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Evidence for the Existence of Granzyme-Like Serine Proteases in Teleost Cytotoxic Cells

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Abstract. Granzymes are granule-associated serine proteases, which are important effector molecules in NK cell and CTL functions. The granzyme family poses a perplexing problem in phylogenetics due to the lack of nonmammalian sequence information. We now report the identification of a cDNA that codes for a granzyme homologue, channel catfish granzyme-1 (CFGR-1), from nonspecific cytotoxic cells (NCC) of a teleost. NCC are the first identified and extensively studied cytotoxic cell population in teleosts. *Ictalurus punctatus* (channel catfish) granzyme cDNA encodes a protein with ~50% similarity to granzymes A and K. Highly conserved catalytic triad residues of serine proteases and other motifs common to granzymes were also identified. Conserved amino acid sequences, structure–function data available for the serine protease family, and the crystal structure of human granzyme K supported a model of CFGR-1. It suggested an Arg/Lys primary substrate specificity that is shared with granzymes A and K. Furthermore, CFGR-1 has the four conserved disulfide bonds of granzymes A, K, and M. Phylogenetic analysis suggested that this molecule is a member of the granzyme family. Expression of CFGR-1 in NCC was confirmed by RT-PCR analysis. Presence of a granzyme-like molecule that might play an important role in the effector functions of NCC indicates that cell-mediated immunity with granule exocytosis and Fas pathways have been conserved for more than 300 million years.

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Introduction

Mammalian cytotoxic T cells (CTL) and natural killer (NK) cells play an important role in immunosurveillance against virus-infected and tumor cells. Although recognition of target cells differs greatly, the mechanisms by which CTL and NK cells cause target cell death following formation of conjugates appear to be the same. Two main cytotoxic pathways have been described that are independent from each other (Waterhouse and Trapani 2002). One pathway involves the cross-linking of death receptors on the target cell (members of the TNF receptor family such as Fas) by death ligands present on effector cells (FasL, TRAIL). This interaction leads to apoptotic target cell death (Trenn et al. 1987; Kagi et al. 1994). The other pathway requires granule exocytosis, with the resulting secretion of two abundant granular proteins, perforin and granzymes, which appear to act in concert to induce target cell death (Henkart 1985; Podack 1985; Young and Cohn 1986; Peters et al. 1990; Podack and Kupfer 1991; Doherty 1993). Both pathways act synergistically on many target cells, but the exocytosis pathway alone is sufficient to kill target cells, which do not express death receptors.

The mechanisms of action of perforin and granzymes have been the subject of intense studies (Henkart 1985; Podack 1985; Young and Conn 1986;

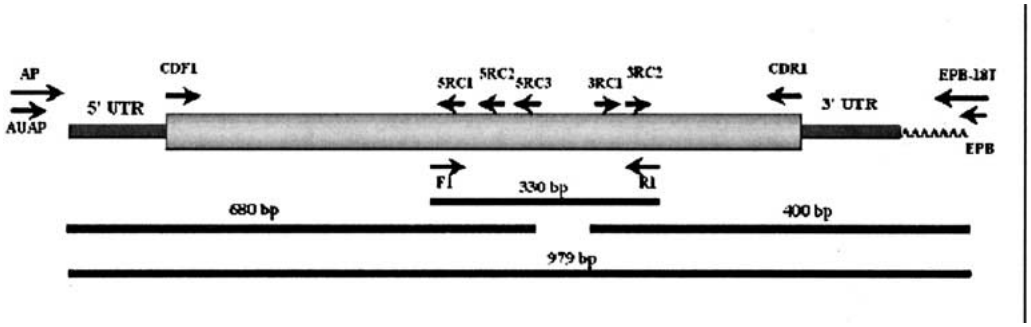

Fig. 1. Cloning strategy used to identify and characterize the cDNA for catfish granzyme. The primer sequences are listed in Table 1.

Table 1. Oligonucleotide primers used in the cloning of catfish granzyme and for expression studies by RT-PCR

Name	Sequence	Use
F1	5′-GCTGCAGTAGCATGATGTCA-3′	Initial screening
R1	5′-GTTACACACAAGAGGGCCTCCA-3′	Initial screening
5RC1	5′-GCATGATGTCATTGGAGAAGTTC-3′	5′ RACE
5RC2	5′-GGGAATCTTCTTAACGTCTAC-3′	5′ RACE
5RC3	5′-GTTCCCCACCCTCTTACTTCAC-3′	5′ RACE
3RC1	5′-CAAGCAGAGGGACAAAGATGCA-3′	3′ RACE
3RC2	5′-TGGAGGCCCTCTTGTGTGTAAC-3′	3′ RACE
CDF1	5′-CCCGGATCCATGCACGTGCAACAACGTTC-3′	ORF amplification
CDR1	5′-GGGGTTCTAGATGCTTTTTAATTATCTTGTTGATCCAGTCA-3′	ORF amplification
EPB-18T	5′-GCGATTTCTGCAGGATCCAAACTT$_{(17)}$-3′	3′ RACE RT
EPB	5′-GCGATTTCTGCAGGATCCAAACT-3′	3′ RACE
AP[a]	5′-CUACUACUACUACACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′	5′ RACE
AUAP[a]	5′-CUACUACUACUACACGCGTCGACTAGTAC-3′	5′ RACE
GRRTF1	5′-GTTTCAAGCAACAGCTTGCTCAGGCA-3′	RT PCR
GRRTR1	5′-GCATAGGACTTTCACTCGCATAGCA-3′	RT PCR
ACTINF	5′-GACAATGGTTCCGGTATGTGCA-3′	RT PCR
ACTINR	5′-GGTGCCAGATCTTCTCCATGTCA-3′	RT PCR

[a] AP (5′ RACE abridged anchor primer) and AUAP (abridged universal amplification primer) were obtained as a component of the 5′ RACE system (Invitrogen, CA). AP and AUAP are specifically engineered for the optimal amplification of dC-tailed cDNA. It contains strategically positioned deoxyinosine (I) and U residues to maximize specific priming at the dC-tailed end.

Peters et al. 1990; Podack and Kupfer 1991; Doherty 1993). Perforin is a pore-forming molecule that, in addition to inducing the lysis of cells by osmotic leakage, acts synergistically with granzymes to facilitate their entry into target cells. Granzymes are members of a granule-associated serine proteases family expressed in CTL and NK cells that induce apoptosis of target cells either through caspase activation or through caspase-independent pathways. Recent evidence suggests that even in the absence of perforin, granzymes can enter target cells by receptor-mediated endocytosis via the cation-independent mannose-6-phosphate receptor. However, internalization of granzyme in the absence of perforin is without toxic consequences (Froelich et al. 1996; Jans et al. 1998; Browne et al. 1999; Motyka et al. 2000). Four orthologous members of the granzyme family with different substrate specificities have been identified in humans and rodents (A, B, K, M) and additional nonorthologous genes exist in the human (H), rat (J), and mouse (C, D, E, F, G, N) genomes (Trapani 2001; Barry and Bleackley 2002). The granzyme loci have been placed into three groups by their chromosomal location and structural similarities. They have four primary specificities, "aspase," "chymase," "tryptase," and "metase," referring to the type of activities of the proteases encoded for in their genes. Granzymes A and B have different substrate specificities (tryptase and aspase, respectively) but they are both proapoptotic. Although the function of Granzyme M, a metase, has not been determined, it is believed to play a role in NK cell function, as it is expressed only in those cytotoxic cells. The substrate specificity of other family members remains unknown.

The diversity of enzymatic activities of the granzyme family members has prompted speculation that these proteases may play a role in other immune functions such as lymphocyte migration and extravasation as well as amplification of cytokine function (Simon et al. 1991; Sayers et al. 1992; Vettel et al. 1993; Smyth et al. 2001). It is apparent that in spite of their biological importance, many questions remain unanswered about granzymes. At present, only murine, rat, and human sequence information for granzymes is available and

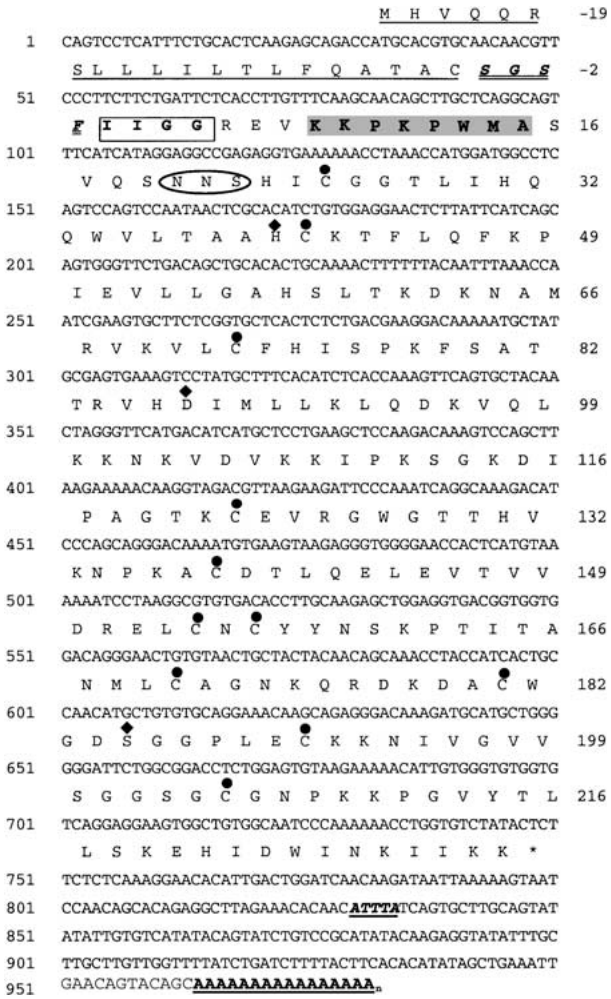

Fig. 2. Compiled full-length catfish granzyme cDNA sequence. Predicted signal sequence at the N-terminus is underlined. Putative tetrapeptide propeptide is double underlined. The amino acid numbering starts at the amino terminal of predicted mature protein as determined by sequence homology to other granzymes. The highly conserved IIXG motif at the N-terminal is boxed, while a region similar to the PHSRPYMA motif in other granzymes is highlighted. Putative glycosylation site is circled. Cysteine residues in the mature protein are marked with (•), while catalytic triad residues are marked with (♦). The ATTTA motif in the 3′ UTR is italicized and underlined, and the poly(A) tail is underlined.

the identification of evolutionary precursors would provide an important step in the elucidation of their biological activities. This is the first report of the characterization of a nonmammalian granzyme sequenced from lower vertebrates.

Nonspecific cytotoxic cells (NCC) are the teleost equivalent to mammalian NK cells. Since their initial description in the channel catfish (Graves et al. 1985; Evans et al. 1988, 1998; Jaso-Friedmann et al. 1990; Evans and Jaso-Friedmann 1999), these cells have been well characterized in a number of lower vertebrates (Faisal et al. 1989; Greenlee et al. 1991; McKinney and Schmale 1994; Suzumura et al. 1994; Jaso-Friedmann and Evans 1999; Jaso-Friedmann et al. 2002). Although originally described as agranular lymphocytes (Graves et al. 1985), NCC from catfish, tilapia, and trout have subsequently been shown to kill target cells by granule exocytosis and use both necrotic and apoptotic pathways (Faisal et al. 1989; Jaso-Friedmann et al. 1990; Greenlee et al. 1991). We have previously reported the importance of the Fas ligand-mediated killing mechanism of mammalian target cells by NCC (Bishop et al. 2000; Jaso-Friedmann et al. 2000). While others have also reported that catfish cytotoxic T cell lines kill target cells by the granzyme–perforin pathway, the lack of molecular tools did not allow the authors to identify these proteins (Zhou et al. 2001). In the present study we have identified a granzyme from a cDNA library constructed from cytotoxic cells of the lower vertebrate *Ictalurus punctatus*. Sequence analysis showed that this novel teleost protein shares that highest similarity with the human and murine granzymes K and A. Primers designed from the sequenced enzyme in catfish were used to identify another granzyme homologue from NCC of tilapia (*Oreocromus niloticus*). Based on the phylogenetic sequence analysis we conclude that granzyme-like homologs already exist in teleost fish. Characterization of granzyme precursors from lower vertebrates in turn will advance our understanding of the multiple roles of granzymes in the mammalian immune system.

Table 2. Amino acid identity and similarity of mature catfish granzyme to other known granzymes and related proteins

	% identity	% similarity
Human GRNZ A	**41.0**	**53.1**
Mouse GRNZ A	**39.7**	**53.2**
Human GRNZ B	32.1	40.9
Mouse GRNZ B	35.9	46.8
Rat GRNZ B	37.0	46.6
Mouse GRNZ C	30.3	41.2
Mouse GRNZ D	31.6	43.0
Mouse GRNZ E	30.3	42.0
Mouse GRNZ F	31.1	42.4
Mouse GRNZ G	32.4	43.7
Human GRNZ H	32.9	43.9
Rat GRNZ J	34.6	45.4
Human GRNZ K	**43.3**	**55.4**
Mouse GRNZ K	**45.8**	**55.8**
Rat GRNZ K	**45.4**	**55.0**
Human GRNZ M	33.1	47.5
Mouse GRNZ M	32.5	42.9
Rat GRNZ M	33.9	47.1
Human cathepsin G	31.2	44.5
Human mast cell chymase	37.4	49.2

Materials and Methods

Experimental Animals and Isolation of NCC

Outbred channel catfish (*Ictalurus punctatus*) of both sexes weighing 10–25 g were obtained from local commercial farms and

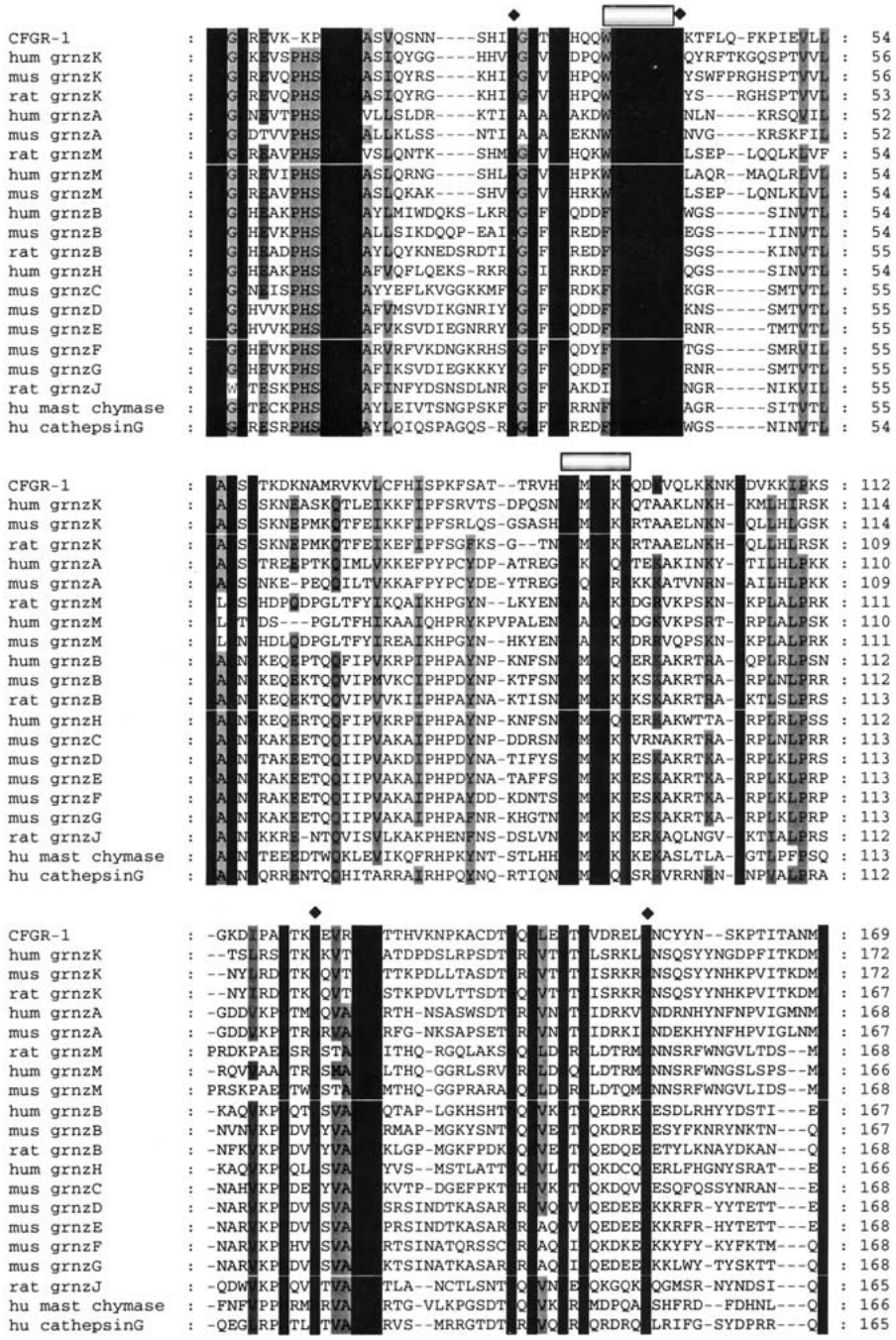

Fig. 3. Multiple sequence alignment of predicted catfish granzyme sequence (mature protein) with known granzymes and related proteins using CLUSTAL W. Highly conserved regions around the catalytic triad residues are indicated as boxes above the alignment. Cysteine residues marked (♦) are highly conserved among all known granzymes, while those marked (•) are conserved among granzymes A, K, and M. Residues marked with an asterisk are predicted to contribute to the primary substrate specificity of the protease. Mature portions of the following proteases were used in this comparison: human granzyme K (accession No. AAH35802), mouse granzyme K (accession No. AAC17930), rat granzyme K (accession No. NP_058815), human granzyme A (accession No. AAH15739), mouse granzyme A (accession No. NP_034500), rat granzyme M (accession No. Q03238), human granzyme M (accession No. AAH25701), mouse granzyme M (accession No. NP_032530), human granzyme B (accession No. AAH30195), mouse granzyme B (accession No. NP_038570), rat granzyme B (accession No. NP_612526), human granzyme H (accession No. A32692), mouse granzyme C (accession No. NP_034501), mouse granzyme D (accession No. NP_034502), mouse granzyme E (accession No. NP_034503), mouse granzyme F (accession No. AAA37741), mouse granzyme G (accession No. NP_034505), rat granzyme J (accession No. AAC53168), human mast cell chymase (accession No. KYHUCM), and human cathepsin G (NP_001902). Darker shading represents identical or residues with similar properties in 100% of the sequences and lighter shading represents identical or residues with similar properties in 80% or more of all the sequences.

outbred tilapia (*Oreochromis niloticus*) of both sexes weighing 60–100 g were obtained from Americulture, Inc., Animas, NM. Fish were maintained in fiberglass aquaria equipped with a constant flowthrough system at ambient water temperatures for channel catfish and 23–25°C for tilapia. Tilapia and catfish were fed a commercial diet of pelleted fish food (Southern States Co-operative Inc, VA). All animals were acclimatized for a minimum of 3 months prior to experimentation and were free from any active infections. NCC were purified from anterior kidney (bone marrow equivalent in fish) of channel catfish and peripheral blood of tilapia as described before (Jaso-Friedmann et al. 1990; Bishop et al. 2000). Purity of cell preparation was verified by flow cytometric analysis using 5C6 (a monoclonal antibody detecting NCCRP-1, which is an activation marker found exclusively on NCC [Evans et al. 1998, Jaso-Friedmann et al. 2001]).

Construction of a cDNA Library

Total RNA was isolated from purified NCC with TRIzol (Invitrogen) and mRNA was purified by the PolyATtract mRNA isolation system (Promega). The purified mRNA was used to construct an expression cDNA library using ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene) according to the manufacturer's instructions. The library was amplified and recombinant phages were converted into pBluescript plamids by in vivo excision according to

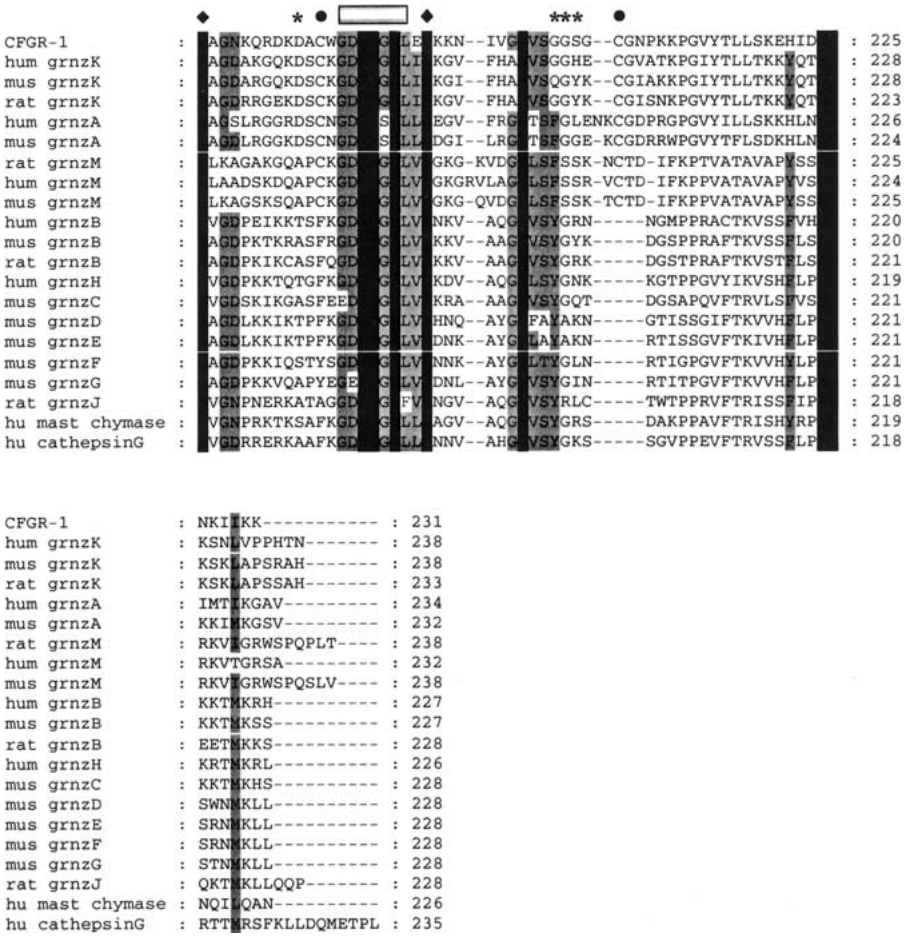

Fig. 3. Continued

the manufacturer's instructions. This was used as template for the PCR reactions in the initial screening for granzyme cDNA.

Screening for Granzyme cDNA

The strategy used in sequencing granzyme mRNA of channel catfish is illustrated in Fig. 1. Multiple sequence alignments were done using Clustal W provided with the vector NTI package, version 6 (InforMax Inc), to identify conserved domains in the known mammalian granzymes. Primers were designed (Table 1) and polymerase chain reactions were done to amplify specific regions of the cDNA. YT-INDY, a human natural killer-like leukemic cell line (Montel et al. 1995), cDNA was used as a positive control. Amplicons were TA cloned into pDrive cloning vector using a PCR cloning kit (Qiagen). Inserts were sequenced in two directions and compared with the known sequences in the DDBJ/EMBL/GenBank databases using BLAST version 2.2.5 (Altschul et al. 1997).

Rapid Amplification of cDNA Ends (RACE)

Sequence from one of the amplicon which had closest similarity to the known granzyme sequences was used to design primers to amplify the entire 5′ and 3′ends of the catfish granzyme cDNA. Fresh RNA was purified from catfish anterior kidney NCC for RACE. For 5′ RACE, purified mRNA (500 ng) was reverse transcribed using granzyme specific primers using a Generacer Superscript II RT module (Invitrogen). First-strand cDNA was purified using a PCR purification kit (Qiagen) followed by homopolymeric tailing with cytosine using terminal transferace (Roche). Tailed cDNA was purified using the PCR purification kit (Qiagen). Later dC-tailed cDNA was amplified using an abridged anchor primer and gene-specific nested primer, followed by a reamplification using a nested abridged universal amplification primer (AUAP) and another nested gene-specific primer. The amplicons were purified with the gel extraction kit (Qiagen) and TA cloned for sequencing. For 3′ RACE, catfish anterior kidney RNA was reverse transcribed using an anchor primer, EPB-18T (Evans et al. 1998), to generate first-strand cDNA. Using gene-specific nested primers and EPB, the 3′ end of the mRNA was amplified and TA cloned for sequencing. The sequences were edited and assembled to obtain the complete sequence of catfish granzyme. Later forward and reverse primers were designed at the two ends of the open reading frame to amplify the coding region, which was TA cloned to verify the sequences.

Phylogenetic Analysis

Analyses similar to those previously done with NCCRP-1 of zebrafish were performed (Jaso-Friedmann et al. 2002). Briefly, multiple sequence alignments were done using Clustal W, using only the mature portion of various proteases. Phylogenetic analysis was done using Mega version 2.1 (Kumar et al. 2001). The aligned data set was analyzed by the criteria of maximum parsimony using the branch-and-bound algorithm. The reliability of the trees was tested using 1000 bootstrap replicates. The alignment was also analyzed by the neighbor joining method as implemented by Mega with 1000 bootstrap replications. For the neighbor joining method, Poisson correction was used with the complete deletion option. The tree was rooted on a subtree consisting of trypsin and chymotrypsin sequences to resolve the differences between highly similar granzyme sequences.

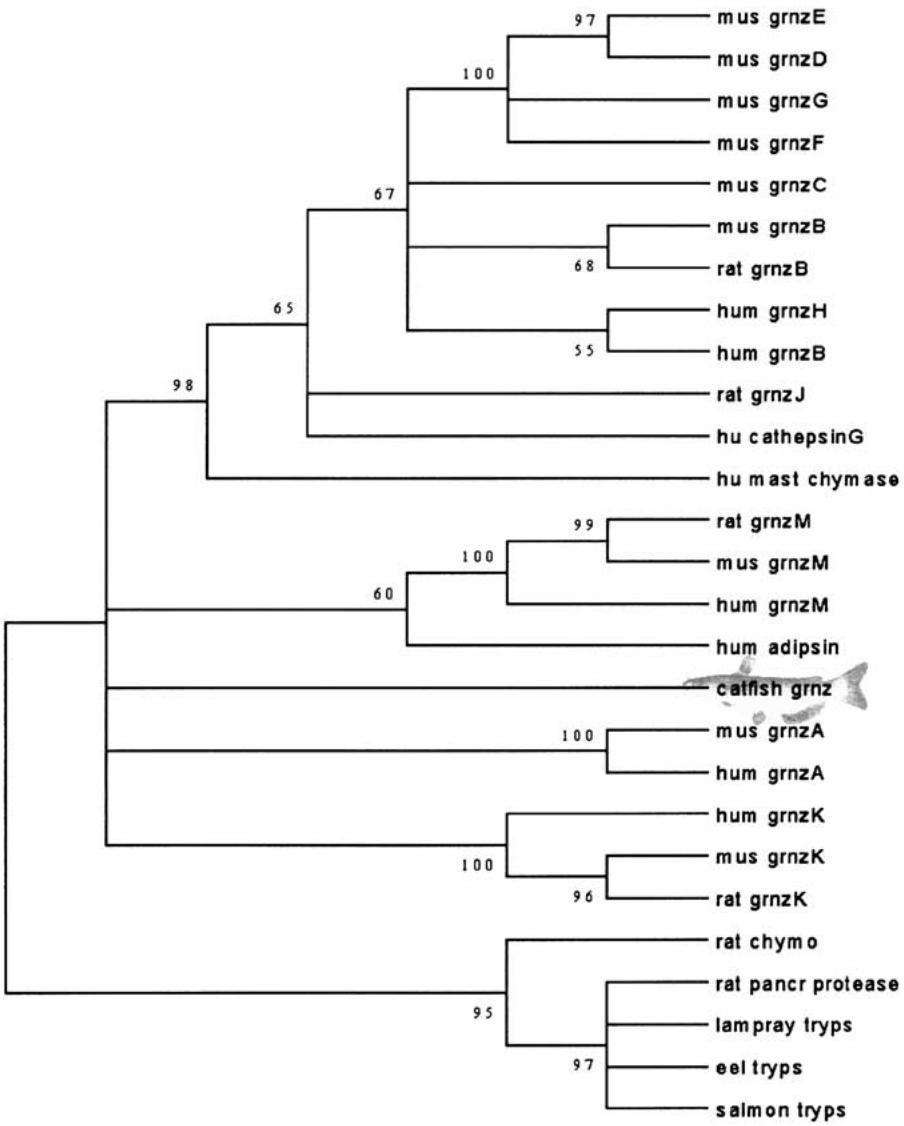

Fig. 4. Phylogenetic analysis of catfish granzyme: phylogram showing relationships of catfish granzyme to other known serine proteases. The tree was derived by parsimony analysis, with Mega version 2. Numbers shown above the branches are bootstrap values based upon 1000 replicates for parsimony. A separate analysis using maximum likelihood and neighbor joining methods produced a tree with similar topology. The tree was rooted on a subtree containing trypsin and chymotrypsin of fish and murine origin to determine the clustering of catfish granzyme with very similar known granzyme sequences. In addition to the previously described granzyme sequences, mature sequences of the following proteases were used to obtain the tree human adpsin (accession No. NP_001919), rat chymotrypsin B (accession No. AAA98732), rat pancreatic protease (accession No. NP_036861), lampray (*Petromyzon marinus*) trypsin (accession No. AAB69656), eel (*Anguilla japonica*) trypsin (accession No. BAB85634), and salmon (*Salmo salar*) trypsin (accession No. CAA49679).

Protein Modeling

The three-dimensional structure of catfish granzyme was modeled using SWISS-MODEL in the first approach mode accessible via the Internet (http://www.expasy.org/swissmod). The known crystal structures of serine proteases whose crystal structure has been resolved and had high sequence identity upon pairwise alignments (protein databank entries: 1MZA [Hink-Schauer et al. 2002], human granzyme K; 1DST [Jing et al. 1998], complement factor D) were used as template for the modeling. The coordinate files were imported to RasWin software version 2.6 for analyzing bond lengths and other conformational features of the molecule.

RT-PCR

Total RNA was extracted from channel catfish NCC (from anterior kidney, spleen, and peripheral blood), unfractionated head kidney, spleen, blood, gill, heart, muscle, and trunk kidney using TRIizol (Life Technologies). The purified RNA (3 μg) was reverse transcribed into cDNA using the Generacer Superscript II RT module (Invitrogen). The final volume of cDNA synthesis reaction was 50 μl, and 2 μl of the same was used in a 50-μl PCR reaction. β-Actin was used as a normalization control for RT-PCR. PCR was done with initial denaturation at 94°C for 2 min, followed by 30 cycles as follows: 30 s of denaturation at 94°C, 30 s of annealing at 57°C, and 30 s of extension at 72°C. The products were

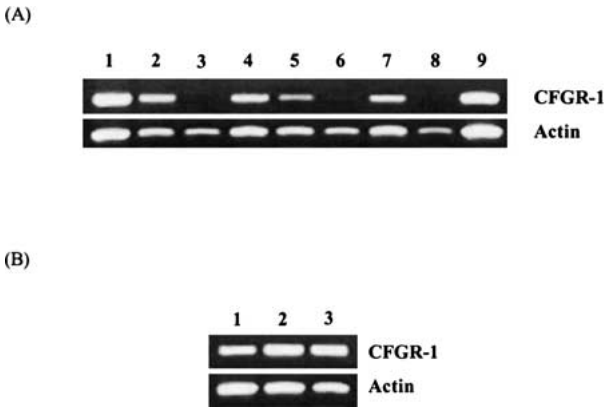

Fig. 5. Analysis of tissue expression of catfish granzyme. **A** RT-PCR analysis was performed with catfish granzyme specific primers using cDNA from various tissues. Lane 1, anterior kidney; lane 2, spleen; lane 3, total blood; lane 4, liver; lane 5, gill; lane 6, muscle; lane 7, trunk kidney; lane 8, heart; lane 9, NCC cDNA library (positive control). **B** NCC were enriched from three tissues to analyze the granzyme expression. Lane 1, NCC purified from peripheral blood; lane 2, NCC purified from anterior kidney; lane 3, NCC purified from spleen.

resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Results

Nucleotide and Deduced Amino Acid Sequence of Catfish Granzyme

An expression cDNA library generated from catfish anterior kidney NCC was used as template, with different primers designed based on conserved (functional) domains in human and murine granzymes. YT-INDY cDNA was used in the PCR reactions as a positive control. The amplicons were analyzed on 1.5% agarose gel. Among the different products, one with identical size to a product generated by the same primer set from YT cDNA was selected for sequencing. The sequence was compared and verified for the similarity to known granzymes. Based on the obtained product, additional nondegenerate primers were synthesized to obtain the sequence for the complete transcript by 5′ and 3′ RACE. The transcript of the catfish granzyme cDNA was 978 base pairs (bp) in length, with a single open reading frame yielding 768 bp (Fig. 2). One putative start codon was identified near the 5′ end of the full-length cDNA determined by 5′ RACE. The catfish granzyme cDNA encodes a putative mature protein with 231 amino acids. The predicated molecular mass of the unglycosylated protein was calculated as 25,526 daltons, with an isoelectric point of 9.56, suggesting a highly basic nature of the enzyme.

Initial analysis of the translated cDNA sequence by comparison to other known granzymes in the database suggested that the product was a member of the granzyme family of serine proteases (Fig. 2). The catfish sequence was subsequently submitted to GenBank (accession number AY286012). The three key amino acid residues representing the catalytic triad (charge relay system) of serine proteases as well as their neighboring residues are well conserved in catfish granzyme (Fig. 2; boxes). The three residues, His at position 57, Asp at 102, and Ser at 195 (chymotrypsinogen numbering system), are in locations similar to those of other serine proteases. A summary of these results showed that the mature granzyme sequence from catfish had 29–43% identity and 39–52% similarity to other granzymes (Table 2).

Primary Sequence Analysis for Identification of Signature Motifs of Known Granzymes

A signal sequence of 20 amino acids was identified at the N-terminal end, indicating that this enzyme is sorted to the secretory pathway (Fig. 3). The mature protein begins at +1, with a highly conserved Ile–Ile–Gly–Gly motif found in the majority of the granzymes. The most likely cleavage site for the signal sequence was predicted between residues at −5 (Cys) and −4 (Ser) as determined by the weight matrix analysis of von Heijne (1986). There is a single putative glycosylation site at the N-terminus of the mature protein (Asn^{20}–Asn–Ser).

Prediction of Tertiary Structure and Substrate Specificity

The three-dimensional structure of catfish granzyme was modeled using SWIS-MODEL. The templates used for modeling were based on known crystal structures of serine proteases. Disulfide linkages are crucial in correct folding of serine proteases and their catalytic ability. The mature catfish granzyme has an unusually high number of Cys residues (11 in total). Of these, six are highly conserved in all serine proteases (positions 42, 58, 136, 168, 182, and 201; chymotrypsinogen numbering). By analogy and based on results from other granzymes, the formation of disulfide bonds can be predicted as follows: 42 with 58, 136 with 168, 182 with 201). Cys191 and Cys220 (chymotrypsinogen numbering) could be analogous to a fourth disulfide linkage which bridges active-site serine, as in chymotrypsin. Additional comparisons with other granzymes yielded more similarities, like the formation of an internal salt bridge between the characteristic N-terminal motif (IIGG) and $Asp_{chymotrypsinogen\ 194}$. The overall structure of the predicted model for catfish granzyme was similar to that of other granzymes, especially granzyme K (data not shown). The model was also instrumental to

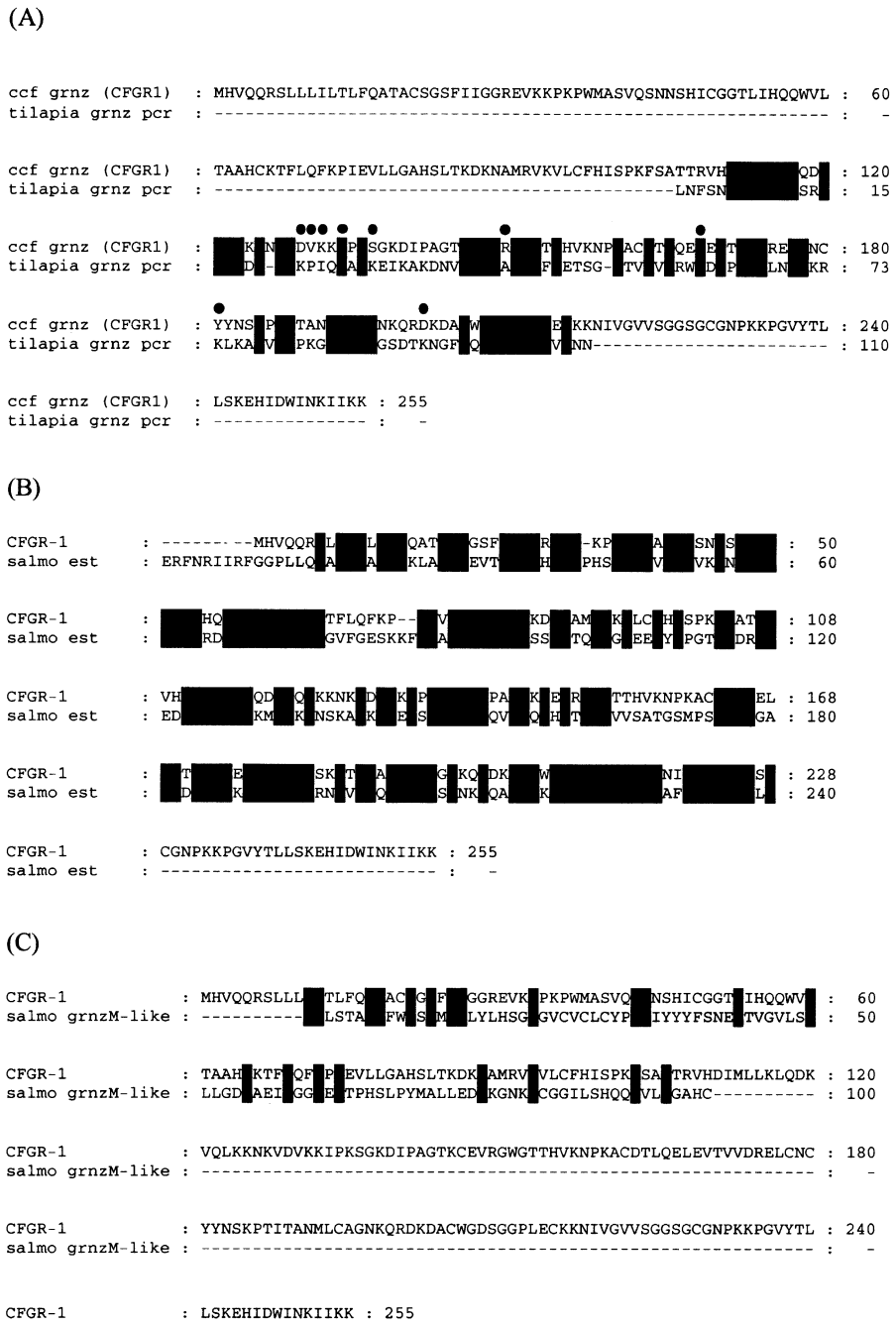

Fig. 6. Alignment of other fish granzyme sequences with catfish granzyme. **A** A portion of tilapia granzyme was amplified with primers used in cloning of catfish gene. The nucleotide sequence was edited, translated, and aligned with catfish granzyme. Residues marked (•) above the alignment differ from catfish granzyme but are highly similar to different mammalian granzymes. **B** Partial sequence of a *Salmo salar* granzyme derived from an EST (accession no CB516537) was edited and translated to compare with mature catfish granzyme. **C** N-terminal region of a partial sequence from *Salmo salar* (accession No. AF434669) which resembles human granzyme M was edited and translated to compare with the mature catfish granzyme.

predict the active site conformation of catfish granzyme. Based on the structure of trypsin (Rypniewski et al. 1994), the residues which determine the configuration of the active site were identified in the model (Gly43, Gly44, Gly140, Trp141, Gly142, and Leu155; chymotrypsinogen numbering). Those residues are in close proximity of the catalytic triad suggesting a highly conserved trypsin like active site structure. Four different enzymatic activities (tryptase, chymase, aspase, and metase) have been assigned to the granzymes identified so far. The combination of amino acid residues that are found to determine the primary substrate specificity of granzymes (−6, +16, +17, and +18 relative to active-site serine) were analyzed in catfish granzyme by examining the primary sequence and predicted model. The most critical residues determining the S1 subsite are found at positions 189, 216, and 226 (chymotrypsin numbering). Comparing these residues to that, e.g., in granzymes, A and B, cathepsin G, and leukocyte elastase, clearly indicates a Lys/Arg specificity (like granzyme A and K) for CGL-1 (Jackson et al. 1998).

Phylogenetic Analysis

Initial multiple alignments and visual examination of the catfish granzyme exposed many conserved

domains common to the mammalian granzymes. The aligned data set was analyzed by different methods to obtain reliable phylogenetic data. Similar results were obtained by the methods used and a representative tree is shown in Fig. 4. The similarity to multiple granzymes at different regions of the molecule posed problems for the grouping with the known enzymes. In order to resolve the differences between highly similar granzyme sequences, the tree was rooted on a subtree consisting of trypsin and chymotrypsin sequences. Catfish granzyme did not cluster with any of the granzyme families but branched out as a separate group, indicating that the three major granzyme branches (granzyme AK, granzyme BCDEFGH, granzyme M/elastase-2/proteinase 3/factor D) started to diverge just before or after the emergence of teleost fish.

Expression Pattern of the Transcript for the Catfish Granzyme

The presence of granzyme transcripts was analyzed in different tissues. RT-PCR was performed with primers designed based on the least conserved sequences between the teleost granzymes and other known granzymes in order to selectively obtain the predicted amplicon. Figure 5A shows a representative experiment suggesting that the tissue with the highest concentration of NCC (head kidney, spleen, and liver) had a higher level of granzyme mRNA than whole blood, muscle, and heart tissue. However, there was a significant increase in the expression levels of granzymes when the NCC were enriched from the whole blood (Fig. 5B), indicating that NCC are the main source of CFGR-1 in catfish.

Expression of Granzymes in Other Fish Species

An expression cDNA library was constructed from tilapia NCC purified from peripheral blood. An initial primer pair, which amplified a 330-bp amplicon from the catfish library, was used to screen the tilapia library by PCR. An amplicon of similar size as obtained from catfish and YT-INDY cDNA was sequenced. The sequence obtained was compared with known sequences in the DDBJ/EMBL/GenBank databases using BLAST version 2.2.5. The PCR product had a high similarity to known granzyme sequences. The tilapia granzyme PCR product was translated and aligned with catfish granzyme using Clustal W (Fig. 6A). There are considerable differences between the corresponding regions of the two molecules (36% identity and 46% similarity between the corresponding regions), but at the same time tilapia sequence retains the signature motifs common to all granzymes. This is suggestive evidence for the presence of different members of the granzyme family in other fish species. In order to identify potential representatives of granzyme family in other fish species, the catfish cDNA was compared with sequences in the EST database of NCBI. A *Salmo salar* EST (accession number CB516537) was found to be similar to the catfish granzyme sequence. The EST sequence was edited and translated to obtain the predicted protein sequence. Although this EST is lacking a few residues at both the ends and the two untranslated regions, considerable similarity (53% identity and 63% similarity) between the catfish granzyme molecule and the translation of the EST was found (Fig. 6B). Additional searches showed that the NCBI database has listed a partial sequence of a granzyme-like molecule from *Salmo salar* (accession number AF434669), similar to human granzyme M. The N-terminal portion of the mature protein from that sequence was compared with catfish granzyme. The N-terminal 43-amino acid domain of that protein has 56% identity and 65% similarity to the corresponding region of catfish granzyme (Fig. 6C).

Discussion

Although several laboratories have functionally shown the existence of the granzyme/perforin pathway in the cytotoxic activity of teleost CTL and NK-like cells (Greenlee et al. 1991; Evans and Jaso-Friedmann 1999; Zhou et al. 2001), this is the first report of the identification of a nonmammalian granzyme in catfish and tilapia NCC. The primary sequence characteristics that place this protein in the granzyme family include the presence of signature motifs shown in Figs. 2 and 3; the catalytic triad amino acids, each of them with surrounding conserved residues; the presence of a propeptide that must be removed to convert the inactive zymogen to an active protease; the phylogenetically conserved N-terminus amino acids; the cysteine residues important in disulfide bond formation; and the necessary leader sequence to place the nascent protein in the endoplasmic reticulum. The novel enzyme's predicted tertiary structure is consistent with serine proteases, and phylogenetic analysis indicates that CFGR-1 may be a member of the granzyme family (Fig. 4). Furthermore, we show that the expression of the catfish granzyme is restricted to lymphoid tissue (Fig. 5).

Homology comparisons with known mammalian granzymes alone were not enough to determine the substrate specificity of catfish granzyme because of the lack of predominant similarities to a particular member of the family. However, based on residue identities and residue similarities (Table 2), CFGR-1 appears to be most similar to the mammalian granzymes A and K. Both of these proteases are the only granzymes with tryptase activity sequenced to date.

Support for the idea that the teleost granzyme cleavage sites may be after arginine and lysine was provided by analyzing the putative substrate specificity pocket amino acids, which were determined by comparison to similar residues in other granzymes. The catalytic triad residues (histidine, aspartic acid, and serine) are conserved across different granzyme species, suggesting similar active-site structures. VLTAA**H**C around His 57 and GD**S**GGPL around Ser 195 are highly conserved, while **D**IML is conserved in many granzymes. Interestingly, the tilapia product had closer similarity to granzymes from the metase family. Although it is still early to conclusively assign substrate specificity to this tilapia granzyme, the significance of this finding is that there may be a fish ortholog to granzyme M. Granzyme M is uniquely expressed by NK cells, which suggests that the NCC are the NK-like cells (or their precursors) in teleosts. Other indications that these two teleost granzymes may belong to different families are that RT-PCR using catfish granzyme specific primers failed to amplify the tilapia cDNA.

In addition to the charge relay system, other granzyme signature motifs are present in the catfish homolog. As has been shown in all mammalian granzymes (Kam et al. 2000; Smyth et al. 1996), the catfish enzyme is produced as a propeptide. The leader sequence is a necessary requirement for the translated message of these enzymes to be compartmentalized to the rough endoplasmic reticulum and their final destination in the exocytic granules (Jenne and Tschopp 1988; Bleackley et al. 1988). Application of von Heijne's algorithm would leave a tetrapeptide as the inactivating portion of the enzyme. Although the propetide portion of most granzymes is a dipeptide, the only mammalian granzyme that is exclusively expressed in NK cells, granzyme M, has a hexapeptide at this position (Smyth et al. 1993).

Mammalian granzymes vary significantly in the degree of glycosylation, from none (granzyme C) to accounting for as much as 50% of their total mass (granzyme D). The possibility of glycosylation for the catfish granzyme is interesting in that it could have functional significance. It has been shown that granzymes A and B are glycosylated with phosphorylated mannose-rich residues, which recognize the mannose-6-phosphate receptor. This is an important pathway for targeting of these enzymes to secretory granules. Moreover, glycosylation could allow target cell entry of the catfish granzymes in the absence of perform by binding to the mannose-6-P receptor followed by endocytosis, an important point since the presence of teleost perforin has not been reported. Although the actual glycosylation status of the catfish granzyme is not presently known, it is noteworthy that the predicted protein has at least one putative glycosylation site at position 36 (chymotrypsinogen numbering). It has been demonstrated that glycosylation of granzymes occurs preferentially at Asn residues rather than Ser–Thr residues (Griffiths and Isaaz 1993).

Based on the sequence information and expression pattern, this novel molecule can be predicted as a new member of the granzyme family. This is the first report of a nonmammalian granzyme and there is convincing evidence for the presence of more granzyme-like molecules in fish cytotoxic cells. By definition, granzymes are found within the granules of cytotoxic cells. Characterization of granzymes from lower vertebrates sheds more light on the evolutionary patterns of serine proteases.

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