Multiple high activity cysteine proteases of *Leishmania mexicana* are encoded by the *lmcpb* gene array

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The interrelationship of the multiple cysteine proteases (CPs) found characteristically at high activity in *Leishmania mexicana* amastigotes has been investigated. The mature forms of the five enzymes of groups B and C, which have subtly different substrate preferences, are the same size. Enzymically deglycosylated group A CP proteins also have the same molecular mass. Proteases of all three groups are specifically recognized by antisera raised against the group B or group C CPs. In addition, CPs of groups A, B and C have highly similar N-terminal amino acid sequences. The consensus sequence matches that predicted from the sequenced *lmcpb* gene, which occurs in a tandem array of over ten similar genes. Thus, the results are consistent with the groups A, B and C CPs being products of different *lmcpb* genes within the array, the different genes encoding CPs with identical N-termini, but with limited amino acid substitutions within the mature enzyme accounting for the different properties of the CPs. Evidence is also presented to indicate membrane-association of proteolytically active but less processed forms of *lmcpb* products.

Keywords: *Leishmania mexicana*, cysteine proteases, *lmcpb* genes, amino acid sequence

INTRODUCTION

The intracellular amastigote form of the protozoan parasite *Leishmania mexicana* has a much greater cysteine protease (CP) activity than promastigote forms (North & Coombs, 1981; Coombs, 1982; Lockwood *et al.*, 1987; Coombs *et al.*, 1991; Robertson & Coombs, 1992). It has been proposed that this high CP activity is crucially important for the survival of the amastigote in its host mammalian macrophage, and, hence, that the enzymes may be targets for novel anti-leishmanial drugs based on specific enzyme inhibitors or pro-drugs preferentially activable by the amastigote CPs.

Previous studies employing substrate-SDS-PAGE have indicated that the high amastigote CP activity is due to multiple enzymes (North & Coombs, 1981; Lockwood *et al.*, 1987; Coombs *et al.*, 1991; Robertson & Coombs, 1990, 1992). The enzymes have been segregated into three groups, A, B and C, on the basis of their physical properties and substrate preferences (Robertson & Coombs, 1990). The group A CPs are glycosylated as shown by their specific association with concanavalin A Sepharose, whereas the groups B and C CPs do not bind to the immobilized lectin. The groups B and C CPs differ in terms of their net charge at pH 6.0 (the group B enzymes elute from anion-exchange columns at lower salt concentrations), and also in terms of their substrate preferences, with the group C CPs being less able to accommodate the bulky amino acid tyrosine in the substrate P1 position.

The interrelationship of these enzymes was, however, uncertain, and it was not apparent whether the enzymes are encoded by different genes or are post-transcriptionally or post-translationally modified forms of just one gene product. It is known, for example, that differential proteolytic processing at the N-terminus of the mature enzyme of a barley aleurone layer CP is responsible for multiple CP activities detected using substrate-SDS-
PAGE gels (Koehler & Ho, 1988). This investigation was undertaken with the aim of resolving this uncertainty.

Two CP genes have been cloned from a *L. mexicana* amastigote cDNA library and sequenced (Mottram et al., 1992; Souza et al., 1992). One of these, *lmcpa*, is a single-copy gene, and, although it is expressed at higher levels in amastigotes than in multiplicative promastigotes, it does not appear to encode any of the multiple high-activity CPs of the amastigote (Mottram et al., 1992). The other gene, *lmcpb*, is present as a tandem array of more than ten copies. Its mRNA is much more abundant in amastigotes than promastigotes (Souza et al., 1992), indicating a transcriptional regulation that parallels the expression of the multiple CP activities.

Here we report evidence, including N-terminal sequence data, which indicates that the multiple high-activity CPs of *L. mexicana* amastigotes are products of the *lmcpb* gene array, and supports the notion that expression of slightly different copies of *lmcpb* within the gene array accounts for the different CP activities detected. In addition, data are presented to show that other similar CP activities are membrane-associated.

**METHODS**

**Parasites.** Promastigotes of *L. mexicana* (MNVC/BZ/62/M379) were grown axenically in *vitro* at 25 °C as described previously (Mallinson & Coombs, 1986). Multiplicative promastigote populations were harvested 2 d after culture initiation at 10⁷ parasites ml⁻¹, and stationary-phase populations (containing putative metacyclic promastigotes) were harvested 9 d after culture initiation. The parasites were harvested and washed as described previously (Mallinson & Coombs, 1986).

Amastigotes of *L. mexicana* were purified essentially as described previously (Mottram & Coombs, 1985) from cutaneous lesions raised in CBA mice.

Purified parasites were stored as washed pellets at −70 °C until required.

**CP purification.** CPs of groups A, B, and C were purified from *L. mexicana* amastigotes using the chromatography procedures and enzyme assays described previously (Robertson & Coombs, 1990). Briefly, the supernatant fraction (11,600 *g*, 5 min) of an amastigote lysate prepared in 0.25 M sucrose/0.25% (v/v) Triton X-100 was passed through a Pharmacia HR 10/30 Superose 12 gel-filtration column. Fractions with activity towards BzPFRpNA were pooled and applied to a Pharmacia Mono Q HR 5/5 column and eluted with a non-linear NaCl gradient. After gelatin-SDS-PAGE analysis of the eluted activities, fractions having CP activities of groups A, B, and C, and groups A and C, were pooled separately. These were then passed through a 1 ml column of concanavalin A Sepharose to which the group A CPs bound specifically. The three separate pools containing CPs of groups A, B, and C, respectively, were concentrated by ultrafiltration on an Amicon (w/v) with respect to SDS and incubated at 4 °C in 25 mM Tris/192 mM glycine/20% (v/v) methanol for 60 min at 100 °C (Bio-Rad mini-transblotter). The blots were routinely stained with Ponceau S to allow molecular mass standard (Sigma MW SDS-70L) positions to be marked. The blots were blocked overnight at 4 °C in 25 mM Tris/0.15 M NaCl/2.7 mM KC1 0.02% Tween 20, pH 7.5 (TBS-Tween), containing 5% (w/v) non-fat dried milk and 10% (v/v) horse serum, and then incubated in the same solution for 2 h at 4 °C with 1/500 anti-B CP antiserum or 1/1000 anti-C CP antiserum. The blots were washed three times with TBS-Tween and once with 50 mM Tris/0.15 M NaCl, pH 7.4 (Tris-NaCl), before being incubated at ambient temperature for 60 min in Tris-NaCl containing 5% (w/v) non-fat dried milk and 1.7500 goat anti-rabbit IgG (Fc) alkaline phosphatase conjugate (Promega). The blots were then washed with four changes of Tris-NaCl and incubated in alkaline phosphatase assay buffer (0.1 M Tris/0.1 M NaCl/5 mM MgCl₂/0.9 mM BCIP/0.2 mM NBT, pH 9.5) until stained, and the reaction stopped in 0.5 M EDTA/NaOH, pH 8.

**Enzymatic N-deglycosylation.** Samples of purified CPs (0.02 ml) were made 1% (w/v) with respect to SDS and incubated at 100 °C for 2 min. The samples were mixed with 0.18 ml 55% (w/v) *n*-octylglucoside in 20 mM sodium phosphate/10 mM Na₂SO₄/50 mM EDTA, pH 7.2, and again incubated for 2 min at 100 °C. The sample was split in two, and 2 µl of stock buffer. This material represents the crude preparation of group E CPs. Experiments to solubilize the group E CP activity used a fraction of this crude preparation, pelleted by centrifugation (11,600 *g*, 5 min) and washed twice with storage resuspension buffer. The pellet was then resuspended with solubilization buffer.

**SDS-PAGE.** Mini (0.75 mm thickness) 11% (w/v) acrylamide SDS-PAGE gels were used for protein analyses and for protein separation prior to Western blotting. Analysis of CP activity towards both copolymerized gelatin and fluorogenic peptide substrates after SDS-PAGE was done as described previously (Robertson & Coombs, 1990).

The following modifications were used for SDS-PAGE separation of CP proteins for sequence analysis. The gels were prepared with PDA (Bio-Rad) substituted weight for weight with N,N'-methylene-bisacrylamide. This did not significantly alter the mobilities of the proteins in the gels. Prior to loading the sample, the gel was run for 60 min at 3 mA with 0.05 mM reduced glutathione in the cathode buffer. The sample was separated with fresh electrophoresis buffer, with 0.1 mM sodium thioglycolate included in the cathode buffer.

**N-terminal sequence analysis.** Purified proteins for sequencing were separated by SDS-PAGE as described above, and electroblotted to Problott PVDF membrane (Applied Biosystems) for 30 min at 50 V in 10 mM CAPS/NaOH pH 11, 10% (v/v) methanol. The blotted proteins were stained on the membrane with Amido black and excised for sequence analysis (Mar-I-suada, 1987). Sequencing was performed by Mr B. Dunbar at the SERC Protein Sequencing Facility, Department of Molecular and Cell Biology, University of Aberdeen, UK.

**Production of antisera.** Antisera against group B CP proteins (anti-B CP antiserum) and against group C CP proteins (anti-C CP antiserum) were produced in New Zealand White rabbits following standard procedures (Harlow & Lane, 1988). Reduced and denatured CP proteins (approx. 10 µg for each group) were excised from SDS-PAGE gels and homogenized with Freund's complete adjuvant for the first inoculation, and Freund's incomplete adjuvant for subsequent boosts. Antisera were collected on day 10 after the third boost.

**Immunostaining of Western blots.** SDS-PAGE gels were blotted to 0.22 µm nitrocellulose in 25 mM Tris/192 mM glycine/20% (v/v) methanol for 60 min at 100 V (Bio-Rad mini-transblotter). The blots were routinely stained with Ponceau S to allow molecular mass standard (Sigma MW SDS-70L) positions to be marked. The blots were blocked overnight at 4 °C in 25 mM Tris/0.15 M NaCl/2.7 mM KC1 0.02% Tween 20, pH 7.5 (TBS-Tween), containing 5% (w/v) non-fat dried milk and 10% (v/v) horse serum, and then incubated in the same solution for 2 h at 4 °C with 1/500 anti-B CP antiserum or 1/1000 anti-C CP antiserum. The blots were washed three times with TBS-Tween and once with 50 mM Tris/0.15 M NaCl, pH 7.4 (Tris-NaCl), before being incubated at ambient temperature for 60 min in Tris-NaCl containing 5% (w/v) non-fat dried milk and 1.7500 goat anti-rabbit IgG (Fc) alkaline phosphatase conjugate (Promega). The blots were then washed with four changes of Tris-NaCl and incubated in alkaline phosphatase assay buffer (0.1 M Tris/0.1 M NaCl/5 mM MgCl₂/0.9 mM BCIP/0.2 mM NBT, pH 9.5) until stained, and the reaction stopped in 0.5 M EDTA/NaOH, pH 8.

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*Flavobacterium meningosepticum* N-glycosidase F (Boehringer Mannheim) was mixed with one aliquot. Both samples were incubated for 24 h at 37 °C, then concentrated by centrifugal evaporation at ambient temperature to about 25 μl, mixed with an equal volume of reducing SDS-PAGE sample buffer, and incubated for 3 min at 100 °C before analysis.

**RESULTS**

**Purification of groups A, B and C CPs**

The procedures used to purify the *L. mexicana* amastigote CPs of groups A, B and C do not result in individual activities, but in groups of CPs free from other proteins (Robertson & Coombs, 1990). Purified CPs of groups A, B and C were prepared as described in Methods, and are shown silver-stained in Fig. 1. Of particular interest is the observation that when reduced and denatured, the CP proteins of each of the three groups run together in SDS-PAGE gels as single bands with lower mobility than the multiple bands observed using non-reduced, partially denatured samples.

**Comparison of N-terminal amino acid sequences**

At present, electrophoresis is the only way to separate the individual activities of each group of CPs. To investigate whether N-terminal heterogeneity occurred between the multiple enzymes (as with the barley aleurone layer CPs: Koehler & Ho, 1988), the CP proteins were separated by SDS-PAGE, and blotted to a PVDF membrane from which individual bands could be excised for sequencing (Matsudaira, 1987). To minimize the risk of blocking the N-terminus of the CPs during electrophoresis, reducing agents, as polymerization radical scavengers, were included in the electrophoresis cathode buffer during both the electrophoretic separation and a pre-run of the gel without sample. As a result of the pre-run, the pH discontinuity between the stacking and resolving phases of the gel was lost, and so too was the separation of the individual proteins of each group of CPs. Indeed, under such conditions, the CP proteins seemed to behave as their reduced and denatured forms. Thus, it was only possible to obtain N-terminal sequences for the reduced and denatured pools of purified CPs containing, respectively, several group A, three group B and two group C CP proteins (Fig. 2). By taking the risk that the proteins might become N-terminally blocked, sequence data were obtained for homogeneous preparations of CPs B1 and B2 (Robertson & Coombs, 1990), purified by excision of bands of activity towards PFR-AMC after separation of the purified group B CPs by SDS-PAGE (Fig. 2). (The fragments of excised gel having the single CP activity were incubated at 100 °C in reducing SDS-PAGE sample buffer and electrophoresed in an SDS-PAGE gel prepared for sequencing proteins.) In all but one of the cases, the sequence was identical for the first ten amino acids, and matched exactly that predicted for the same N-terminal region of the mature *lmcpb* product (Souza et al., 1992). One of the C proteins had an additional alanine residue at the N-terminus and this was also predicted by *lmcpb*. The amino acid sequences subsequent to residue 10 were also very similar, although some differences were apparent.

**Western blot analysis of *L. mexicana* lysates and CP groups**

Antisera were raised in rabbits against the reduced and denatured group B and group C CP proteins. Both antisera recognized the same molecules in Western blots of crude *L. mexicana* amastigote lysates. Fig. 3 shows the data with anti-C CP antiserum; anti-B CP antiserum gave identical results. The predominant molecule recognized was the reduced and denatured form of the groups B and C CP proteins. This is consistent with results from substrate-SDS-PAGE gels that show these to be the most abundant activities (Robertson & Coombs, 1990). A molecule of the same apparent molecular mass was also recognized in lysates prepared from stationary-phase
promastigote populations. Again, this is consistent with the presence of groups B and C CP activities in such parasite populations (Robertson & Coombs, 1992). A molecule of lower apparent molecular mass was also recognized in this sample, and seems to be absent from amastigotes. It is possible that this protein represents the reduced and denatured form of the metacyclic promastigote-specific group F CPs (Robertson & Coombs, 1992).

The reduced and denatured group A CPs were also recognized by these antisera (Fig. 4), again indicating a close similarity between the groups A, B and C CPs. The group A CPs were recognized, even after enzymic deglycosylation by N-glycosidase F. The enzymic deglycosylation shows that the group A CPs are N-glycosylated, and that the carbohydrate-free group A CP proteins have a similar molecular mass as the groups B and C CPs. The latter enzymes, and the group E CP proteins (see below), were unaltered by N-glycosidase F treatment (data not shown), indicating that they are not N-glycosylated.

**Characteristics of group E CPs**

Two further molecules in the amastigote lysate specifically recognized by both antisera appear to belong to a group of CPs that we have designated group E. These molecules have molecular masses of 36.3 kDa and 32.5 kDa, and have the peculiarity that they are found associated with the pellet fraction after centrifugation of amastigote lysates prepared by repeated cycles of freezing and thawing or in 0.25% (v/v) Triton X-100 detergent. Such a pellet fraction has two distinct activities with lower mobilities than the groups A, B or C CPs in gelatin-SDS-PAGE gels (Fig. 5). These activities (group E CPs) are released from the lysate pellet fraction by 0.1% (w/v) SDS (hence they are solubilized by SDS-PAGE sample buffer, and run in the gels), 2 M NaCl and high pH. The E molecules recognized by the antisera show the same solubility profile. The E activities detected in gelatin-S...
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Lysates of *L. donovani* and *L. major* exponential- and stationary-phase promastigote populations were also analysed on Western blots with the anti-C CP anti-serum (not shown). Molecules of around 30 kDa were specifically recognized in these samples, and suggest the presence of CP activities similar to the *lmcpb* products in these other two *Leishmania* species, although none were detected previously using gelatin-SDS-PAGE gels (Lockwood *et al.*, 1987).

**DISCUSSION**

The prime aim of this study was to provide more information on the multiple *L. mexicana* amastigote CP activities detected in gelatin-SDS-PAGE gels. The N-terminal sequence data obtained in this study for the purified CP groups suggest that the CPs of groups A, B and C are very closely related and do not differ in the same way as barley aleurone layer CPs (Koehler & Ho, 1988). The group C CP preparation that was sequenced gave two overlapping sequences, indicating that at least part of the reason for the heterogeneity of the two group C CPs is due to differential processing at the N-terminus. The additional amino acid at the N-terminus of the protein (alanine) is that predicted from the *lmcpb* gene.

The N-terminal amino acid sequence determined for all the CP proteins in this study closely matches that predicted for the N-terminus of the mature *lmcpb* gene product (Souza *et al.*, 1992). This gene forms a tandem array of over ten copies, and its transcript is found at much higher levels in amastigotes than in metacyclic promastigote populations, and not at all in multiplicative promastigote populations. This mirrors the expression of the groups A, B and C CP activities and proteins in these parasite populations (Lockwood *et al.*, 1987; Robertson & Coombs, 1992; Fig. 3). Recently, an *lmcpb* homologue, *lpmdb*, has been isolated and sequenced from *L. pifanoi* (Traub-Cseko *et al.*, 1993). It too forms a tandem array of 8–20 copies, and has higher transcript levels in amastigotes than promastigotes. These tandem arrays of CP genes seem to lack restriction fragment length polymorphisms, suggesting that the gene copies are very similar (Souza *et al.*, 1992).

The data presented here suggest that the sizes of the CP proteins of all three groups, A, B and C, are very similar, as the individual proteins of groups B and C run as single bands of 24 kDa mobility in denaturing SDS-PAGE gels (Fig. 1), and the deglycosylated form of the group A CPs is apparently the same size as the groups B and C CP proteins (Fig. 4). The predicted molecular mass of the protein encoded by the central region of the *lmcpb* gene is 23578 Da. This suggests that the groups A, B and C CPs are encoded almost entirely by this part of the gene. The *lmcpb* gene predicts a CP with a long C-terminal extension, as has been reported for a group of CPs in other trypanosomatids and some plants (North *et al.*, 1990; Watanabe *et al.*, 1991). The data presented here suggest that this C-terminal extension is cleaved from the central domain and not present in the mature CP protein. A
Trypanosoma brucei rhodesiense CP has been heterologously expressed in Escherichia coli without its C-terminal extension, yet it remains enzymically active (Pamer et al., 1991). Thus, the C-terminal extension of this class of trypanosomatid CPs does not appear essential for activity and may have some other function.

The groups B and C CPs show some different preferences for peptidyl fluorogenic substrates after SDS-PAGE (Robertson & Coombs, 1990), yet their protein sizes are the same (Fig. 1), they react with the same anti-sera (Fig. 4), and have closely similar N-terminal amino acid sequences (Fig. 2). Thus, the substrate-preference differences would appear to be due to small differences in the protein structures. Indeed, glycine or lysine is found at residue 11 in the different CP protein sequences (Fig. 2). Site-directed mutagenesis of the gene for the papaya CP, papain has altered the engineered enzyme’s preference for peptide substrates away from that of papain towards that of the mammalian CP cathepsin B (Khoury et al., 1991). This was done simply by substituting two amino acid residues (Val133Ala/Ser205Glu) in the papain S2 binding pocket. It is therefore possible that limited amino acid substitutions in the different lmcpb products will produce CPs with different substrate preferences. This implies that a number of different lmcpb genes exist which account for the multiple CP activities, and hence that comparison of their sequences with the properties of the expressed enzymes would yield interesting insights into the molecular basis of these leishmanial CP substrate-binding specificities.

Further support for the idea that the groups A, B and C CPs are closely related comes from the studies using antisera raised separately against the groups B and C CPs. Both antisera recognized the groups A, B and C CP proteins in Western blots, indicating that they have shared epitopes (Fig. 4). Consistent with their high activity in amastigotes, the groups B and C CP proteins produce the largest signal in Western blots of amastigote lysates. The same molecule is recognized in stationary-phase promastigotes, of a molecule with faster mobility than the groups B and C CP proteins suggests the specific recognition, only in Western blots of stationary-phase promastigotes, of a molecule with faster mobility than the groups B and C CP proteins suggests that the group F CPs could be related to the lmcpb products.

Two other molecules specifically recognized by the anti-C CP anti-serum are solubilized by the same procedures as the E CP activities. These results suggest that the E CP activities could also be products of lmcpb genes. How can their higher apparent molecular mass in SDS-PAGE gels be explained? They are not apparently N-glycosylated, so it seems unlikely that their high molecular mass and pellet association is due to highly glycosylated forms binding with membrane-bound mannose 6-phosphate receptors. Phosphorylated mannose residues were not found in the related protozoon, T. cruzi (Cazzulo et al., 1990), and the release of the E CPs from the pellet fraction at pH 9.5 also argues against this possibility, as mannose 6-phosphate receptors usually release their ligands at low pH (Gonzalez-Noriega et al., 1980). The apparent molecular masses of the two molecules recognized by the anti-C CP antisera in Western blots of E CP samples are 36.3 and 32.5 kDa (Fig. 4). Their relatively large size could be due to their being less processed gene products. The predicted molecular mass of pro-Lmcpb lacking the C-terminal extension is 35863 Da, whereas the predicted molecular mass of the Lmcpb central domain plus C-terminal extension is 34071 Da (Souza et al., 1992; North et al., 1990). Retention by the E CPs of the predicted C-terminal extension could explain the location of the enzyme. There are a number of conserved cysteine residues in the CP C-terminal extensions described so far, suggesting a conserved and compact secondary structure in this domain (Souza et al., 1992); this may be involved in the enzymes’ membrane-association. An alternative possibility is that the C-terminal extension could be a signal for intracellular targeting. The C-terminal pro-peptide of barley lectin is involved in the protein’s targeting to the plant vacuole (Bednarek & Raskhel, 1991), and the short C-terminal extension of the T. brucei glycosomal phosphoglycerate kinase is necessary for targeting of this enzyme to the glycosome (Sommet et al., 1993).

Data have been presented that the pro-forms of the mammalian lysosomal proteases cathepsin L and cathepsin D are membrane-associated (McIntyre & Erickson, 1991). Like the L. mexicana group E CPs, this association can be disrupted by high ionic strength or high pH. The
association is reversible, dependent on the pro-region of the protein, and is proposed to occur via a lysosomal protein receptor in microsomal membranes. Thus, pro-forms of lysosomal enzymes can be specifically translocated to the lysosome independently of the mannose 6-phosphate receptor pathway. It is feasible that such a pathway is involved in the targeting of the \( \text{lmc}_{\text{lp}} \) products to their subcellular location in the lysosome-like megasome (Pupkis et al., 1986), and that group E CPs represent such enzymes. Purification and N-terminal sequence analysis of the E CPs should indicate whether these proteins are pro-enzymes or mature enzymes with the retained C terminal extension.

The predicted molecular mass of the entire \( \text{lmc}_{\text{lp}} \) product is 47840 Da (Souza et al., 1992), and molecules in this molecular mass range were specifically recognized by the anti-C CP anti-serum in Western blots of amastigote lysates (Fig. 3). This indicates that the whole of the \( \text{lmc}_{\text{lp}} \) transcript is likely to be translated before being processed to the mature active enzymes. In addition, molecules in the molecular mass range 20–14 kDa were also specifically recognized by the antiserum at higher loadings of amastigote lysate (Fig. 3, lane 3). These were probably proteolytic fragments of the mature CPs.

The findings that molecules of around 30 kDa were specifically recognized by the anti-C CP antiserum in Western blots of \( L. \) \textit{donovani} and \( L. \) \textit{major} lysates, and that homologues of the \( L. \) \textit{pifanoi \textit{lp}_{\text{cy}2} \) gene (which is homologous with \( \text{lmc}_{\text{lp}} \)) have been detected in other leishmanias, albeit at lower levels (Traub-Csako et al., 1993), suggest that \( \text{lmc}_{\text{lp}} \) homologues are common to all leishmanias, but that the enzymes occur at much greater levels in the \( L. \) \textit{mexicana} sub-class.

Further delineation of the interrelationship of the multiple \( \text{lmc}_{\text{lp}} \) products may best be probed using genetic manipulation, studies which are now under way. In particular, the expression of a single \( \text{lmc}_{\text{lp}} \) copy in a \( \text{lmc}_{\text{lp}} \)-free background, followed by biochemical analysis of the expressed product, will allow individual \( \text{lmc}_{\text{lp}} \) copies to be matched to individual CP activities. Similarly, expression of engineered \( \text{lmc}_{\text{lp}} \) products lacking the C-terminal extension may give an insight into the role of this protein domain. The \( \text{lmc}_{\text{lp}} \) genes and their products therefore provide an excellent system in which to study CP structure–function relationships and their roles in the parasite’s life.

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