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Bm-CPI-2, a cystatin from *Brugia malayi* nematode parasites, differs from *Caenorhabditis elegans* cystatins in a specific site mediating inhibition of the antigen-processing enzyme AEP

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Abstract

The filarial parasite *Brugia malayi* survives for many years in the human lymphatic system. One immune evasion mechanism employed by *Brugia* is thought to be the release of cysteine protease inhibitors (cystatins), and we have previously shown that the recombinant cystatin Bm-CPI-2 interferes with protease-dependent antigen processing in the MHC class II antigen presentation pathway. Analogy with vertebrate cystatins suggested that Bm-CPI-2 is bi-functional, with one face of the protein blocking papain-like proteases, and the other able to inhibit legumains such as asparaginyl endopeptidase (AEP). Site-directed mutagenesis was carried out on Bm-CPI-2 at Asn-77, the residue on which AEP inhibition is dependent in vertebrate homologues. Two mutations at this site (to Asp and Lys) showed 10-fold diminished and ablated activity respectively, in assays of AEP inhibition, while blocking of papain-like proteases was reduced by only a small degree. Comparison of the *B. malayi* cystatins with two homologues encoded by the free-living model organism, *Caenorhabditis elegans*, suggested that while the papain site may be intact, the AEP site would not be functional. This supposition was tested with recombinant *C. elegans* proteins, Ce-CPI-1 (K08B4.6) and Ce-CPI-2 (R01B10.1), both of which block cathepsins and neither of which possess the ability to block AEP. Thus, *Brugia* CPI-2 may have convergently evolved to inhibit an enzyme important only in the mammalian environment.

Keywords: Papain-like proteases; *Caenorhabditis elegans* proteins; Cathepsins

1. Introduction

Cysteine proteases and their inhibitors (the cystatins) are well-established as key players in the breakdown of pathogen proteins for host immune recognition. For example, different families of cysteine proteases are involved in the MHC class II antigen-processing pathway [1–5]. Most prominent among these are the C1 papain-like proteases (such as cathepsins B, L and S), and the C13 legumain-like asparaginyl endopeptidase (AEP) [6]. These enzymes are required both for maturation of the class II receptor (for example by breakdown of the invariant chain chaperone, Ii) and for the proteolysis of exogenously derived antigens endocytosed by antigen-presenting cells (APC). Activation of proteases occurs by post-translational proteolytic cleavage of pro-enzyme forms, and the activity of cysteine proteases is further regulated by a family of cystatin inhibitors expressed in APC populations.

The precise pattern of protease and cystatin expression is highly dependent upon cell type and the maturational state of the cell in question. Most APCs, other than in the thymic

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epithelium, express cathespin S (CatS) and chemical inhibition or genetic knockout of CatS function has the most profound effect on invariant chain breakdown and peptide loading [7–10]. However, antigen presentation by thymic epithelial cells is dependent on CatL [11], and while macrophages express both CatL and CatS, only CatS is upregulated on stimulation [12]. Superimposed on this differential expression of papain-like cathepsins, APCs also produce AEP, shown to initiate Ii breakdown [13] and to be required for the processing of certain protein antigens such as the tetanus toxin (TT) fragment [14]. The importance of AEP is underscored by the finding that proteolytic activation of papain-like cathepsins is lost in mice in which the AEP gene is nonfunctional [15].

Within the immune system, cystatins act to control and negate, in a specific fashion, the action of these cysteine proteases. In the case of cystatin C, an inhibitor of CatS, expression is high in immature DCs resulting in deficient Ii cleavage and the direction of MHC class II/Ii complexes to lysosomal compartments. Down-regulation of cystatin C accompanies DC maturation, leading to complete Ii cleavage and class II transit to the surface membrane [16]. A broader analysis of cystatins has determined that some bear two inhibitory sites: one, shared by all cystatins, blocks classical cysteine proteases such as papain. The second, limited to particular mammalian cystatins (such as expressed in DCs) is a novel site able to block AEP, which cleaves proteins at aspargine positions [6]. Significantly, the AEP-inhibitory site contains a crucial asparagine residue.

Given the dependence of host immune function on appropriate expression and control of cysteine proteases and their inhibitors, it is to be expected that extracellular parasites such as the filarial nematode *Brugia malayi* may target this pathway to interfere with host immune recognition. We have previously reported that *Brugia* expresses two homologues of mammalian cystatins, Bm-CPI-1 and CPI-2, and that the latter acts to inhibit antigen processing by human B cells [17]. Similar products have also been discovered across a range of helminth parasite organisms [18]. A fascinating aspect of this interplay is that the cystatin inhibitors are evolutionary related members of the same gene families shared between host and parasite. We therefore addressed the question of whether the functional ability of parasite cystatin to impede immunity is an ancestral property common to all nematode cystatins, or an adaptation evolved by parasites to enhance immune evasion.

In this report, we test the hypothesis that cystatins from a human parasite, but not homologues from a free-living species, possess a functional AEP-inhibiting motif. We use site-directed mutagenesis to confirm that the same motif as found in mammalian proteins is involved in enzyme inhibition. These data lead us to suggest that Bm-CPI-2 displays micro-convergent evolution, having acquired a mammalian-like motif to inhibit a host enzyme, which is inserted into an evolutionary background from which the motif is altogether absent.

2. Materials and methods

2.1. Recombinant cystatin cloning

The *B. malayi* cystatin Bm-CPI-2, was isolated as described elsewhere [19]. Two cystatin homologues were identified in searches of the complete *Caenorhabditis elegans* genome, Ce-CPI-1 (K08B4.6) and Ce-CPI-2 (R01B10.1). The complete open reading frame encoding each protein, without predicted signal peptides, was subcloned into the *Bam*H1/*Not*1 site of pET22b (Novagen). For Ce-CPI-1, the insert corresponded to amino acids 20–121, and for Ce-CPI-2, aa 20–125. Each protein was expressed together with the pEL-B amino terminal 31 amino acids, and an 11-aa C-terminus with a terminal hexa-histidine tag.

2.2. Site-directed mutagenesis

Two mutants of Bm-CPI-2 were constructed, based on the mature sequence of amino acids 26–161, in which Asn-77 was altered to Asp (clone JM-002) and Lys (clone JM-003), respectively. The Quikchange Site-Directed Mutagenesis Kit from Stratagene was used for the mutagenesis reactions. Forward and reverse primers designed for the Asn-77 to Asp-77 substitution were, GTA AAT CAA CAA TCA *GAC* GAT GAG TAC CAT TTG ATG CC and GG CAT CAA ATG GTA CTC ATC *GTC* TGA TTG TTG ATT TAC. Primers designed for the Asn-77 to Lys-77 substitution were GTA AAT CAA CAA TCA *AAA* GAT GAG TAC CAT TTG ATG CC and GG CAT CAA ATG GTA CTC ATC *TTT* TGA TTG TTG ATT TAC. In each case, the codon underlined and italicised denotes the altered sequence. Thermocycling conditions were as follows: 1 cycle at 95 °C for 30 s followed by 12 cycles of 95 °C for 30 s, 55 °C for 1 min, 68 °C for 12 min. The length of the extension period is determined by the size of the plasmid. Both mutant sequences were cloned into pET-22b with the same pEL-B and hexa-histidine flanking sequences as described above.

2.3. Cystatin expression and purification

pET22b plasmids encoding wildtype and mutant CPI homologues were used to transform *E. coli* BL21 (DE3) cells. Protein expression was induced with 1 mM IPTG (isopropyl-α-D-thiogalactopyranoside) for 3 h at 37 °C. Cells were centrifuged (6000 × *g*) and the pellets incubated with 5 ml Bugbuster$^{\text{TM}}$ reagent (Novagen, UK) and 125 U Benzonase nuclease (Novagen, UK) per gram of cells for 20 min at room temperature. Following centrifugation (15,800 × *g*), soluble fractions were dialysed against binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole) before loading on HIS-bind resin columns on an automated AKTAprime (Amersham Pharmacia). Gradient elution with 20 mM sodium phosphate, 0.5 M NaCl, 1 M imidazole was used to recover His-tagged recombinant protein. The presence of soluble recombinant protein was checked by SDS-

PAGE and Western blotting. Purified fractions were then dialysed into phosphate buffered saline (PBS) and aliquotted at −70 °C for experimental use.

2.4. Enzyme assays

Enzyme inhibition assays were performed with a human B cell lysosomal extract, taken from the human B cell line EDR, prepared on 27% Percoll density gradient as described [20] using β-hexosaminidase activity to identify lysosomal fractions. A range of specific substrates was used to distinguish each category of cysteine protease. Substrates used were Z-Ala-Ala-Asn-NH methyl coumarin (substrate favoured by AEP); Z-Phe-Arg-NHMec (substrate favoured by cathepsins B/L); Z-Val-Val-Arg-NHMec (substrate favoured by cathepsin S). Substrates were incubated with lysosomal protein and increasing concentrations of inhibitors, and release of 7-amido-4-methyl coumarin (NHMec) was measured on a Cytofluor 4000 Fluorimeter (Applied Biosystems) at 460 nm wavelength.

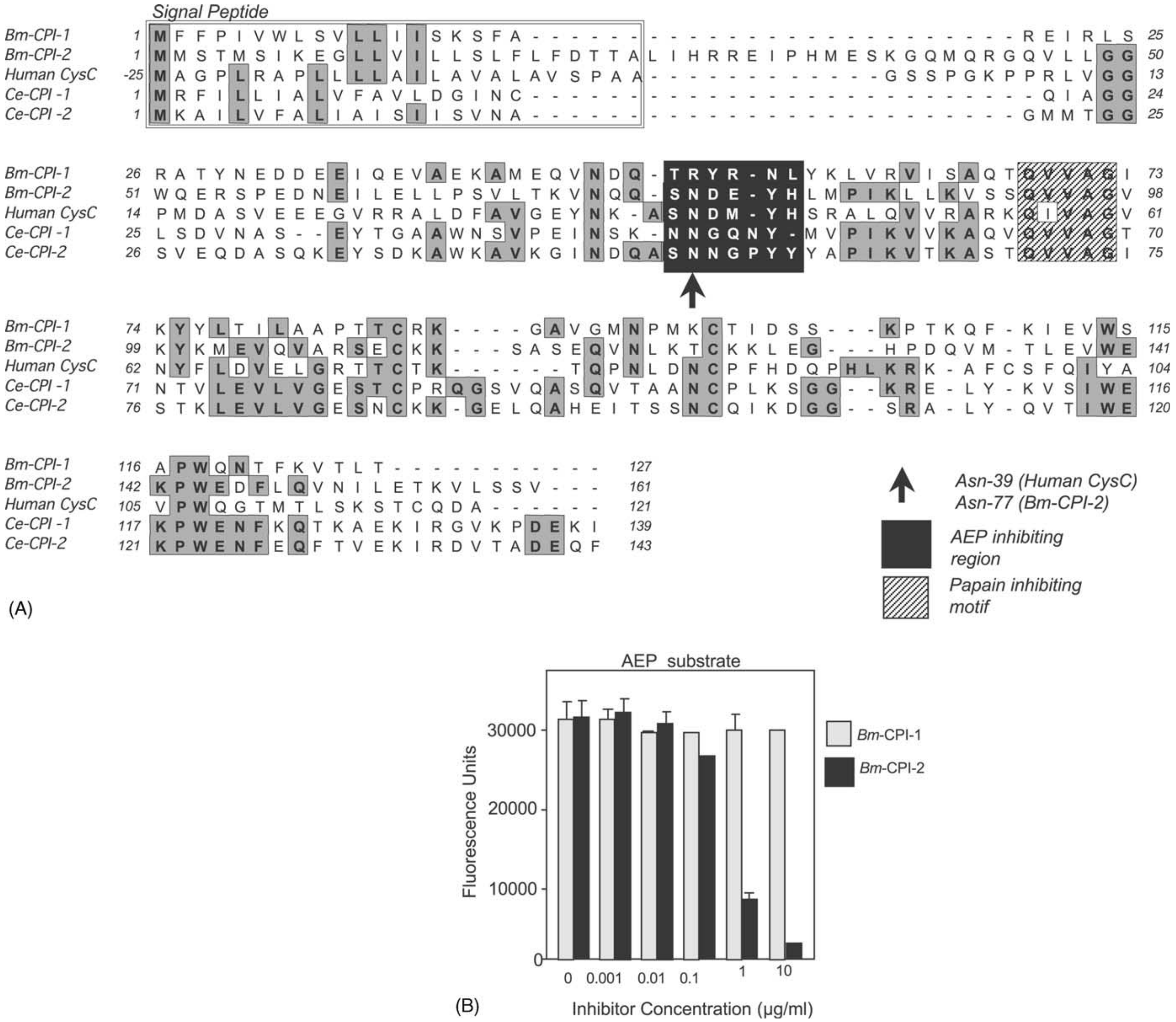

Fig. 1. (A) Alignment of *Brugia*, *C. elegans* and human cystatin C. The double-lined box designates predicted signal peptides. The solid box designates the potential motif for inhibition of aspariginyl endopeptidase, SND in cystatin C and Bm-CPI-2. The hatched box denotes the conserved QVVAG papain inhibitory site. Shaded residues are those shared by three or more of the homologues shown. Site-directed mutants of Bm-CPI-2 had alterations at residue 77 from asparagine to aspartic acid or lysine. Note that cystatin C numbering commences at the first aa of the mature protein, so that Asn-77 or Bm-CPI-2 corresponds to Asn-39 of human cystatin C. Ce-CPI-1 is K08B4.6; Ce-CPI-2 is R01B10.1; these correspond to previous designations Celecys-1 and Celecys-2 [21], respectively. Bm-CPI-2, but not Bm-CPI-1, inhibits AEP. Recombinant proteins were tested for inhibition of the ability of lysosomal proteases to cleave the AEP substrate Z-Ala-Ala-Asn-NHMec. Increasing concentrations of cystatins were added, as described in Section 2. Duplicate determinations were made, and data shown include standard deviations.

3. Results

3.1. Sequence comparison of nematode cystatins

The *B. malayi* cystatins are part of a gene family which is prominent among tissue-dwelling filarial nematode parasites, and has related products in vertebrate organisms [19]. In addition, the complete *C. elegans* genome contains two clear homologues of this gene family [21]. Alignments of these homologues show that all are small (<200-aa) proteins with key conserved features (Fig. 1). For example, all contain signal peptide sequences, indicating that activity in an extracellular or lysosomal locale is common across the phylogenetic scale. Among the more prominent contrasts observed was that filarial cystatins contain a ∼23-aa N-terminal extension absent from vertebrate and *C. elegans* homologues.

Detailed comparisons of the cystatins show similarities are focussed around active loops: for example all contain the QVVAG motif associated with papain inhibition (Fig. 1A, hatched box). In contrast, the second motif (SND) associated with AEP inhibition is restricted to a subset of vertebrate cystatins (Fig. 1A, solid box). Interestingly, this motif is reproduced in Bm-CPI-2, but is absent from Bm-CPI-1, consistent with functional AEP inhibition by CPI-2 but not CPI-1 (Fig. 1B). Inspection of *C. elegans* homologues in this region reveals related peptide sequences (e.g. SNN for Ce-CPI-2) raising the possibility that this product can also block AEP.

3.2. Mutagenesis analysis of Bm-CPI-2

In human cystatin C, mutation of Asn-39 greatly diminishes its ability to inhibit AEP; we therefore performed similar site-directed mutagenesis on the corresponding residue, Asn-77, of Bm-CPI-2. Mutants and parental products were tested for inhibition of a human B cell lysosomal extract which contains a combination of the key antigen-processing enzymes including cathepsins B, L, S and AEP [14].

Replacement of Asn-77 with Asp (N77D) had no effect on degradation of either the cathepsin B/L substrate Z-Phe-Arg-NH-methylcoumarin or the cathepsin S substrate Z-Val-Val-Arg-NHMec (Fig. 2A and B). However, AEP digestion was reduced approximately 10-fold (Fig. 2C). A more profound reversal was achieved by substituting Lys for Asn-77 (N77K); this completely removed AEP inhibition (Fig. 2F), and although cathepsin S activity was unaffected (Fig. 2E), there was significant impairment of cathepsin B/L degradation by this change (Fig. 2D).

3.3. Functional expression of C. elegans cystatins

The two cystatin homologues from *C. elegans* shown in Fig. 1 have not previously been described with respect to their natural expression at either the mRNA or protein level. We therefore probed mRNA from mixed-stage animals by RT-PCR, and found positive products for both genes (Fig. 3A). Because filarial CPIs show variable expression through the life cycle, and to test whether *C. elegans* CPIs are constitutively present, we used quantitative PCR on timed samples taken at consecutive stages in the *C. elegans* life cycle. *C. elegans* cultures were allowed to hatch and develop to the L1 stage. At this point they were arrested to allow the culture to become synchronous. Samples were taken from the culture every 2 h over the next 40 h. We found that Ce-CPI-2 expression varied little throughout the life history of *C. elegans* (Fig. 3B) and obtained similar results with Ce-CPI-1 (data not shown).

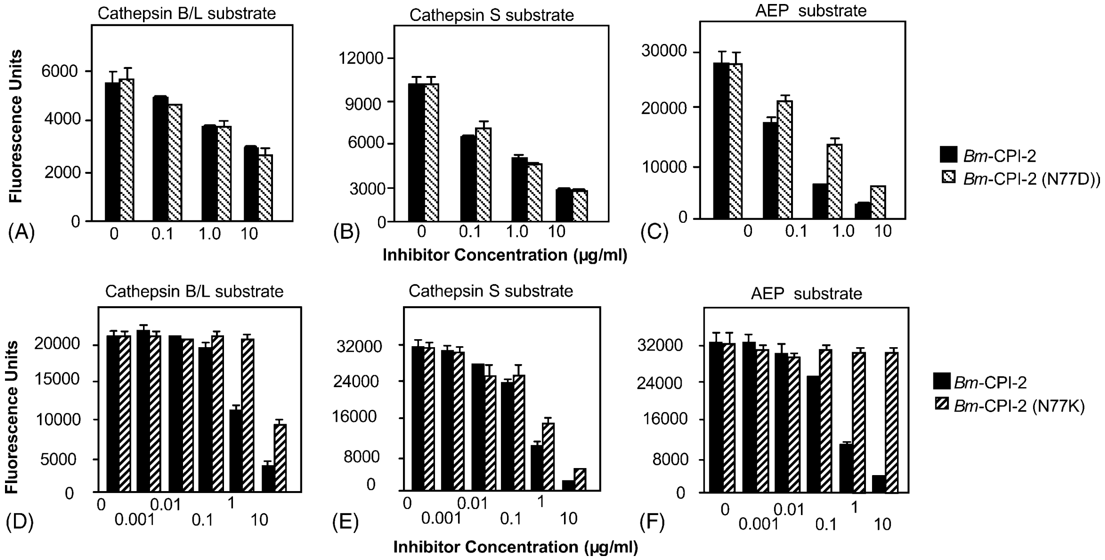

Fig. 2. Effect of mutation of the AEP-reactive site in fim-CPI-2 on inhibition of lysosomal proteases. Recombinant proteins were expressed in *E. coli* from the two CPI-2 site directed mutants N79D (Panels A–C) and N79K (Panels D–F) and tested alongside the wild-type. The substrate for CatB/L (Panels A, D) was Z-Phe-Arg-NHMec; for CatS (Panels B, E): Z-Val-Val-Arg-NHMec; for AEP (Panels C, F): Z-Ala-Ala-Asn-NHMec. In each case, release of 7-amido-4-methyl coumarin (NHMec) was measured. Duplicate determinations were made, and data shown include standard deviations.

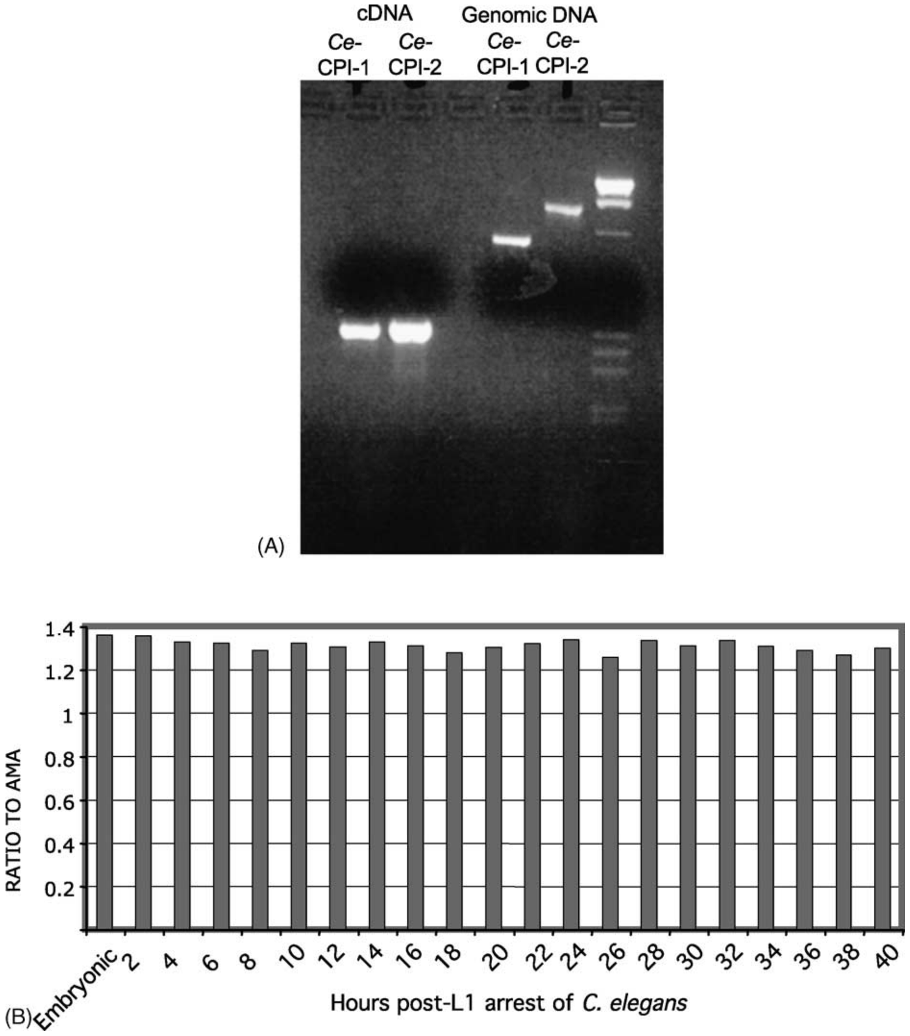

Fig. 3. Expression of Ce-CPI-1 (K08B4.6) and Ce-CPI-2 (R01B10.1) in *C. elegans*. (A) RT-PCR of mixed-stage cDNA showing presence of transcripts for both CPI homologues in *C. elegans*. Primers corresponded to the full-length mature open reading frame. (B) Real-time PCR quantification of Ce-CPI-2 transcript levels at successive times around the life cycle of *C. elegans*. First-strand cDNA was made from organisms taken at 2 h intervals following synchronization by L1 arrest. Primers were designed to nt 58–75 (forward) and 412–419 (reverse) of Ce-CPI-2 cDNA, spanning two introns of total 1004 nt. All products were analysed by agarose gel electrophoresis, and a single band of ∼370 nt was observed in every case.

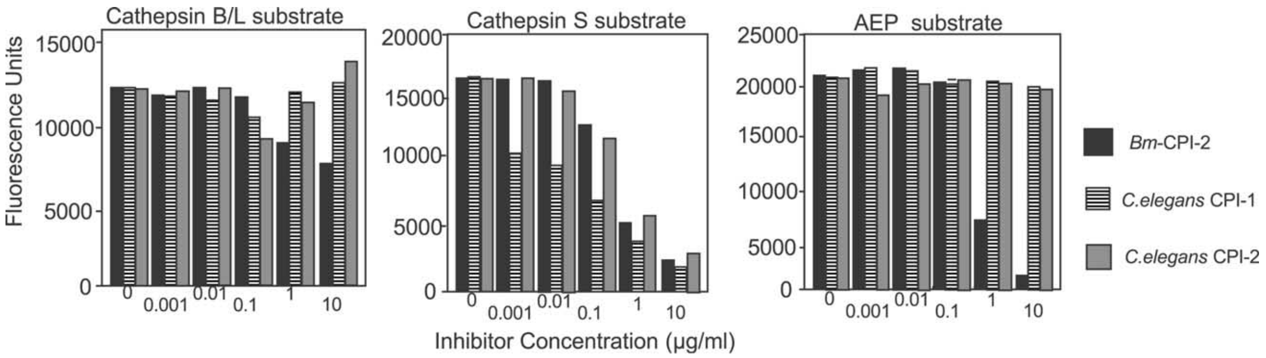

Fig. 4. Lack of AEP inhibition by *C. elegans* proteins Recombinant *C. elegans* cystatins inhibit CatL and CatS-like enzymes, but not AEP. Assays were performed as described in Section 2. Data represent single points for each concentration on the titration scale.

To test for functional activity, we cloned each into the same bacterial expression vector, and tested recombinant proteins in the same assays for inhibition of lysosomal cysteine proteases. As shown in Fig. 4, neither *C. elegans* CPI had any activity against AEP, even at 10 μg/ml, and also failed to act on cathepsin B/L (Fig. 4). However, both blocked cathepsin S, Ce-CPI-2 at concentrations similar to Bm-CPI-2, and Ce-CPI-1 proving much more effective on a molar basis (Fig. 4). Thus, while both *C. elegans* cystatins are functional members of the gene superfamily, they show no activity against AEP.

4. Discussion

Pathogens are thought to have evolved a myriad of immune evasion mechanisms, many of which are aimed at blocking the MHC-linked antigen processing pathways [22]. Most microbial MHC class II inhibitors so far described, such as the viral [23] and bacterial [24] molecules which interfere with class II processing and availability for peptide loading, are novel structures with an unknown evolutionary history. In contrast, the *Brugia* cystatin CPI-2 is derived from a common ancestor to host inhibitors charged with control of the immune system's own proteolytic machinery. The demonstration that Bm-CPI-2 can inhibit host AEP and the class II-dependent process of antigen presentation [17] raises the question of whether this is a universal property of nematode cystatins or a specific adaptation for immune evasion by the tissue-dwelling filarial parasites.

We show here that closely related cystatins from the free-living nematode, *C. elegans*, share a common papain-inhibitory site, but have no ability to inhibit AEP. Inspection of the amino acid sequence in the putative AEP-inhibitory site suggested that *Brugia* has evolved a similar motif to that demonstrated in human cystatin C to block AEP activity. Using site-directed mutagenesis, we confirmed that this site in Bm-CPI-2 is indeed responsible for AEP inhibition, and postulate that micro-evolution at this site has produced a convergent function between vertebrate and filarial cystatins. Supporting this hypothesis, SND motifs associated with AEP inhibition are present in other filarial cystatins, such as those from *Acanthocheilonema viteae* [25], *Litomosoides sigmodontis* [26] and *Onchocerca volvulus* [27]. In contrast, the sequences of cystatins so far described from geohelminths, such as *Haemonchus contortus* [28] and *Nippostrongylus brasiliensis* [29], are very similar to Ce-CPI-2 in this region of the molecule.

The inhibition of AEP and papain-like enzymes may not be the sole function of AEP. We have noted a novel 23-aa N-terminal extension, the role of which has yet to be ascertained. Studies with the filarial cystatins, Onchocystatin from *O. volvulus* [27], and Av17 from *A. viteae* [25] demonstrate an ability to directly induce IL-10 production [25,30] as well as pro-inflammatory outcomes such as elevated nitric oxide release [31]. Moreover, these properties are not observed with the *C. elegans* cystatins [18,21]. Additional reports have now been made on the CPI homologues from the filaria *L. sigmodontis* [26], the gastrointestinal nematodes *H. contortus* [28] and *N. brasiliensis* [29,32], and the platyhelminth *Schistosoma mansoni* [33].

Studies of the filarial cystatins are the only ones, as yet, to combine a structural analysis with evidence for a range of immunological functions. Future work will aim to examine if any of these immunological features can be directly related to the ability to block AEP activity. In our own preliminary studies, no difference between the wild-type CPI-2 and the forms mutated at Asn-77 could be observed in aspects such as nitric oxide generation or IL-10 release from murine splenocytes (B. Martynoga, J. Murray, A. Balic, unpublished). Further studies in this direction are now underway.

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