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A 170 kDa multi-domain cystatin of *Fasciola gigantica* is active in the male reproductive system☆

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ABSTRACT

Cystatins are functional as intra- and extracellular inhibitors of cysteine proteases and are expressed as single or multi-domain proteins. We have previously described two single domain type 1 cystatins in the trematode *Fasciola gigantica* that are released into the parasite's intestinal tract and exhibit inhibitory activity against endogenous and host cathepsin L and B proteases. In contrast, the here presented 170 kDa multi-domain cystatin (FgMDC) comprises signal peptide and 12 tandem repeated cystatin-like domains with similarity to type 2 single domain cystatins. The domains show high sequence divergence with identity values often <20% and at only 26.8% between the highest matching domains 6 and 10. Several domains contain degenerated QVVAG core motifs and/or lack other important residues of active type 2 cystatins. Domain-specific antisera detected multiple forms of FgMDC ranging from <10 to >120 kDa molecular mass in immunoblots of parasite crude extracts and ES product with different banding patterns for each antiserum demonstrating complex processing of the proprotein. The four domains with the highest conserved QVVAG motifs were expressed in *Escherichia coli* and the refolded recombinant proteins blocked cysteine protease activity in the parasite's ES product. Strikingly, immunohistochemical analysis using seven domain-specific antisera localized FgMDC in testis lobes and sperm. It is speculated that the processed cystatin-like domains have function analogous to the mammalian group of male reproductive tissue-specific type 2 cystatins and are functional in spermiogenesis and fertilization.

1. Introduction

In 2005, Khaznadji et al. [1] identified a protein with six cystatin-like domains in the liver fluke *Fasciola hepatica* and demonstrated inhibition of recombinant parasite cathepsin L1 by a single, recombinant expressed domain. The authors reported that several of the six domains showed degeneration or absence of core motifs/residues commonly found in active cystatins but did not attempt further investigation of the protein. Due to our general interest in the biological roles of cystatins in *Fasciola* we decided to follow up the analysis of this protein in the closely related species *Fasciola gigantica*. In this species we have previously analyzed two single domain type 1 cystatins, FgStefin-1 and FgStefin-2 [2,3]. Both are secreted from the parasite's gut epithelial cells into the gut lumen and they are thought to be functional in the regulation of the abundantly released cysteine proteases cathepsin B in the early juvenile and cathepsin L in later stages. FgStefin-2 is present in larger amounts in metacercariae and early juveniles and shows higher efficacy in inhibition of cathepsin B. FgStefin-1 is also expressed in tegumental type tissues where it may be protective against surface-associated proteases. FgStefin-2 has additional regulative function in the seminal fluid as it is also abundantly expressed in the prostate gland. Interestingly, published *Fasciola* transcriptome data [4,5] seems to be devoid of single-domain type 2 cystatins, this is an important finding as members of this subclass have been shown to possess immunomodulatory properties in parasitic nematodes. Of course such type 2 cystatins may yet exist in *Fasciola*, as (1) transcriptome data is incomplete and (2) derived from adult stage parasites only, (3) novel cystatins might show high sequence diversity with only few residues conserved and could be easily missed in sequence similarity screens.

☆ Note: Nucleotide sequence data reported in this paper is available in the EMBL, GenBank and DDJB databases under the accession number KM217580.

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Table 1
RT-PCR primers used for cloning of the complete FgMDC coding sequence.

Position	Forward primer	Position	Reverse primer
1–30	ATGAACTATCTACTGATTATTCTATCTAGC	2028–2047	TATACAGCTTTTCGGCTTGC
1601–1622	CCGTTGAGTCAATCAACGTTAC	4450–4470	TTAACAGCTTATAGATAAATC

Multi-domain cystatins (multicystatins) have been described from diverse organisms. In *Homo sapiens* kininogens, fetuins, and histidine-rich glycoprotein (HRG) are well studied with three cystatin domains for the first and two each for the latter proteins. These secreted proteins carry additional domains/motifs, secondary modifications, may undergo processing and are involved in important biological processes, e.g., blood coagulation, angiogenesis, bone formation in man (for a review see [6]). The cystatin domains of fetuins and HRG do not show inhibition of protease activity. The human multicystatins have been implicated in a diverse set of pathologies, e.g., cancer, inflammation, diabetes. Remarkably, their genes are colocated in a small stretch of DNA on chromosome 3q27 [7]. In the insect *Manduca sexta* (tobacco hornworm) MsCPI contains 13 cystatin-like domains, nine of which are tandemly repeated and highly conserved [8,9]. The huge 2676 amino acid residues preproprotein contains a C-terminally located procathepsin F-like domain and additional interspersed domains of unknown function. The protein undergoes processing by which the nine conserved cystatin domains are released as single domains and they have been shown to be efficient inhibitors of the cognate cathepsin F. In the intracellular parasitic nematode *Trichinella spiralis* a secreted multicystatin containing three cystatin domains has been described [10]. The sequences of the first two domains are conserved while the third shows only low similarity and is cleaved from the proprotein prior to secretion. Inhibition of papain by a recombinantly expressed protein could not be detected and the authors suggested that degenerated core motifs might have led to loss of inhibitory properties.

Considering (1) that degenerated core motifs point to novel functions of cystatin-like proteins, (2) the multitude of processes in which human multicystatins participate and (3) the preliminary finding that the reported *F. hepatica* multi-domain cystatin contained not six but 12 cystatin-like domains, we presumed functions of this protein in *Fasciola* greatly different from those of FgStefin-1 and FgStefin-2. In the present work we demonstrate that FgMDC is post-translationally processed, that four of the domains inhibit cysteine protease activity, and that it has potentially function in the male reproductive system.

2. Materials and methods

2.1. Parasites

Adult *F. gigantica* were freshly collected from bile ducts and gall bladders of naturally infected cattle killed at a local slaughterhouse. Parasites not used immediately were washed in 0.85% normal saline solution and kept frozen in liquid nitrogen until processed in further experiments.

2.2. Molecular cloning and sequence analysis

RT-PCR was used to amplify the complete 4470 bp coding sequence of FgMDC using adult stage total RNA of *F. gigantica* as template. Due to the size of the transcript the amplification was done in two steps yielding two overlapping cDNA fragments with sizes of 2047 bp (nucleotides 1–2047) and 2870 bp (nucleotides 1601–4470). The overlapping region of the cDNAs spanning base pairs 1601–2047 demonstrates their origin from a common transcriptional product. The primers used in the RT-PCR are detailed in Table 1. They were designed based on the sequences of uncharacterized FgMDC EST sequences (contigs 21790, 11926, 21564) and the orthologous *F. hepatica cys1* gene (GenBank: AJ312374, contig 691) identified in BLAST searches of transcriptome data of these parasites [4,5] accessible at the Gasser laboratory at http://research.vet.unimelb.edu.au/gasserlab/index.html. The RNA was extracted in TRIzol reagent (Invitrogen) and treated with DNase (Promega) at 37 °C for 30 min. The DNase was then heat-inactivated in the presence of 20 mM EGTA at 65 °C, 10 min and the DNA-free RNA reverse transcribed by RevertAid^{TM} M-MuLV reverse transcriptase (Fermentas Life Sciences) using the reverse primers listed in Table 1 for 1 h at 42 °C. The RT-products were then used as template for standard PCR (30 cycles at 94 °C, 45 °C, 72 °C, 1 min each step) with the primers listed in Table 1. The PCR products were cloned into pGEM-T Easy (Promega) and the recombinant plasmids were maintained in *Escherichia coli* XL1-Blue. The DNA sequences of the cDNA inserts were determined (1st BASE Sequencing Asia) and editing and analyses of molecular sequences were done in EMBOSS 6.6.0 [11], SignalP 4.0 [12], and NCBI CDD. Sequences of mouse cystatins expressed in the testis were retrieved from UniProtKB: Q9D264 Cystatin E2, Q9EPX9 Cystatin C (Cystatin-3), Q9D1B1 Cystatin E/M (Cystatin-6), Q9QWL5 CMAP (Cystatin F, Cystatin-7), P32766 CRES (Cystatin-8), Q9Z0H6 Cystatin-9, Q9D269 Cystatin-11, Q9DAN8 Cystatin-12, Q80ZN5 Cystatin-13, Q8VII3 Cystatin-14, Q80Y72 Cystatin-like 1 as was the sequence of human cystatin C (UniProtKB: P01034). Clustal Omega 1.2.0 [13] was used to calculate a multiple alignment and TEXshade [14] was used for formatting of the alignment.

2.3. Expression of recombinant FgMDC domains in E. coli and production of polyclonal anti-FgMDC domain-specific antisera

The cDNA sequences of FgMDC domains 1, 3, 4, 6, 9, 10, 12 were amplified by PCR and subcloned into the expression vector pQE30 (QIAGEN) using the introduced terminal restriction recognition sites as listed in the description of the PCR primers in Table 2. The DNA sequences of all inserts were verified (1st BASE Sequencing Asia). The cloning process added the vector-derived residues MRGSHHHHHHAS to the N-terminus of each recombinant protein. *E. coli* M15 was used as bacterial host and expression was induced with IPTG at a final concentration of 1 mM. The recombinant proteins were expressed in insoluble form and had to be purified by Ni-NTA affinity-chromatography under denaturing conditions following protocols 10 and 17 as outlined in the QIAExpressionist manual (QIAGEN). Antisera against the purified FgMDC domains were raised in ICR mice by intraperitoneal immunization three times in 3-week intervals with 10 μg recombinant protein in each immunization. Pre-immune and immunized sera were collected for further immunological studies. The use of experimental animals in this study was approved by the Thammasat University Animals Ethics Committee (21 December 2012, Project No. 010/255).

2.4. Parasite antigen preparation

Excretory/secretory (ES) product was prepared from freshly collected adult parasites. The parasites were washed, preincubated in PBS, pH 7.2 for 1 h at 37 °C, and transferred into fresh PBS for 4 h at 37 °C. Insoluble material and eggs in the buffer were removed by centrifugation at 5000 × *g* and 4 °C for 20 min. The ES product in