greatly by the time of initial exposure to the DMK (Table 2); but was reduced more by 7 days than by 3 days.

To determine whether use of Z-FA-DMK mimicked deleting the *CPB* array, the effect of the inhibitor on the infectivity of wild-type lesion amastigotes to PECs was examined (Table 3). After 7 days incubation with Z-FA-DMK, the number of infected macrophages was again reduced to a very low level compared with the control parasites (and, as with promastigotes, this reduction in infectivity was not as pronounced after just 3 days); as was the number of amastigotes per macrophage (Table 3). Again, Z-LVG-DMK had less effect.

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Table 2. Effect of Z-FA-DMK on the infectivity of wild-type promastigotes to PECs

(Stationary phase promastigotes were either pre-incubated for 15 min with 10 μg/ml Z-FA-DMK and exposed to the inhibitor throughout, or the inhibitor was added 4 h or 24 h after the PECs were first exposed to the parasites. Controls contained 0·1 % Me_2SO. The data are the means ± S.D. from 3 independent experiments. The percentage infected PECs after 3 days for pre- and 4 h incubations were significantly different from the control (P values of $< 0{\cdot}05$ and $< 0{\cdot}02$, respectively) but not from each other. The infection rates at 7 days for pre-, 4 h and 24 h incubations were all highly significantly different from the control (P values $< 0{\cdot}01$). The number of amastigotes/infected PEC after 3 days with pre-, 4 h and 24 h incubations were all significantly different from the control (P values of $< 0{\cdot}02$, $< 0{\cdot}02$, and $< 0{\cdot}05$, respectively) but not from each other. The number of amastigotes/infected PEC at 7 days with pre-, 4 h and 24 h incubations were still significantly different from the control (P values of $< 0{\cdot}05$, $< 0{\cdot}05$ and $< 0{\cdot}01$, respectively).)

	Infected PECs (%)		Number of amastigotes/PEC	
Inhibitor schedule	3 days	7 days	3 days	7 days
Present throughout	19·0 ± 6·2	0·7 ± 1·1	1·6 ± 0·3	0·5 ± 0·9
From 4 h post-infection	20·0 ± 1·5	1·0 ± 1·3	1·7 ± 0·1	0·8 ± 0·7
From 24 h post-infection	22·0 ± 12·0	1·9 ± 1·7	1·8 ± 0·4	1·1 ± 0·9
None	35·0 ± 6·6	29·0 ± 3·6	2·8 ± 0·4	2·5 ± 0·3

Table 3. Effect of CP inhibitors on the infectivity of wild-type lesion amastigotes to PECs

(The parasites were pre-incubated for 15 min with the inhibitors, which were also present in the culture medium at 10 μg/ml for the duration of each experiment (3 or 7 days). The data are the means ± S.D. from 3 independent experiments. The percentage infected PECs after 3 days with either Z-FA-DMK or Z-LVG-DMK were not significantly different from the controls; after 7 days the percentage infected PECs with the controls and Z-LVG-DMK were highly significantly different from that with Z-FA-DMK (P values $< 0{\cdot}01$), but not from each other. The numbers of amastigotes/PEC after 3 days with Z-FA-DMK and Z-LVG-DMK were significantly different from the controls (P values of $< 0{\cdot}02$ and $< 0{\cdot}01$, respectively) but not from each other; after 7 days the values with Z-FA-DMK and Z-LVG-DMK were still significantly different from the controls (P values of $< 0{\cdot}05$ and $< 0{\cdot}01$, respectively). The results for the untreated and 0·1 % Me_2SO controls were not significantly different from each other.)

	Infected PECs (%)		Number of amastigotes/PEC	
Inhibitor	3 days	7 days	3 days	7 days
None	40·0 ± 14·0	41·0 ± 1·4	4·3 ± 0·1	6·7 ± 1·4
Z-LVG-DMK	33·0 ± 12·0	29·0 ± 8·5	2·6 ± 0·1	2·6 ± 0·3
Z-FA-DMK	18·0 ± 13·0	0·4 ± 0·2	2·8 ± 0·3	2·3 ± 1·2
None (0·1 % Me_2SO)	31·0 ± 10·0	37·0 ± 2·0	4·0 ± 0·4	4·3 ± 1·4

DISCUSSION

The principal aim of this study was to improve our understanding of the roles of *L. mexicana* CPs in the interaction of the parasite with its host. The results add support to the previous contention that the CPB enzymes are virulence factors (Mottram *et al*. 1996) and the evidence presented suggests that the CPB enzymes not only aid the initial survival of promastigotes as they differentiate into amastigotes in macrophages but also play other roles that facilitate infections in mice.

Time-course experiments showed that promastigotes of the *CPB* null mutants initially infect PECs as successfully as wild-type parasites, thus showing that the enzymes do not have a role in the invasion process itself, but most are then killed within 24 h. This intracellular death could be due to the CP-

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deficient mutants being less able to transform to amastigotes sufficiently rapidly, or being less able to withstand the initial microbicidal action of the macrophage, or both. The differentiation of the promastigote to the amastigote requires considerable cell remodelling that also involves a large amount of protein turnover. It is possible that the CPs play a role in this process and that the lack of the CPB enzymes compromises the ability of the parasite to survive the microbicidal action of the macrophage during the differentiation process. The discovery that lesion amastigotes of the *CPB* null mutants differed considerably from promastigotes in being able to infect macrophages *in vitro* as efficiently as wild-type lesion amastigotes, adds strong support to the hypothesis that the lack of the CPB enzymes makes promastigotes vulnerable to the microbicidal activity of macrophages. Unfortunately, however, the finding does not distinguish between the two postulates for the death of the promastigotes lacking CPB – either they are more sensitive *per se* to the microbicidal activities of macrophages, or they transform to amastigotes more slowly and so are less able to avoid being killed as promastigotes.

The findings of the importance of CPB for promastigote survival in macrophages but apparent unimportance for amastigote survival would appear to be inconsistent with the perceived importance of the enzymes for amastigotes, a belief arising from the amastigote specificity of most of the CPB enzymes (Robertson & Coombs, 1990; Mottram *et al*. 1997). However, it is now known that the first 2 *CPB* genes of the array are metacyclic-specific in their expression pattern (Mottram *et al*. 1997), which is consistent with an important role in this stage. In addition, the *in vivo* infectivity data obtained in the current study clearly show that the CPB enzymes play an important part in the amastigote's growth in a mammal even if not in isolated macrophages.

The poor ability of Δ*CPB* promastigotes to infect macrophages could be one reason why they show low virulence in mice. Clearly, however, the situation is not as simple as this for the lesion amastigotes also were poor at infecting mice. This suggests that at least some isoenzymes of CPB have other roles in the host–parasite interaction. One possibility is that the amastigote CPBs help the parasite circumvent the host's immune system by the destruction of effector molecules that would not be of consequence in *in vitro* macrophage infections. Indeed it has been reported that the CPB enzymes degrade MHC II of the host macrophage (Leao *et al*. 1995). Parasite CPs have also been reported to have direct actions on other components of the mammalian immune system, such as immunoglobulins (Bontempi & Cazzullo, 1990) and complement factors (Reed, Keen & McKerrow, 1989). In some cases they are thought to interfere with antigen presentation and processing (Arholdt & Scharfstein, 1991) and it has also been reported that CPs may modulate cytokine responses in a way that is beneficial to the parasite's survival (Finkelman & Urban, 1992). In support of this contention, we have shown that mice mount a Th1-associated immune response to Δ*CPB* mutants in contrast to the Th2-associated response to wild-type parasites (Alexander *et al*. 1998). Another possible reason for the low infectivity of Δ*CPB* lesion amastigotes is that they have reduced capacity to invade or survive in cell types of low phagocytic potential such as Langerhan's cells and fibroblasts which could play an important part in the parasite's interactions *in vivo* (Blank *et al*. 1993; Bogdan & Röllinghoff, 1998).

There are several possible explanations for the finding that Δ*CPB2.8* mutants also produced relatively small lesions in mice, although it is notable that this line re-expressing 1 *CPB* gene consistently produced lesions several weeks before those of Δ*CPB*. *CPB2.8* expression dropped substantially during lesion formation, when G418 drug selection pressure could not be maintained, and this could account for the smaller lesions. It was anticipated that if the *CPB2.8* gene was advantageous to the parasite *in vivo* then there would have been selective pressure to maintain a high level of expression. However, it is possible that the pTEX plasmid used in this study produces a lower level of expression in amastigotes compared to promastigotes, as has been noticed previously for other plasmids (McGwire & Chang, 1994). At least some parasites retained the episome as CPB2·8 expression returned to high levels when parasites recovered from the animals were grown under G418 drug selection. Nevertheless, the possibility that many amastigotes lost the episome when in mice cannot be excluded. An alternative explanation is that CPB2.8 is not by itself sufficient to allow optimal parasite survival in mice and the other isoenzymes are also required.

The two CP inhibitors Z-FA-DMK and Z-LVG-DMK used in this study were shown to inhibit CPB in both parasite lysates and the living parasites and could be used to mimic, to some extent, deletion of the genes. Thus their lack of effect on promastigote growth was to be expected. The inhibitors also had no obvious effect on transformation of the parasite, either from promastigote to metacyclic or from metacyclic to amastigote, consistent with a previous report on metacyclic to amastigote transformation of *L. mexicana in vitro* (Bates, Robertson & Coombs, 1994). This suggests that if CPB does play a role in these processes, it is a subtle one. These findings clearly show that *L. mexicana* differs in this respect from the related trypanosomatid *Trypanosoma cruzi*. The differentiation of this parasite from epimastigote to metacyclic form is susceptible to the same peptidyl-DMKs (Bonaldo *et al*. 1991; Mereilles *et al*. 1992; Franke de Cazzulo *et al*. 1994).

The gelatin–SDS–PAGE analyses of wild-type

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cells grown with the two DMK inhibitors not only confirmed that CPB isoenzymes were inhibited but also revealed that the CPB pro-enzymes were accumulated. It is likely that DMK inhibitors interfere with the catalytic processing of the pro-forms of CPB and so results in the build up of pro-enzymes. The lack of inhibition of the 35–40 kDa CP enzymes when the inhibitors are added to cultures of parasites has been reported previously (Pral *et al*. 1993). Possible explanations are that in the living cells the enzymes are inactivated by endogenous inhibitors, or predominantly exist as larger molecular weight precursors. However, it is also possible that these lower mobility CPs are in a cell compartment impervious to the diazomethylketone CP inhibitors tested. However, perhaps the most likely explanation is simply that *in vivo* they are not active (as would be expected for pro-enzymes) and so would not bind the inhibitors and are only activated upon or subsequent to cell lysis. The increase of these precursors in the drug-treated parasites is suggestive of the inhibitors reducing the processing of the pro-enzymes to the mature form and indicates that a CP is at least partially responsible for this.

Previous studies had shown amastigote growth in macrophages to be hindered by the CP inhibitors antipain and leupeptin (Coombs & Baxter, 1984), although it is now known that the activities of these compounds are not restricted to CPs. The results of the current study clearly indicate that specific peptidyl-DMK CP inhibitors are active against *L. mexicana* growing intracellularly *in vitro*. Our finding that Z-FA-DMK reduced the infectivity of wild-type promastigotes to PECs by $> 80\%$ by day 7 of infection is consistent with a role for CPs in the intracellular survival of the parasite and mirrors the effect of deleting the *CPB* genes (Mottram *et al*. 1996). However, there are important differences between the results obtained with Δ*CPB* and the use of the inhibitors. In particular, the inhibitors affected the infectivity of amastigotes to PECs and they were effective even when only added to the cultures 24 h after initiation; also parasite clearance was slower. It seems very likely that the explanation for these findings is that the inhibitors were affecting other CPs in addition to CPB. These could either be CPs of the parasite or the host macrophage. This is an important finding, for it could be that parasite CPs other than CPB offer even better opportunities for chemotherapeutic exploitation. The recent report (Selzer *et al*. 1999) that both promastigotes and amastigotes of *L. major* are killed by inhibitors of CPs not only reinforces the potential of leishmanial CPs as drug targets but also shows that there are distinct differences between species in their susceptibility to CP inhibitors.

The findings of this study reaffirm both that the CPB isoenzymes of *L. mexicana* play important roles in aiding the parasite's survival and proliferation in mice and also that CP inhibitors have potential as anti-leishmanial drugs. However, the evidence would suggest that it might be advisable to target enzymes in addition to CPB, either other essential enzymes or perhaps several enzymes. The report (Engel *et al*. 1998*b*) that CP inhibitors kill *T. cruzi* in an indirect way by causing an accumulation of unprocessed pro-enzyme that is lethal to the cells shows that inhibitors can be effective even without targeting an essential enzyme. CP inhibitors may also be beneficial against leishmaniasis by inhibiting the pathological effects of released amastigote proteinases (Ilg *et al*. 1994). The anti-leishmanial activity of peptidyl-based CP inhibitors *in vitro* is promising, but there seemed to be reasonable concern that their pharmacokinetic properties *in vivo* may be poor. Thus the recent reports of *in vivo* anti-parasite activity are encouraging (Engel *et al*. 1998*a*; Selzer *et al*. 1999).

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