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A new multi-domain member of the cystatin superfamily expressed by *Fasciola hepatica*☆

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Abstract

Cystatins are cysteine protease inhibitors that are widespread in the plant and animal kingdoms. Cystatins are expressed by helminth parasites that may employ these proteins to regulate parasite cysteine protease activity and to modulate host immune responses. Here, we describe the cloning of a cDNA encoding a high molecular weight protein of *Fasciola hepatica* that contains two domains with significant identity to the cardinal cystatin signatures and four domains with degenerated cystatin signatures. This is the first report of a multi-domain cystatin in an invertebrate species. While cystatins are divided into three evolutionary related families, our phylogenetic analysis shows that all cystatin domains within this protein, like several other helminth cystatins, belong to the cystatin family 2. The DNA region encoding the domain 4 that is the best conserved at the level of its cystatin signatures was expressed in *Drosophila* cells and a recombinant protein was produced and purified. This protein was a potent inhibitor of the papain and of the major cysteine protease of *F. hepatica*, the cathepsin L1.

Keywords: Cystatin; Cysteine protease inhibitor; Proteases; Cathepsin L1; Parasites, *Fasciola hepatica*; Newly excysted juvenile

1. Introduction

Fasciolosis is an infection caused by the parasitic trematode *Fasciola hepatica*. This parasite is widespread and infects a wide range of wild and domestic mammals including sheep and cattle (Torgerson and Claxton, 1999). The disease causes poor grow performances in sheep and cattle that lead to major annual economic looses in agricultural communities in both the developed and developing world. Moreover, human fasciolosis is a public health problem in several regions including the Peru, Bolivian Altiplano and the Nile Delta of Egypt (Mas-Coma et al., 1999a,b). Identification of *F. hepatica* antigens as candidates for vaccines and the study of host–parasite interactions has become a major research focus since the parasites resistant to chemical treatments have emerged in several countries including Australia, the United Kingdom, Ireland and the Netherlands (Gaasenbeek et al., 2001). Several vaccination experiments have been performed using proteases, anti-oxidants or fatty acid binding proteins from adult and immature flukes and elicited significant protection rates (30–80%) after challenge infection (for review see Spithill and Dalton, 1998).

There are few data concerning proteins from newly excysted juvenile (NEJ) flukes due to the minute amount of material that can be obtained from these stages. In efforts to identify potential targets for vaccination in this developmental stage, we screened a subtractive library and identified a large number of mRNA fragments specifically or over-expressed by NEJ. One of the specific mRNAs was found to possess similarities with motifs that are signatures for cystatins. Cystatins are tight-binding inhibitors of

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papain-like cysteine proteases and are widespread in plants and animals. Members of the cystatin super-family are classified into three evolutionary related families (Rawlings and Barrett, 1990; Turk and Bode, 1991). Members of family 1, termed Stefins, are low molecular weight, single domain cystatins that do not contain disulphide bridges. Members of family 2 also possess a single cystatin domain, but their structure contains at least two disulphide bridges. In contrast, the members of family 3 contain three cystatin domains and are typically represented by the mammalian blood plasma kininogens. Cystatins are expressed by filarial nematodes such as *Acanthocheilonema vitae*, *Brugia malayi* (Maizels et al., 2001) and *Onchocerca volvulus* (Lustigman et al., 1992) and intestinal nematodes such as *Nippostrongylus brasiliensis* (Dainichi et al., 2001). Recent work also shows that *Schistosoma mansoni* express a cystatin related to the family 1 (Morales et al., 2004). Filarial cystatins are pathogenicity factors and are thought to play a key role in balancing the host–parasite immune relationship (Shierack et al., 2003). Here, we describe the characterisation of a cDNA encoding a protein that contains six cystatin-like domains, two of which are well conserved while four are degenerate. One of the conserved domains, domain 4, suspected to be an active protease inhibitor, was expressed as a recombinant protein in *Drosophila* Schneider 2 cells and shown to be a potent inhibitor of papain and of the major cysteine protease, cathepsin L1, of *F. hepatica*. Phylogenic analyses revealed that the cystatin domains all belong to a lineage of cystatins that consist mainly of helminth cystatins that belong to the family 2. This protein is the first multi-domain cystatin to be described in invertebrates.

2. Material and methods

2.1. Parasites

Fasciola hepatica metacercaria were obtained from in vitro-infected snails *Lymnaea truncatula* maintained in our laboratory. Excystment of juvenile flukes was performed as previously described (Wilson et al., 1998). Adult *F. hepatica* flukes were recovered from naturally infected bovine livers at a local abattoir.

2.2. Construction of cDNA library from newly excysted juvenile

Total RNA was isolated from three adults and from 5000 NEJ *F. hepatica* with a modified caesium chloride purification method of Sambrook (Sambrook et al., 1989). Reverse transcription was performed with 1 μg of adult total RNA or 500 ng of juvenile worm total RNA with the Smart™ PCR cDNA synthesis kit (Clontech) and the Super Script II MMLV reverse transcriptase (Invitrogen). NEJ cDNAs (10 μg) were digested with proteinase K (Promega), purified, blunted with T4 DNA polymerase (Promega) and size fractionated through a Chromaspin 400 column (Macherey Nagel). The cDNAs of more than 500 bp were collected, precipitated with ethanol and dissolved in water. The resulting cDNAs were immediately used for ligation with *Eco*R 1 adaptators (Promega). The reaction was phosphorylated with T4 polynucleotide kinase (Promega) and excess adaptator was removed by passage through a Chromaspin 400 column. The fractions containing cDNAs were treated with ethanol and the resulting precipitated cDNAs were dissolved in water. Two hundred nanograms of cDNA were used for ligation with 500 ng of λTriplEx vector (Clontech) that had been previously digested with *Eco*R 1. The ligation was mixed with 50 μl of packaging extract (Promega) and incubated for 3 h at 22 °C to obtain the phage library. The resulting library was plated and titrated using standard protocol.

A subtractive cDNA library enriched with specific juvenile cDNAs was made using the PCR Select™ Subtraction kit (Clontech). The subtraction products were subcloned into the pGEM-T easy vector and the recombinant vectors transfected into competent cells (Promega). One hundred and fifty colonies were individually collected and each of the corresponding DNA was used to probe adult and juvenile total cDNAs in Southern blot. A 472 bp clone that hybridised cDNA from juvenile worms and not from adult worms was selected, sequenced and studied further.

2.3. Screening of the NEJ library

The 472 bp DNA (100 ng) was labelled with 25 μCi dCTP [αP^{32}((ICN France) using random priming kit (Promega) and used to screen the NEJ cDNA library prepared in λTriplEx vector and also used to probe cDNAs from adult or NEJ in Southern blot. A total of 4000 colonies were plated and transferred to nylon membranes (Porablot NY plus, Macherey Nagel). These membranes were incubated overnight at 55 °C in hybridisation solution (6×SSC, 1% SDS, 5×Denhardt's and 50% formamide containing 100 μg/ml denatured herring sperm DNA) to which the radiolabelled 472 bp probe was added. The membranes were then washed twice for 30 min in 3× SSC/1% SDS at 55 °C and twice for 30 min in 1.5× SSC/1% SDS at 55 °C and then placed in contact with X-OMAT AR5 Film (Kodak) for 48 h. Following a second round of screening, as described above, a 2359 bp cDNA clone was isolated and its sequence determined.

2.4. Southern blot analysis

Genomic DNA (gDNA) was extracted from adults using the Aquapure genomic DNA isolation kit (Bio-Rad) and 3 μg were digested with *Eco*R 1 and *Rsa* 1 and the products were separated on a 1% agarose gel. Electrophoresis of the cDNAs was performed using the same conditions except