Processing and trafficking of cysteine proteases in *Leishmania mexicana*

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SUMMARY

Removal of the pro-domain of a cysteine protease is essential for activation of the enzyme. We have engineered a cysteine protease (CPB2.8) of the protozoan parasite *Leishmania mexicana* by site-directed mutagenesis to remove the active site cysteine (to produce CPB\(^{C25G}\)). When CPB\(^{C25G}\) was expressed in a *L. mexicana* mutant lacking all CPB genes, the inactive pro-enzyme was processed to the mature protein and trafficked to the lysosome. These results show that auto-activation is not required for correct processing of CPB in *in vivo*. When CPB\(^{C25G}\) was expressed in a *L. mexicana* mutant lacking both CPA and CPB genes, the majority of the pro-enzyme remained unprocessed and accumulated in the flagellar pocket. These data reveal that CPA can directly or indirectly process CPB\(^{C25G}\) and suggest that cysteine proteases are targeted to lysosomes via the flagellar pocket. Moreover, they show that another protease can process CPB in the absence of either CPA or CPB, albeit less efficiently. Abolition of the glycosylation site in the mature domain of CPB did not affect enzyme processing, targeting or in vitro activity towards gelatin. This indicates that glycosylation is not required for trafficking. Together these findings provide evidence that the major route of trafficking of *Leishmania* cysteine proteases to lysosomes is via the flagellar pocket and therefore differs significantly from cysteine protease trafficking in mammalian cells.

Key words: Cysteine protease, Protease, Cathepsin L, Trypanosomatid, *Leishmania*, Transfection, Enzyme processing, Trafficking, Flagellar pocket

INTRODUCTION

Cysteine proteases of the papain superfamily, designated Clan A, family C1 (Barrett and Rawlings, 1996), are synthesised as zymogens that are activated by cleavage of the pro-domain to generate mature enzymes located predominantly within lysosomes. Trafficking of the mammalian proteases is mediated primarily through binding of mannose-6-phosphate residues present on the surface of the enzymes to appropriate receptors (Kornfeld and Mellman, 1989). A mannose-6-phosphate-independent mechanism based upon recognition of membrane-associated receptors has also been described for cathepsin D and cathepsin L (McIntyre and Erickson, 1993; Zhu and Conner, 1994). Cleavage of the pro-domain of the mammalian proteases appears to occur either in late endosomal compartments or in the lysosomes themselves (Mach et al., 1994). The pro-domains of many cysteine proteases contain a short conserved region centred on a heptapeptide. Mutational analysis of this motif of the plant cysteine protease papain has indicated that cleavage of the pro-domain is triggered by changes in the electrostatic charge of the motif at acidic pH (Vernet et al., 1995) and that completion of the activation process is entirely auto-catalytic (Vernet et al., 1991). In vitro studies with prokaryotic (Burns et al., 1997), mammalian (Mason et al., 1987), trypanosomal (Hellman et al., 1991; Eakin et al., 1993) and leishmanial (Sanderson et al., 2000) cysteine proteases have also suggested that maturation of these enzymes can occur auto-catalytically. However, definitive studies of in vivo processing were lacking and the participation of other enzymes remained a possibility. It seems likely that the mechanisms of processing and trafficking operating in lower eukaryotes such as trypanosomatids may differ significantly from those in mammals, and the lack of mannose-6-phosphate on lysosomal cysteine proteases of *Trypanosoma cruzi* (Cazzulo et al., 1990) supports this postulate. The current study of in vivo processing and trafficking of leishmanial cysteine proteases was undertaken to test this hypothesis.

*Leishmania mexicana* contain many cathepsin L-like cysteine proteases that are classified as members of the papain superfamily (Berti and Storer, 1995). The most abundant enzymes occur within the large lysosomes, known as megasomes, which characteristically are present in the amastigote (mammalian) stage of the life-cycle (Pupkis et al., 1986; Duboise et al., 1994). They are encoded by the *CPB* genes, which are arranged as a tandem repeat of 19 copies (Mottram et al., 1996; Mottram et al., 1997). The genes of the array that have been analysed to date are highly conserved (about 99% identical) in the pre-, pro- and mature domains. However, only gene copies *CPB3-CPB18*, which are expressed predominantly in amastigotes, encode isoenzymes with
unusual 100 amino acid C-terminal extensions (CTE) that are characteristic of the most abundant group of trypanosomatid cysteine proteases. The finding that the first two genes of the CPB array encode isoenzymes with a truncated CTE and yet become located in lysosomes refutes the possibility that the CTE plays a part in intracellular trafficking of the enzyme (Mottram et al., 1997). Moreover, a second leishmanial cathepsin L-like cysteine protease, CPA, lacks a CTE (Mottram et al., 1992) but also occurs in lysosomes (Duboise et al., 1994). A recent study on the effects of cysteine protease inhibitors on Trypanosoma cruzi showed that there was an accumulation of unprocessed pro-enzyme of the major cysteine protease in late-Golgi vesicles, which suggests that cleavage of the pro-domain is necessary for successful trafficking to lysosomes (Engel et al., 1998). Moreover, it has been shown (Huete-Pérez et al., 1999) that the pro-domain is both sufficient and necessary for targeting of both Leishmania and T. cruzi cysteine proteases to lysosomes. Nevertheless, the mechanisms of processing and trafficking of cysteine proteases in these parasites remains uncertain (Engel et al., 2000).

We have generated a series of cysteine protease-deficient mutants of L. mexicana. ΔCPA lacks the single copy CPA gene (Souza et al., 1994), whereas ΔCPB lacks the entire CPB gene array (Mottram et al., 1996). Double mutants lacking both CPA and CPB (ΔCPA/CPB) have also been generated (Mottram et al., 1996). Re-expression of different copies of CPB in ΔCPA demonstrated that the isoenzymes encode enzymes with different substrate specificities (Mottram et al., 1997). We have now exploited this ability to re-express CPB isoenzymes in the cysteine protease-deficient mutants to investigate the mechanisms whereby the enzyme is processed to the mature, active form and at the same time trafficked to lysosomes. To this end, we have engineered an inactive CPB, by mutation of a cysteine residue in the active site that is essential for activity, and a CPB that lacks the only N-linked glycosylation site on the mature region of the enzyme. The data generated from the re-expression of these mutated genes provide new insights into the processing and trafficking of these leishmanial enzymes.

MATERIALS AND METHODS

Parasites

L. mexicana (MN50/BZ/62/M379) promastigotes were grown in HOMEM medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, pH 7.5, at 25°C as described previously (Mottram et al., 1992). Axenic amastigote forms (axenic amastigotes) were grown in Schneider’s Drosophila medium (Life Technologies) with 20% (v/v) heat-inactivated fetal calf serum, pH 5.5, at 32°C and in the presence of 5% (v/v) CO₂ (Bates et al., 1992). The required antibiotics were added to the cultures of the cysteine protease null mutants of L. mexicana as follows: hygromycin B (Sigma) at 50 μg/ml, phleomycin (Cayla, France) at 10 μg/ml, nourseothricin (Hans-Knöll Institute, Germany) at 25 μg/ml, puromycin (Calbiochem) at 10 μg/ml, and neomycin (G418, Geneticin, Life Technologies) at 25 μg/ml.

Mutagenesis, constructs and transfections

Mutations of the CPB active site and glycosylation site were incorporated into pGL28, a pBluescript SK– plasmid containing the CPB2.8 gene (Mottram et al., 1996), using the QuikChange site-directed mutagenesis kit (Stratagene) and the following reverse phase-purified oligonucleotides (only the sense strand primer is shown and the mutated sites are given in bold):

OL 422 to generate pGL38 (encoding CPB(C25G)), 5′-GGTGCCTGAGCGCTCGAGCTC-3′; and OL 424 to generate pGL39 (encoding CPB(N103S)), 5′-GCCCGAGTGCTCGAGCAGC-3′.

These mutant constructs were verified by sequence analysis using an ABI 373 automated DNA sequencer (Perkin-Elmer). The native and mutant CPB2.8 genes were excised from pBluescript SK– as 2.0 kb EcoRV fragments and ligated to the Smal site of the pX episomal shuttle vector (LeBowitz et al., 1990) to generate pGL46 (pXCPB2.8), pGL47 (pXCPB(C25G)) and pGL48 (pXCPB(N103S)). The Trypanosoma brucei cysteine protease cDNA (Mottram et al., 1989) was cloned into the HindIII/EcoRI site of the pTEX shuttle vector (Kelly et al., 1992). Plasmids were transfected into the L. mexicana ΔCPB and ΔCPA/CPB mutants as described previously (Souza et al., 1994; Mottram et al., 1996). Briefly, plasmids were prepared using Qiagen Tip100 columns as outlined by the manufacturer. Transfection used 10 μg of DNA and 4×10⁷ late-log phase ΔCPB or ΔCPA/CPB promastigotes. Following electroporation, cells were allowed to recover in 1 ml HOMEM medium with serum for 24 h at 25°C and then transformants were selected by transfer of cells into HOMEM medium with serum containing 25 μg/ml G418.

Analysis of cysteine proteases

Parasite cysteine protease activity was determined using substrate-SDS-PAGE as previously described (Robertson and Coombs, 1990; Mottram et al., 1996). Briefly, parasite cell lysates (10⁷ cells) were subjected to electrophoresis under reducing conditions using 12% (w/v) acrylamide gels incorporating 0.2% (w/v) gelatin. Following electrophoresis, the gel was washed for 1 hour with 2.5% (v/v) Triton X-100 and then incubated in 0.1 M sodium acetate, pH 5.5, containing 1 mM DTT. Hydrolysis of gelatin was detected by staining with 0.25% (w/v) Coomassie Blue R-250. Western blotting used polyclonal anti-CPB antiserum (1 in 2500) raised against recombinant CPB2.8 expressed in and purified from Escherichia coli as a hexahistidine fusion protein (Sanderson et al., 2000).

Immunoelectron microscopy

Axenic amastigotes were processed and immunostained as previously described (Mottram et al., 1997). Briefly, pelleted cells were fixed in 2% (w/v) p-formaldehyde/0.1% glutaraldehyde for 30 min at 4°C, processed cold to −20°C in increasing alcohol concentrations, and finally embedded in LR White Medium Resin (BioCell International, UK) at 20°C, polymerising under 365 nm light for 2 days. Sections were collected on nickel grids and immunostained with anti-CPB polyclonal antibody (1:500) raised against recombinant CPB2.8 conjugated to 10 nm gold (Aurion, The Netherlands) at 1 in 20. Labelling was observed and recorded with a Zeiss 902 EFTEM at 80 kV, using zero-loss imaging to enhance contrast.

RESULTS

In vivo processing of cysteine proteases

The L. mexicana CPBs are similar to other cathepsin L-like cysteine proteases in being produced as inactive precursors that are processed to produce the mature, active enzyme (Sanderson et al., 2000). Processing of the papain family enzymes is thought to occur via autocatalytic and predominantly intramolecular cleavages (Vernet et al., 1991). We addressed the mode of processing of CPB by mutating the active site cysteine (C25G) of one CPB isoenzyme with a well-defined substrate specificity (CPB2.8; Mottram et al., 1997) and then expressing this inactive protease, from the pX episome, in the ΔCPB null.
Cysteine proteases in *L. mexicana*

Fig. 1. Expression of cysteine proteases in ΔCPB. Cysteine protease activity was analysed by gelatin-SDS-PAGE (A,C) and protease expression confirmed by western blotting (B). Extracts from 10^7 stationary phase promastigotes were used for gelatin-SDS-PAGE analysis and 5×10^6 stationary phase promastigotes were used for western blotting with anti-CPB antiserum (1 in 2500). (A,B) Wild-type *L. mexicana* (lane 1). ΔCPB (lane 2). ΔCPB[pXCPB2.8] (lane 3) and ΔCPB[pXCPB^{C25G}] (lane 4). Note that the lower mobility activities (>42 kDa) are not encoded by CPB genes. (C) ΔCPB (lane 1) and ΔCPB[pTEXtb] (lane 2).

mutant (to give cell line ΔCPB [pXCPB^{C25G}]). The lack of cysteine protease activity in the mutated protein CPB^{C25G} was confirmed by substrate-SDS PAGE analysis (Fig. 1A). Stationary phase wild-type *L. mexicana* had two dominant cysteine protease activities towards gelatin (with apparent molecular masses of approximately 23 and 25 kDa) (lane 1) that were absent in ΔCPB (lane 2) and ΔCPB[pXCPB^{C25G}] (lane 4). Analysis of ΔCPB[pXCPB2.8] (lane 3) demonstrated that when native CPB2.8 was re-expressed in the null mutant a dominant CP activity of ~23 kDa is produced. In addition, there were slower mobility activities (approx. 33-38 kDa). These correspond to intermediate processing forms of the zymogen that have been activated in situ (Sanderson et al., 2000). Western blotting (Fig. 1B) confirmed the presence of CPB^{C25G} in ΔCPB (lane 4). Moreover, CPB^{C25G} was processed to the same size, 28 kDa, as the mature active enzyme (Fig. 1B lanes 3 and 4), indicating that its maturation in vivo was due to the action of one or more protease other than CPB. The respective precursor forms of CPB2.8 and CPB^{C25G} were detected at lower abundance than the mature proteins, and the profile of intermediates appeared to differ slightly (Fig. 1B, lanes 3 and 4, 38-48 kDa bands). The African trypanosome, *T. brucei*, also contains a multicopy array of cysteine protease genes that encode enzymes with a CTE (Mottram et al., 1989). When expressed in ΔCPB (cell line ΔCPB[pTEXtb]) the active trypanosome cysteine protease was detected at approx. 32 kDa (Fig. 1C, lane 2), which is the size found in procyclic trypanosome cell extracts (not shown). This shows that this cysteine protease from a closely related trypanosomatid can be correctly processed and activated in *Leishmania*.

To investigate the possibility that the cysteine protease CPA was responsible for the processing of CPB^{C25G} in ΔCPB, the ΔCPA/CPB double null mutant (Mottram et al., 1996) was transfected with pGL47, and cells expressing CPB^{C25G} were analysed by western blotting (Fig. 2A, lane 2). A single dominant precursor form of CPB^{C25G} of approx. 45 kDa was detected in addition to fully processed enzyme other than 28 kDa, the relative abundance being approximately 9:1. This indicates that CPA is capable of participating in the in vivo processing of CPBs in *L. mexicana*. Interestingly, however, when native CPB2.8 was expressed in ΔCPA/CPB, western blot analysis showed that the protease was processed to the size of the mature enzyme with no unprocessed protein being detected (Fig. 2A, lane 1). Moreover, gelatin SDS-PAGE analysis (Fig. 2B) showed that CPB2.8 was fully active when expressed in ΔCPA (Souza et al., 1994). This indicates that CPB zymogen processing is not dependent upon an intermolecular activation by CPA and is also suggestive of a degree of redundancy between CPA and CPB. The protease responsible for processing the approx. 10% of CPB^{C25G} in ΔCPA/CPB remains to be characterised.

**Fig. 2.** CPA is able to process CPB^{C25G} in vivo. (A) Western blot analysis of CPB2.8 (lane 1) and CPB^{C25G} (lane 2) re-expressed in ΔCPA/CPB. Extracts from 5×10^6 stationary phase promastigotes were used for western blotting with anti-CPB antiserum (1 in 2500). (B) Gelatin-SDS-PAGE analysis confirming that CPB is active in ΔCPA. Extracts from 10^7 stationary phase promastigotes were used for substrate gel analysis: wild-type *L. mexicana* (lane 1), ΔCPA (lane 2) and ΔCPB (lane 3).

** Trafficking of cysteine proteases in *L. mexicana***

In wild-type *L. mexicana* amastigotes, mature CPBs are localised to large lysosomes (Mottram et al., 1997). In agreement with the detection of fully processed and active protease, immunoelectron microscopy of ΔCPB and ΔCPA/CPB re-expressing CPB2.8 showed that this enzyme was efficiently targeted to the lysosomes (Fig. 3a and 3b). There was little labelling of other cellular compartments (for example, the Golgi complex), as might have occurred if there had been a low efficiency of targeting. The inactivated CPB^{C25G} was also localised to the lysosomes of ΔCPB (Fig. 3c), again with little labelling of other cellular compartments. In contrast, expression of CPB^{C25G} in ΔCPA/CPB resulted in
the protease accumulating predominantly in the flagellar pocket and vesicles subtending this compartment (Fig. 3d and 3e). A low level of labelling was apparent in the lysosomes, which correlates with correct targeting of the minor proportion of fully processed CPB(C25G) (Fig. 2A, lane 2).

Some of the native L. mexicana CPBs are known to be glycosylated (Robertson and Coombs, 1990) and a potential N-linked glycosylation site is present on a surface loop in the mature domain of all the CPBs characterised to date. The CPB2.8 glycosylation site (N103) was mutated to a serine (N103S) in order to address any potential role that this motif may have in trafficking of the enzymes. Following transfection of ΔCPB with pGL48, immunolocalisation of CPB(N103S) clearly showed this enzyme to be correctly targeted to the lysosomes (Fig. 3f). Again, no labelling was detected in other cellular compartments. Consistent with a correctly targeted enzyme was the observation that removal of this glycosylation site had no effect on the processing of the precursor to the mature protein (Fig. 4B) or on the activity of the enzyme (Fig. 4A).

**DISCUSSION**

One aim of this study was to determine the mechanism whereby the cathepsin L-like cysteine proteases of L. mexicana that are encoded by the CPB gene array are processed in vivo to the active, mature form. Studies on similar enzymes of a number of other eukaryotes have demonstrated that in vitro the proteases are able to autoactivate under acidic conditions (Vernet et al., 1991; Eakin et al., 1993; Mason et al., 1987; Burns et al., 1997). The precise mechanism of this autocatalytic process is still unclear, but the finding that the pro-region binds to the active site cleft in the opposite orientation to that of a standard substrate suggested that cleavage might not be catalysed by the enzyme’s usual mechanism (Coulombe et al., 1996). Work with human cathepsin L has suggested that in vitro autocatalysis is exclusively an intramolecular event (Nomura and Fujisawa, 1997). In contrast, Menard and co-workers concluded that the autocatalytic processing of recombinant human pro-cathepsin L was effected via both intermolecular and intramolecular cleavages (Menard et al., 1998). Some trypanosomatid cysteine proteases can also autoactivate in vitro – this was observed with both the major cysteine protease of T. cruzi (Eakin et al., 1993) and also recombinant L. mexicana CPB2.8 (Sanderson et al., 2000). However, these findings do not show if autoactivation occurs, or is the sole means of precursor processing, in the living cell. Indeed, the initial cleavage of the recombinant T. cruzi cysteine protease is thought to be mediated by an uncharacterised E. coli enzyme (Eakin et al., 1993).

Indirect support for the involvement of another enzyme in the natural processing of cysteine proteases has been obtained previously with plants. It was found that the N-terminal sequence of in vitro processed recombinant papain was heterogeneous, whereas the N-terminus of the naturally
occurring enzyme is homogeneous (Vernet et al., 1990). This implies that another protease may predominate during the maturation process in vivo. Heterologous proteases are also thought to be responsible for the activation of several other cysteine proteases. For instance, a second cysteine protease is required for in vitro activation of the barley cysteine protease aleurain (Holwerda et al., 1990). Furthermore, pro-papaya protease IV, a highly abundant cysteine protease from *Carica papaya*, is also unable to process autocatalytically as a consequence of steric crowding of the active site cleft (Baker et al., 1996). Most recently, in vitro activation of human cathepsin X has been shown to require the addition of cathepsin L (Nagler et al., 1999). We have now shown that other proteases are able to mediate maturation of leishmanial CPBs in vivo and that autoactivation is not the only way in which these cysteine protease precursors can be processed.

Our results from using *L. mexicana* ΔCPA/CPB clearly demonstrate that CPB can be activated in vivo by another cathepsin L-like cysteine protease, CPA. It is likely that CPA can cleave the pro-domain of CPB directly as the two cysteine proteases are closely related (Mottram et al., 1996), although the possibility that CPA could act indirectly through processing of an intermediate protease cannot be excluded at this stage. However, CPA is not essential for in vivo processing of CPB as CPB was still efficiently processed in the absence of CPA (Fig. 2B, lane 2). Moreover, the mature forms of both the mutated, inactive CPB and the native protease following re-expression in ΔCPB were shown by western blotting to be the same size. This suggests that the same final cleavage site is used by both CPA and CPB. However, the western blots revealed several higher molecular mass proteins, which were not identical in the different lines (Fig. 1B, lanes 3 and 4). This indicates that multiple cleavages occur in vivo, as has been shown for recombinant CPBs in vitro (Sanderson et al., 2000), and that some cleavage sites used by CPB and CPA differ.

The mechanism mediating trafficking appears not to be glycosylation. Our finding that a mutant CPB with its only N-linked glycosylation site on the mature domain abolished was localised in an identical fashion to the native enzyme provides good evidence on this point. The same conclusion was reached for the major cysteine protease of *T. cruzi*, which has been shown to lack mannose-6-phosphate (Cazzulo et al., 1990), and also more recently for *L. amazonensis* (Boukai et al., 2000). There is another potential glycosylation site some 30 amino acids from the start of the CTE of the CPB2.8 isoenzyme (N246). However, this motif is absent from some other CPBs that lack a long CTE and yet are still targeted to the lysosomes (Mottram et al., 1997), which suggests that the motif plays no role in trafficking. This conclusion, that neither glycosylation nor the CTE mediate CPB trafficking, is supported by the results of Huete-Pérez and colleagues (Huete-Pérez et al., 1999) who showed that the pro-
domain, which is not glycosylated, is both sufficient and necessary for targeting of both *Leishmania* and *T. cruzi* cysteine proteases to lysosomes.

The data showing the accumulation of the unprocessed pro-CPB in or near the flagellar pocket (Fig. 3d,e) suggest that a route for the trafficking of the proteases in *Leishmania* to the lysosomes is via this compartment. It is not at present possible to entirely dismiss the possibility that the unprocessed cysteine proteases accumulated in the flagellar pocket owing to a default mechanism whereby wrongly processed proteins are directed to the compartment for subsequent excretion from the cell. However, this seems unlikely, as the pocket of amastigotes is effectively a sealed environment (Overath et al., 1997) and hence release from this location would not be possible. Moreover, no secretion of CPB<sup>25G</sup> was detected from ΔCPA/CPB promastigotes (data not shown) even though the flagellar pocket acts as the major site of endocytosis/exocytosis in this stage of the life-cycle (Overath et al., 1997). Indeed, rapid turnover of unprocessed CPB would be more likely to occur via direct targeting of CPB<sup>25G</sup> to the lysosomes of ΔCPA/CPB.

As the stage-specific expression of CPA and CPB is generally similar, and it seems likely that both types are trafficked to lysosomes via the same route (Mottram et al., 1997; Duboise et al., 1994), the processing of CPB could occur at any stage during trafficking. Addition of cysteine protease inhibitors to *T. cruzi* resulted in unprocessed zymogen accumulating within the late Golgi/early endosome network, which suggested that the processing of the cysteine proteases of this parasite must normally occur at or before that stage (Engel et al., 1998). However, our results with the mutated and so inactive CPB in ΔCPA/CPB demonstrate a build up of unprocessed precursor cysteine protease in the flagellar pocket and subtending vesicles of *L. mexicana* amastigotes. Treatment of *L. major* with cysteine protease inhibitors also leads to a build up of inactive cathepsin B-like cysteine protease in the flagellar pocket and immediate surrounding vesicles (Selzer et al., 1999). The finding that processed CPB<sup>25G</sup> is targeted to the lysosomes (Fig. 3c) and yet unprocessed CPB<sup>25G</sup> accumulates in the flagellar pocket (Fig. 3d,e) provides further evidence that the pocket is also likely to be the site of cleavage of the pro-domain to produce mature CPB enzyme. Consequently zymogen activation is likely to be a pre-requisite for the delivery of CPBs from the flagellar pocket to the lysosomes. The processed CPB is likely to complete its passage to lysosomes from the flagellar pocket via interaction with a membrane-bound receptor, the result being internalisation of the enzyme into a vesicle destined for the lysosomes (see Fig. 5 for a model of trafficking and processing). Such a receptor may have evolutionary links to the mammalian receptors involved in mannose-6-phosphate independent lysosomal targeting (McIntyre and Erickson, 1993; Zhu and Conner, 1994). Nevertheless, both this study on *L. mexicana* and that on *T. cruzi* (Engel et al., 1998; Engel et al., 2000) clearly demonstrate that cleavage of the pro-region is essential for successful targeting. As studies on *T. cruzi* and *L. major* using cysteine protease-specific inhibitors showed so convincingly (Engel et al., 1998; Selzer et al., 1999), disruption of this event has severe consequences on the parasite. It is interesting that the mutants expressing the CPB that was not processed survive and replicate in vitro, whereas Selzer and co-workers (Selzer et al., 1999) reported that cysteine protease inhibitors that prevent activation of CPB in *L. major* caused parasite death in vitro and in vivo. One possible explanation for this difference is that the *L. major* CPB homologues were not the sole target of the CP inhibitors used, and hence cell death was not due to the observed disturbances in trafficking but rather a consequence of the inhibition of some unknown cysteine protease(s). Furthermore, Selzer and co-workers postulated that the inhibitor blocks parasite replication through inhibition of both cathepsin B-like and cathepsin L-like cysteine proteases. The *L. mexicana* ΔCPA/CPB [pXCPB<sup>25G</sup>] mutant still possesses functional cathepsin B-like protease activity and this may be crucial for the survival and replication of the line. Additional factors that could account for the differences observed include variation in the levels of cysteine protease expression, pro-enzyme accumulation between the *Leishmania* lines and possibly other species-specific characteristics.

The observed differences between the accumulation of precursor cysteine proteases in *Leishmania* (this study and Selzer et al., 1999) and *T. cruzi* (Engel et al., 1998; Engel et al., 2000) point to species variations either in the site of cysteine protease processing or the trafficking route, or perhaps both. The discovery that a lysosomal membrane glycoprotein, p67, of bloodstream forms of *T. brucei* is targeted to this organelle via the flagellar pocket (Brickman and Balber, 1994) indicates that such a trafficking route does exist in at least this species of trypanosome. The involvement of this cell compartment in cellular trafficking in these trypanosomatids suggests that the molecules underpinning this unusual route for trafficking to lysosomes may represent attractive chemotherapeutic targets.

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