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Functional conservation of a natural cysteine peptidase inhibitor in protozoan and bacterial pathogens[1]

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Abstract Cysteine peptidase inhibitor genes (*ICP*) of the chagasin family have been identified in protozoan (*Leishmania mexicana* and *Trypanosoma brucei*) and bacterial (*Pseudomonas aeruginosa*) pathogens. The encoded proteins have low sequence identities with each other and no significant identity with cystatins or other known cysteine peptidase inhibitors. Recombinant forms of each ICP inhibit protozoan and mammalian clan CA, family C1 cysteine peptidases but do not inhibit the clan CD cysteine peptidase caspase 3, the serine peptidase trypsin or the aspartic peptidases pepsin and thrombin. The functional homology between ICPs implies a common evolutionary origin for these bacterial and protozoal proteins.

Key words: Chagasin; Cysteine peptidase inhibitor; Peptidase; Protease; *Leishmania*; *Trypanosoma*; *Pseudomonas*

1. Introduction

A natural cysteine peptidase inhibitor potently effective against the parasite's own cysteine peptidases and mammalian counterparts was discovered in *Trypanosoma cruzi* and given the trivial name chagasin [1]. The lack of significant identity with proteins of the cystatin class suggested that the inhibitor is a representative of a previously unknown class of peptidase inhibitors and this is reflected in its recent classification as clan IX, family I42 in the MEROPS database (http://merops.sanger.ac.uk). Subsequently, gene fragments predicted to encode regions of apparent homology to chagasin were identified in a number of other eukaryotic and prokaryotic genomes [2]. Comparison of the predicted proteins led to postulations on the regions that may be involved in binding, but experimental data on the functioning of the proteins were not obtained to test the hypotheses. Initial modelling studies using the *T. cruzi* chagasin sequence resulted in the suggestion that chagasin has an immunoglobulin-type structure and a mode of interaction with cysteine peptidases similar to that of two unrelated groups of papain inhibitors, the cystatins and the thyropins [3]. However, information from additional chagasin-like gene sequences was not consistent with these proposals [2]. We have now characterised representatives of this class of cysteine peptidase inhibitors (designated ICP, for inhibitor of cysteine peptidase), which we have identified in the parasitic protozoa *Trypanosoma brucei*, *Leishmania mexicana* and *Leishmania major* and the bacterium *Pseudomonas aeruginosa*. In this study we show that these proteins all inhibit clan CA, family C1 cysteine peptidases and thus that there is functional homology between the eukaryotic and prokaryotic ICPs.

2. Materials and methods

2.1. Pathogen materials

T. brucei (EATRO/795), *L. mexicana* (MNYC/BZ/62/M379) and *L. major* (MHOM/JL/80/Friedlin) were cultured and purified as described previously [4–6]. Mid-log phase (multiplicative) and stationary phase (metacyclic) promastigotes of *Leishmania* were harvested from cultures at densities of $\sim 5\times10^6$ ml^{-1} and $\sim 2\times10^7$ ml^{-1}, respectively. *L. mexicana* cysteine peptidase null mutants, Δ*cpa*, Δ*cpb* and Δ*cpc*, were generated previously [7–9]. Genomic DNA of *P. aeruginosa* (PA01) was obtained from Prof. T.J. Mitchell, University of Glasgow, UK.

2.2. Cloning

The regions encoding the *L. mexicana ICP* gene, together with flanking sequences, were amplified from genomic DNA using standard protocols [10] and primers NT103 (TGATGTCGTCGCTGTAGCGCAG) and NT104 (TCTGTGAGGGAGGAAGCCAACG) based on the *L. major* genomic DNA sequence. Products from two independent polymerase chain reactions (PCRs) were cloned into pGEM T EASY and sequenced. Total RNA was prepared from *L. mexicana* promastigotes and axenic amastigotes using TRI Reagent (Sigma). 5′ RACE was carried out using NT105 (TGGTTCCTCTGCGTTCTTCC) to prime cDNA synthesis, NT107 (ACGTTGAGGTGCAACGTGTAC) and the splice leader (SL) primer LMEXSL1 (TAACGCTATATAAGTATCAGTTTC) for the first PCR and NT106 (CCAGGATCTCATCGCTGAGC) and the nested SL primer LMEXSL2 (AGTATCAGTTTCTGTACTTTATTG) for the second PCR.

2.3. Production of recombinant ICPs and cysteine peptidases

Each *ICP* gene was amplified with specific primers from genomic DNA and inserted into the the *Nde*I and *Xho*I sites of pET28(a)$^+$. *L. mexicana ICP* used primers NT109 (TGCTCGAGCTACTTCACGTTGAGGTGCAACG) and NT110 (GCCATATGATCGCCCCGCTCAGTGTGAAGG). *T. brucei* ICP used primers NT90 (GGCTCGAGCTATGCGGTGGCCTCGACGTGAATG) and NT91 (GGCGCATATGTCCCACAACCTATTTACTGAGG). *P. aeruginosa* ICP used primers NT134 (GGCATATGTCCTTTTCCCCCTCCCGTCTGCTG) and NT135 (GGACTCGAGTCAGCGCACCTGGATCGCGCAGG). *Escherichia coli* BL21DE3 was transformed with pBP67 (*L. mexicana* ICP), pBP117 (*T. brucei* ICP) or pBP109 (*P. aeruginosa* ICP). N-terminally $(His)_6$-tagged protein was induced with 1 mM

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IPTG; the *Leishmania* and *Pseudomonas* ICPs induced at 37°C for 4 h, and the *T. brucei* ICP at 15°C overnight. Soluble ICP was purified on fast flow nickel agarose resin using a BIOCAD facility and eluted with 500 mM imidazole. The fractions containing the major protein peak were dialysed against 100 mM Tris–HCl, 100 mM NaCl, 2 mM EDTA, 0.05% Triton X-100, pH 8.3 at 4°C and the sample stored frozen at −20°C. Total protein concentration was determined using the BioRad protein assay.

The N-terminal His-tag was removed using 6 U of thrombin per mg of ICP over 2 h at 25°C (Novagen). The streptavidin-labelled thrombin was removed following the manufacturer's instructions and the released His-tag removed using nickel agarose resin. The sample was then dialysed as above.

Mammalian cathepsins B and L (Calbiochem), papain and caspase 3 (Sigma), and recombinant cysteine peptidase (CPB2.8ΔCTE) of *L. mexicana* [11] were used. The functional molarities of the *Leishmania* enzyme, cathepsin L and cathepsin B were determined by active site titration using *N-trans*-epoxysuccinyl-L-leucine-4-guanidinobutylamide (E-64) [12]. Papain was used to determine the functional molarity of each ICP preparation [13]. The molar ratio of binding between CPB2.8ΔCTE and ICP from *L. mexicana* was determined by active site titration [13] at pH 5 using *N*-benzoyl-PFR-*p*-nitroanilide hydrochloride (Sigma) as substrate.

2.4. *ICP:peptidase interactions and* K_i determination

The K_i assays were carried out for 1 min in a Perkin Elmer LS 55 luminescent fluorimeter at 37°C in 0.1 M sodium phosphate buffer pH 6.5 with 10 μM Z-FR-7-amino-4-methylcoumarin hydrochloride (FR-AMC, Sigma), 2 mM EDTA, 1 mM dithiothreitol (DTT), 0.4 nM functional enzyme and a range of at least 10 different ICP concentrations spanning the estimated K_i value. The K_i was determined from several independent experiments ($n \geq 2$) and derived using the following equation: $K_i = IC_{50}(1/(1+[S]/K_m)$. The K_m of each cysteine peptidase for FR-AMC was determined under the same assay conditions as used for the K_i determinations but in the absence of inhibitor.

ICPs were tested for inhibitory activity versus other classes of peptidases. The aspartic peptidase pepsin was incubated with ICP at a molar ratio of 1:40 (*L. mexicana*) and 1:2 (*P. aeruginosa*) at 37°C in 0.5 M NaCl, 0.1 M acetic acid pH 2.5 in the presence or absence of 2 mg ml^{-1} bovine serum albumin (BSA). Proteolysis of bovine serum albumin and/or ICP was monitored over 2 h by analysis on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and compared with a control sample in which pepsin was omitted to confirm the stability of ICP at acidic pH. The serine peptidase trypsin was incubated with each ICP at a molar ratio of 1:30 at 30°C in 50 mM Tris–HCl, 10 mM $CaCl_2$ pH 7.2 and proteolysis monitored over 2–4 h as above. The activity of trypsin was also assayed at 30°C using 2 mM *N*-α-benzoyl-R-*p*-nitroanilide hydrochloride (Sigma) in the presence of a 30-fold (*P. aeruginosa*) and 3400-fold (*L. mexicana*) molar excess of ICP. The clan CD cysteine peptidase caspase 3 was assayed at 30°C in 20 mM Pipes, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% Chaps, 10% sucrose pH 7.2 using 100 μM Ac-DEVD-AMC (Calbiochem) in the presence of a 30-fold molar excess of each ICP.

2.5. *Detection and quantification of ICP in parasite lysates*

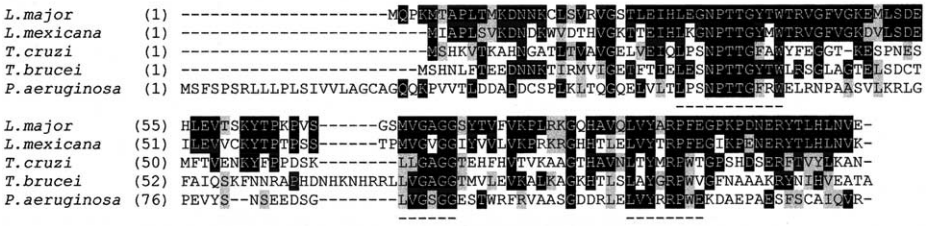
Parasites pellets were lysed at 10^9 cells ml^{-1} in 0.25 M sucrose, 0.25% (v/v) Triton X-100, 1 mM phenylmethanesulphonyl fluoride, 1 μM E-64, 10 μM *Z*-FA-diazomethylketone, 1 μM leupeptin, 1 μM pepstatin and 5 mM EDTA, pH 5.5 at 4°C using five cycles of freeze/thaw and lysates resolved on 15% SDS–PAGE. Western analysis was performed as previously described [11]. Polyclonal antiserum raised against recombinant *L. mexicana* ICP protein (by Scottish Diagnostics) was affinity purified on an *L. mexicana* ICP aminolink column. Anti-rabbit IgG horseradish peroxidase conjugate (Promega) was detected using Supersignal West Pico chemiluminescent reagent (Pierce). To quantify the level of ICP in wild-type *L. mexicana*, Western analysis was performed on known amounts of stationary phase promastigote lysates and the signal corresponding to ICP compared with those of a standard curve comprising known amounts of purified, recombinant non-His_6-tagged *L. mexicana* ICP resolved on the same gel. For comparison, the level of CPB was also determined in this way using antiserum raised against recombinant CPB2.8ΔCTE [11]. The mean values ± standard deviations were determined from three independent experiments.

3. Results

3.1. *Analysis of ICP genes*

The *ICP* genes were identified using Blast searches of the NCBI databases with the *T. cruzi* chagasin sequence. We identified an open reading frame annotated as a hypothetical protein of *P. aeruginosa* (gene PA0778) as *ICP*. The *L. major*, *T. brucei* and *P. aeruginosa ICP* gene sequences were confirmed by analysis of two independent PCR products obtained from appropriate genomic DNA. The *L. mexicana ICP* gene was amplified using PCR primers directed to the flanks of the *L. major* gene. The *ICP* genes of the pathogens predict small proteins: *L. mexicana*, 12.7 kDa; *L. major*, 13.0 kDa; *T. brucei*, 13.5 kDa; *P. aeruginosa*, 14.6 kDa. Comparison of the *ICP* genes revealed 16 identical residues and three motifs

a



b

	L. mexicana	*L. major*	*T. brucei*	*T. cruzi*
L.major	65.0			
T. brucei	35.7	37.3		
T. cruzi	31.0	36.8	30.7	
P. aeruginosa	23.9	23.9	23.7	27.3

Fig. 1. Alignment of the deduced amino acid sequences of ICP proteins. a: Sequences of *L. mexicana* ICP (AJ548776), *T. cruzi* chagasin (AJ299433), *T. brucei* ICP (AJ548777) and *P. aeruginosa* ICP (AAG04167, gene PA0778 [21]) compared with ICP of *L. major* (AJ548878). The sequences were aligned using AlignX (Vector NTI). Identical amino acids are shaded black, conservative amino acid changes are shaded grey. Conserved motifs are underlined. b: Percentage amino acid identities between ICP proteins. The N-terminal extension of *P. aeruginosa* ICP and the insertion in *T. brucei* ICP were removed before comparisons.

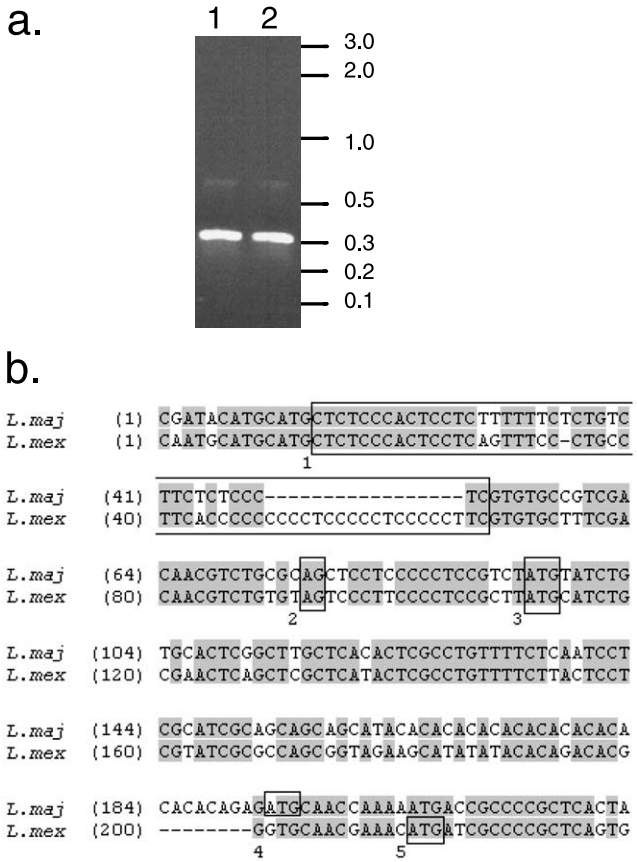


Fig. 2. 5′ RACE analysis of *L. mexicana* ICP cDNA. cDNA from *L. mexicana* promastigotes and amastigotes was analysed by 5′ RACE. a: Agarose gel electrophoresis of PCR products with LMEXSL2 and NT106 primers: lane 1, promastigotes; lane 2, amastigotes. The sizes of molecular weight standards are indicated in kilobase pairs. b: Alignment of *L. mexicana* and *L. major ICP* gene sequences indicating (boxed): 1, pyrimidine-rich sequence; 2, splice acceptor; 3, first ATG codon; 4, initiation codon for *L. major* ICP; 5, initiation codon for *L. mexicana* ICP. Identical nucleotides are shaded.

(motif 1, LXS/GNPTTGY/FXW; motif 2, L/MV/LGA/XGG; and motif 3, LV/XYXRPW/F) that are totally conserved between the four parasitic protozoa and the bacterium (Fig. 1a). ICP of *T. brucei* is unique amongst the trypanosomatid proteins in having a short insertion located between motifs 1 and 2. In addition, ICP from *P. aeruginosa* possesses an N-terminal extension not present in the other ICP sequences. Apart from the motifs, the proteins differed quite considerably. With the exception of the two *Leishmania* sequences, identities were in the order of 20–40% (Fig. 1b). There was, however, no significant identity or similarity at the primary amino acid sequence level with any member of the cystatin family or any other known cysteine peptidase inhibitor.

In order to determine the *trans*-splice site, PCR was performed on cDNA from *L. mexicana* promastigotes and amastigotes with a SL primer and an internal primer. An identical product was detected with each (Fig. 2a) and sequence analysis confirmed the positions of the pyrimidine rich region [14] required for *trans*-splicing (Fig. 2b, box 1), the AG dinucleotide splice acceptor (box 2) and the first ATG codon downstream of the splice site (box 3). 5′ RACE confirmed that these features are conserved in *L. major* and *L. mexicana*. In *L. major*, the first ATG is out of frame with the ICP coding region and translation from the next available ATG (box 4) predicts a protein of 13.0 kDa, which is similar in size to the *T. cruzi* chagasin. Comparison of recombinant and native ICP also confirmed that the second *L. mexicana* ATG (box 5) is the correct initiation codon for this ICP (see below).

3.2. *Expression of ICP in* Leishmania

The affinity purified anti-ICP antibody detected a protein of ~13 kDa in lysates of log phase promastigotes, stationary phase promastigote populations containing metacyclics, and amastigotes of *L. mexicana* (Fig. 3A). ICP was detected with the highest expression in the log phase promastigotes. The same lysates were probed with antibody raised against the CPB cysteine peptidase of *L. mexicana*, which has a greatly increased level of expression in the amastigote form of the parasite (Fig. 3B). Approximately equal loading was confirmed by probing with antibody to a constitutively expressed protein, cdc2-related kinase 3, CRK 3 [15] (Fig. 3C). The relative levels of ICP and CPB were quantified in lysates of stationary phase promastigotes and found to be 63 ± 23 fmol and 252 ± 38 fmol, respectively.

To test whether ICP expression is modulated by cysteine peptidase expression, promastigotes and amastigotes from *L. mexicana* mutants lacking CPA, CPB and CPC cysteine peptidase genes [7–9] were analysed. ICP expression did not appear to be altered in either promastigotes or amastigotes for any of the cysteine peptidase-deficient cell lines when compared with wild-type parasites (data not shown).

3.3. *Recombinant ICPs are functionally active against protozoan and mammalian cysteine peptidases*

Expression of the N-terminally $(His)_6$-tagged version of *L. mexicana* ICP resulted in about 60 mg l^{-1} of *E. coli* culture of a soluble protein of ~14 kDa. The $(His)_6$-tag was selectively removed by thrombin treatment resulting in a protein of ~13 kDa. This co-migrated with the native protein as detected by Western analysis, confirming the correct identifica-

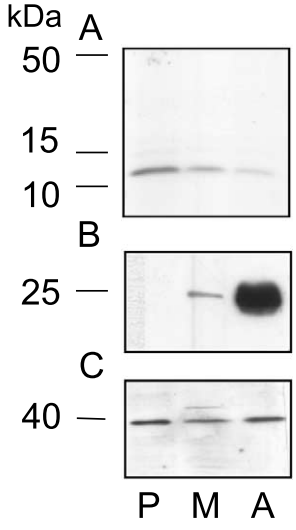


Fig. 3. Western analysis of the stage-related expression of ICP in *L. mexicana*. Lysates (10 μg protein) of wild-type *L. mexicana* promastigotes (P), stationary phase promastigote populations containing metacyclics (M), and mouse lesion-derived amastigotes (A) resolved on 15% SDS–PAGE and probed with anti-*L. mexicana* ICP antibody (panel A), anti-CPB2.8 serum (panel B) or anti-CRK 3 serum (panel C). The positions of marker proteins are shown.

tion of the second ATG as the start codon. Typically, 30–50% of the recombinant protein was functional as an inhibitor. Recombinant forms of the ICP of *T. brucei* were produced with similar yields and functional molarities.

Both forms (with and without the His-tag) of both ICPs were potent inhibitors of *L. mexicana* CPB2.8ΔCTE (Table 1). The presence of the $(His)_6$-tag had little or no effect on interaction, suggesting that the extreme N-terminal portion of ICP does not play a significant role in the interaction with the target cysteine peptidase. The molar ratio of inhibition approximated to a 1:1 relationship and inhibition was competitive (results not shown). Interestingly, the ICP of *T. brucei* was shown to be more potent against the leishmanial enzyme than the leishmanial ICP. High affinity interaction was also observed against mammalian cathepsin L. The ICPs also inhibited mammalian cathepsin B, but the K_is were appreciably higher. ICP of *Pseudomonas* expressed at low levels in *E. coli* (0.5 mg l^{-1} of culture). The purified His-tagged protein of ~17 kDa inhibited CPB2.8ΔCTE and also mammalian cathepsin L, albeit the latter K_i being in the nM range.

3.4. ICPs have specificity towards clan CA cysteine peptidases

None of the three recombinant ICPs inhibited the clan CD peptidase mammalian caspase 3 when tested at a molar ratio of 30:1. All three ICP proteins were also tested for activity against the serine peptidase trypsin and the aspartic protease pepsin. When incubated individually at 2–40-fold molar excesses with the enzymes, the ICPs of *L. mexicana* and *P. aeruginosa* were largely degraded within 1–2 h. The ICPs were also unable to inhibit the proteolysis of BSA by trypsin or pepsin or of *N*-α-benzoyl-R-*p*-nitroanilide hydrochloride by trypsin, even when the molar excess of *L. mexicana* ICP was raised to >3000:1. Furthermore, thrombin treatment was optimal for the removal of the His-tag, indicating a lack of inhibition of this enzyme by the ICPs.

4. Discussion

This study has shown that ICPs from protozoa and bacteria, although sharing low primary amino acid sequence identity, are all effective inhibitors of clan CA, family C1 cysteine peptidases from mammals and protozoa. This suggests that they must have similar tertiary structures, at least in the part that directly interacts with the target peptidases. The three highly conserved sequence motifs identified from the sequence comparisons could well be central to the formation of the interacting domains. The trypanosomatid ICPs have greater potency against CPB2.8ΔCTE and cathepsin L than against cathepsin B, as was also found with chagasin of *T. cruzi* (Monteiro, A.C.S., Abrahamson, M., Juliano, L., Tempone, A., Rumjanek, F., Lima, A.P. and Scharfstein, J., unpublished). This is probably due to the presence of the occluding loop of cathepsin B which obstructs the active site and must dissociate before inhibitors of the cystatin family can bind and so inhibit the enzyme [16].

ICPs appear to be specific for clan CA, family C1 cysteine peptidases and do not inhibit clan CD enzymes. These groups of enzymes are fundamentally quite distinct and only are similar in using cysteine and histidine residues in catalysis [17]. Thus the lack of inhibition of clan CD enzymes by ICP is consistent with ICP acting by insertion into the active site cleft rather than binding to the active site cysteine per se. Clearly such binding will depend upon the complementary tertiary structures of the enzymes and ICP. Cystatins are known to bind in this fashion, the N-terminus of the inhibitor protein being directly involved [18]. The lack of effect upon inhibitory efficacy of ICPs of the presence of a N-terminal $(His)_6$-tag, together with the absence of any significant degree of conservation of the amino acid residues comprising the N-terminus of ICPs, suggests that this may not be the case for ICPs. The total lack of similarity between ICP and cystatins, and indeed thyropins, is consistent with the proteins interacting differently with the target peptidases.

The role of the ICP proteins in the pathogens is yet to be elucidated. Possible functions include interaction with the pathogens' own cysteine peptidases, or inhibition of host enzymes. Several pieces of evidence point to inhibition of host cysteine peptidases being important. Firstly, under the conditions used, the *Leishmania* ICP did not have a higher affinity for its endogenous cysteine peptidases when compared with other ICPs from different species. Notably, the *T. brucei* ICP exhibited higher affinity. Secondly, the trypanosomatid ICPs are almost as potent against cathepsin L as against the *Leishmania* enzymes. Thirdly, there is no direct correlation between the levels of leishmanial ICP and the parasite's major clan CA, family C1 cysteine peptidase CPB (Fig. 3). It was similarly found that the stage regulation of chagasin was inversely proportional to that of the major clan CA, family C1 cysteine peptidases of *T. cruzi* [1]. Quantification of ICP and CPB levels revealed a molar ratio of ~1:4 in stationary phase promastigotes and a predicted molar ratio of at least 1:40 in amastigotes – in which ICP levels are lower and CPB levels are known to be up-regulated greatly [19,20]. Moreover, the expression levels of leishmanial ICP were unaffected by deletion of the clan CA, family C1 enzymes CAP, CPB and CPC. Finally, genomic studies of *P. aeruginosa* have confirmed the absence of any clan CA, family C1 cysteine peptidases. Nevertheless, the pathogen contains an inhibitory ICP. It has been suggested [2] that the true target of this bacterial ICP may be an endogenous serine protease, as the gene for *Pseudomonas* ICP is situated next to a gene encoding a Lon-type serine peptidase. Our finding that ICP does not inhibit the serine

Table 1
K_i values for ICPs versus cysteine peptidases

	CPB2.8ΔCTE (pM)	Cathepsin L (pM)	Cathepsin B (nM)
Lm $(H)_6$ ICP	268 ± 49 (n = 5)	ND	281 ± 30 (n = 2)
Lm (−) ICP	355 ± 49 (n = 5)	496 ± 39 (n = 2)	ND
Tb $(H)_6$ ICP	75 ± 9 (n = 4)	434 ± 83 (n = 4)	75 ± 20 (n = 2)
Tb (−) ICP	71 ± 7 (n = 2)	ND	ND
Pa $(H)_6$ ICP	495 ± 29 (n = 2)	3607 ± 966 (n = 2)	ND

The K_i values are expressed as the mean ± S.E.M. derived from the number determinations given in parentheses. Lm denotes *L. mexicana*; Tb, *T. brucei*; Pa, *P. aeruginosa*; $(H)_6$, His-tagged; (−), His-tag removed; ND, not determined.

peptidases trypsin and thrombin shows that ICPs are not general serine peptidase inhibitors, although the possibility that ICPs inhibit other serine peptidases cannot be currently excluded.

The results of this study show that ICPs with similar inhibitory properties occur in a diverse range of pathogens. It remains to be determined whether or not they perform the same functions within the pathogens and/or in the host–pathogen interactions, but the data presented suggest that they have a common evolutionary origin.

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