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Channel catfish granzyme-like I is a highly specific serine protease with metase activity that is expressed by fish NK-like cells

Michael Thorpe, Srinivas Akula, Lars Hellman*

Department of Cell and Molecular Biology, Uppsala University, Uppsala, Sweden

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ABSTRACT

Here we present the extended cleavage specificity of catfish granzyme-like I, previously identified in fish NK-like cells. This protease has been characterised using substrate phage display and further validated by using a panel of recombinant substrates. A strict preference for Met in the P1 (cleavage) position, indicating metase specificity was observed. A screening of potential in vivo substrates was performed based on the derived P5-P3′ consensus: Arg-Val-Thr-Gly-Met↓Ser-Leu-Val. Channel catfish caspase 6 was one very interesting potential target identified. This site was present in an adjacent position to the classic caspase activation site (Asp179 in human caspase 6). Cleavage of this site (hence potential activation) by the catfish granzyme-like I could reveal a novel mechanism of caspase 6 activation. This poses an interesting idea that the role of granzyme-like proteases in the activation of caspase dependent apoptosis mechanisms has been conserved for over 400 million years.

1. Introduction

There has been an increase in the use of non-rodent animal models with much focus on lower vertebrates including fish species, notably the zebrafish. However, there are few studies on the details regarding the characterization of hematopoietic serine proteases in fish. In mammals, serine proteases play important and diverse roles in number of physiological processes including blood coagulation, food digestion, fertilisation, complement activation and tissue repair (Neurath, 1986). This large chymotrypsin/trypsin family all share a common mechanism for cleaving peptide bonds, based on their catalytic triad, with three vital residues (chymotrypsinogen numbering): His57, Asp102 and Ser195 (Perona and Craik, 1995). These key amino acids are located near a substrate binding pocket (termed S1), typically made up of residues 189, 216 and 226 (Perona and Craik, 1995). Together they form the specificity conferring triplet and provide clues as to the primary specificity of the serine proteases. The substrate specificities of the serine proteases vary between different members of this large group and there are differences in which immune cells express the various enzymes. Of particular interest here are the natural killer (NK) and cytotoxic T-cells expressed granzymes. In humans, there are 5 granzymes: A, B, H, K and M and in mice, 10 are found: A-G, K, M and N (Grossman et al., 2003). In terms of (human) primary specificities defined by cleavage at the P1 site (interacting with the S1 pocket), granzyme A and K are tryptases, cleaving after basic residues Arg/Lys; granzyme B is an aspase, cleaving after Asp residues; granzyme M, a metase, preferring Met and granzyme H, resembling a chymase, cleaving after bulky aromatic amino acids, Tyr/Phe. This information has been used to facilitate what is known about in vivo targets and the ultimate functions of these proteases. Granzyme B has been the most widely characterised and is the archetypal granzyme inducing apoptosis through caspase activation (Martin et al., 1996) as well as through cleavage of Bid in the mitochondrial apoptotic pathway (in humans) (Sutton et al., 2000).

Whilst there is lots of variation regarding true targets of the granzymes and despite some obscurities surrounding all of their involvement in apoptosis induction, it is clear other functions are present. One such function is to modulate inflammatory cytokine production, which is an apparent feature of granzyme A (Metkar et al., 2008; Sower et al., 1996).

Non-lymphocyte cells also contain granzymes as well as many other proteases. Mast cells express granzyme B and have an abundance of tryptase and chymase stored with proteoglycans in

* Corresponding author. Department of Cell and Molecular Biology, Uppsala University, BMC, Box 596, SE 751 24 Uppsala, Sweden.
E-mail address: Lars.hellman@icm.uu.se (L. Hellman).

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their granules. The later is found as a single gene in humans (human chymase), with 5 chymases in mice (mouse mast cell protease-1, -2, -4, -5 and -9), each with varying substrate specificities, adding to the complexity of the proteases. Neutrophils contain four distinct serine proteases: neutrophil elastase, proteinase-3, neutrophil serine protease-4 and cathepsin G. Cathepsin G has a similar cleavage specificity as the human chymase and exists as a single gene in both humans and mice.

The protease genes are found with others clustering on three different chromosomes. Granzyme A and K are found together ('tryptase' locus) on chromosome 13 in mice and 5 in humans. In both humans (on chromosome 19) and mice (chromosome 10), granzyme M clusters with the neutrophil proteases; proteinase 3 and elastase ('metase' locus). Cathepsin G, granzyme B and H as well as the single chymase gene are found together ('chymase' locus) in humans on chromosome 14, where in mice the additional chymases and granzymes are also together (also chromosome 14).

Regardless of their true functions, the fact that so many serine proteases are found in mammals, begged the question whether other such enzymes also existed in other vertebrate lineages. The initial screening of the channel catfish (*Ictalurus punctatus*) for hematopoietic serine proteases resulted in the identification of three proteases termed *granzyme-like I, II* and *III*. These proteases are distantly related to numerous T-cell granzymes (B to H), mast cell chymases, the M8 family of basophil proteases and neutrophil cathepsin G (Wernersson et al., 2006).

In fish, two NK cell homologues exist: NK-like cells and non-specific cytotoxic cells (NCCs). The relationship between the two populations is not entirely clear although one difference between the cell types is their localization, where NK-like cells are derived from peripheral blood and NCCs are tissue based. Granzyme-like I and II are expressed in mixed lymphocyte culture derived NK-like cells and the third member (granzyme-like III) from the autonomous macrophage cell line 42 TA (Shen et al., 2004; Wernersson et al., 2006).

The aim of this study was to characterize the catfish granzyme-like I based on its extended cleavage specificity. A fourth granzyme in this species, termed granzyme-1 (CFGR-1) has been identified earlier and is expressed in NCCs (Praveen et al., 2004). Phylogenetically, this protease clusters with human and mouse granzyme A and K, as well as having Asp-Gly-Gly as the specificity conferring triplet (based on chymotrypsinogen numbering 189-216-226), suggesting tryptase activity (Praveen et al., 2004). Indeed, recombinant granzyme-1 cleaves a tryptase-specific synthetic peptide and likely contributes to cell cytotoxicity induction based on a chromium release assay (Praveen et al., 2006). Alignment of the catfish granzyme-like proteases reveals some fairly varied specificity conferring triplets: Gly-Gln-Gln (granzyme-like I), Gly-Thr-Tyr (granzyme-like II) and Ala-Thr-Gly (granzyme-like III), although none of these match any mammalian serine proteases (Wernersson et al., 2006), which leaves question marks over their true specificities. To address the question of the roles of the various fish proteases in fish immunity here we have characterised the first of these enzymes, the catfish granzyme-like I enzyme by substrate phage display. In order to determine the primary specificity and exact cleavage site of this protease, a synthetic peptide was analysed using mass spectrometry of the cleavage products. Furthermore a large analysis of the cleavage activity was performed on a set of recombinant substrates to determine the importance of the amino acids surrounding the cleavage site for the cleavage efficiency of the enzyme. Using a consensus sequence obtained from the phage display and recombinant substrate analyses, several fish genome sequences and cDNA databases were screened for potential in vivo substrates. The most interesting candidate gene identified in this screening was catfish caspase 6, indicating a role of this protease in apoptosis induction in target cells.

2. Materials and methods

2.1. Catfish granzyme-like I production

The channel catfish granzyme-like I sequence (GenBank accession code: AY942183) was designed and ordered from GenScript (Piscataway, NJ, USA). The synthesised construct was cloned in the pU57 cloning vector, containing EcoRI and XhoI sites. The catfish granzyme-like I sequence was subsequently transferred to a pCEP-Pu2 vector, used for expression in mammalian cells. The enzyme was produced as an inactive recombinant protein, with an N-terminal His_6-tag followed by an EK site. HEK 293 cells were grown to 70% confluency in a 25 cm^3 tissue culture flask (BD VWR) with Dulbecco's Modified Eagles Medium (DMEM) (GlutaMAX, Invitrogen) supplemented with 5% fetal bovine serum (FBS) and 50 μg/ml gentamicin. Following DNA (25 μg of granzyme-like I in pCEP-Pu2) transfection with lipofectamine (Invitrogen, Carlsbad, CA, USA), puromycin was added to the DMEM (0.5 μg/ml) to select for cells which had taken up the DNA along with heparin (5 μg/ml). Cells were expanded and conditioned media collected.

To purify the recombinant enzyme, 750 ml conditioned media was filtered (Munktell 00H 150 mm, Falun, Sweden) and 500 μl nickel nitrilotriacetic acid (Ni-NTA) beads were added. The media with Ni-NTA beads were rotated for 45 min at 4 °C. Subsequently, the Ni-NTA beads were collected by centrifugation and transferred to a column containing a glass filter (Sartorius, Goettingen, Germany). After washing with PBS tween 0.05% + 10 mM imidazole +1 M NaCl, the recombinant protein was eluted in PBS tween 0.05% + 100 mM imidazole fractions. The first fraction volume was half the Ni-NTA bead width (200 μl) and further fractions eluted with a full bead width (400 μl). Individual fractions were run on SDS-PAGE gel, their concentrations estimated from a bovine serum albumin standard (BSA) and the most concentrated were pooled and kept at 4 °C.

2.2. Activation of recombinant catfish granzyme-like I

The recombinant catfish granzyme-like I initial concentration was determined by SDS-PAGE and the level of EK (Roche, Mannheim, Germany) adjusted for activation of the enzyme. A relative concentration was activated depending when it was needed, where for example, 70 μl of the eluted recombinant enzyme was digested with 1 μl EK for 3 h at 37 °C. The activated fractions were stored at 4 °C until use.

2.3. Substrate phage display

A T7 phage library containing 5×10^7 variants, each displaying a unique nine amino acid sequence was used to determine the extended cleavage specificity of the catfish granzyme-like I enzyme. The nine amino acid region has been inserted into the C-terminal of the capsid 10 protein, followed by a His_6-tag. Approximately 10^9 plaque forming units (pfu) were bound to 125 μl Ni-NTA agarose beads via their His_6-tags for 1 h at 4 °C with gentle rotation. Unbound phages were removed by washing ten times with PBS tween 0.05% + 1 M NaCl, followed by two washes with PBS. The beads were re-suspended in 375 μl PBS and approximately 250 ng of recombinant catfish granzyme-like I was added. This reaction was incubated for 2 h at 37 °C with gentle rotation, allowing cleavage of the susceptible phages and their subsequent detachment from the Ni-NTA beads. From this, the supernatant containing released phages was recovered after centrifugation. Thirty μl was used in a plaque assay to determine the number of