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Isolation of two cDNA sequences which encode cytotoxic cell proteases

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Two cDNAs which cross-hybridized with cytotoxic cell protease genes were identified in a library generated from a cytotoxic T cell line. Sequence analysis revealed that the two new members of the family contained the three catalytic triad residues which characterize the active sites of serine proteases. A comparison of the protein sequences revealed not only a high degree of homology but also the conservation of some unusual structural features. These include the lack of a disulphide bond which spans the active site serine, the presence of a signal sequence and the inference of a dipeptide activation sequence.

Serine protease; Cytotoxic T lymphocyte; Gene; cDNA sequence; Cell mediated immunity

1. INTRODUCTION

Cytotoxic T lymphocytes (CTLs) bind to cells which bear foreign antigens and, by a mechanism which is not yet understood, induce them to lyse. Recent evidence has suggested that cytoplasmic granules, present in CTLs, may be primary participants in target cell lysis [1]. Once physical contact is made between CTLs and the target, these granules fuse to the cytoplasmic membrane and thus deliver their contents into the intercellular pocket between the two cells. The target cell is therefore exposed to a high concentration of the granular proteins which set in motion a cascade of events ultimately leading to that cell's demise. Interestingly the CTL itself appears to be relatively resistant to the effects of the granular contents and is spared.

Several granular proteins have been isolated including perforin [2,3], proteoglycans [4] and serine proteases [5]. Perforin is believed to act like the C9 component of complement and create transmembrane channels in the target cell membrane, while the proteoglycans most likely fulfill a role in sequestering and inhibiting the activity of molecules within the granules. Evidence that serine proteases play a role in the lytic process is supported by the observations that specific inhibitors can block target cell lysis [6,7].

Our approach to study this intriguing mechanism has been to identify and isolate genes which are preferentially expressed in activated cytotoxic cells [8]. Two such genes which have been extensively characterized appear to encode serine proteases and we have therefore named them cytotoxic cell protease (CCP) 1 and -2 [9]. Both genes are induced upon CTL activation with kinetics that closely parallel the development of cytotoxicity [8]. Furthermore the proteins encoded are sequestered in the cytoplasmic granules discussed above [10]. Thus we believe that CCP1 and -2 play some role in the granule mediated mechanism of target cell lysis.

From a biochemical point of view CCP1 and -2 exhibit a number of interesting features [9]. These

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include the probable existence of zymogen forms by virtue of the presence of just two extra amino acids at the amino-termini and the lack of a disulphide bond which is normally involved in the stabilization of the substrate binding pocket. In addition the presence of unusual residues in regions involved in substrate binding have led us to suggest that CCP1 may have an unusual substrate specificity [11]. The genomic copies encoding both CCP1 and -2 have been completely sequenced. Their organizations clearly indicate that the two are related but moreover that they belong to a new subfamily of serine protease genes [12]. We report here the cloning and sequencing of cDNAs for two new members of the CCP family.

2. MATERIALS AND METHODS

2.1. *Preparation of RNA*

RNA was prepared by resuspending 2×10^8 cells in 10 ml of 3 M LiCl, 8 M urea and homogenizing with a polytron. The RNA was left in this solution to precipitate overnight, then spun down and resuspended in 0.1 M Tris-HCl, pH 7.5, 0.4 M LiCl. Poly(A)$^+$ RNA was purified on an oligo(dT) cellulose column (Collaborative Research).

2.2. *cDNA library construction*

Double-stranded cDNA was synthesized from 5 μg of poly(A)$^+$ RNA as described by Gubler and Hoffman [13]. The cDNA was methylated with *Eco*RI methylase (New England Biolabs) and then *Eco*RI linkers (Pharmacia) were attached. Size selection was carried out on a Sepharose 4B column. After ligation into λ Zap arms (Stratagene) the cDNA was packaged into phage with Gigapack Plus packaging extract (Stratagene).

2.3. *Filter preparation*

After amplification of the cDNA library in *E. coli* BB4 (Stratagene) 500000 pfu were plated onto 150 mm LB agar plates (50000 per plate) and after 17 h incubation at 37°C transferred onto 137 mm Hybond N nylon filters (Amersham). Two copies of each plate were made. After denaturation in 0.5 M NaOH, 1.5 M NaCl and neutralization in 1 M Tris-HCl, pH 8, 1.5 M NaCl, the filters were baked at 80°C for 1.5 h.

2.4. *Screening with cDNA probes*

The MTL2.8.2 λ Zap cDNA library was screened with cDNA probes which had been prepared by purifying insert DNA on a 5% polyacrylamide gel. This purified insert DNA was labelled by random oligonucleotide priming. Incorporated [^{32}P]dCTP was removed by passage of the sample over a Sephadex G-50 column (Pharmacia). Specific activities were $2–5 \times 10^8$ cpm/μg (Cherenkov). Prior to hybridization the filters were briefly washed in 4 × SSC (1 × SSC is 0.15 M sodium chloride, 0.3 M sodium citrate, pH 7), prehybridized in hybridization buffer (50% formamide, 50 mM phosphate, pH 6.5, 0.1% SDS, 5 × Denhardts, 5 × SSC, 1 mM sodium pyrophosphate, 100 μM ATP, 2.5 mM EDTA) at 42°C overnight. The hybridization buffer was replaced with fresh solution which contained radioactive probe (1×10^6 cpm/ml). The mixture was hybridized for 18 h at 42°C in a shaking waterbath. The filters were first washed for 10 min in 2 × SSC, 0.1% SDS at room temperature and then for 1 h in 0.1 × SSC, 0.1% SDS at 60°C.

Positive plaques were identified by autoradiography using Kodak X-Omat AR film at room temperature overnight. Agar plugs (0.5 cm) containing positive plaques were removed and soaked in SM (0.1 M NaCl, 4 M $MgSO_4$, 20 mM Tris-HCl, pH 7.5, 2% gelatin). Eluted phage were then screened again at a lower density so that positive clones were represented by single well isolated plaques. Agar plugs of 1.5 mm containing the single isolated clone were removed and soaked in SM. Bluescript rescue (Stratagene) was performed to switch the cloned cDNAs from λ Zap into the bluescript phagemid and transformed into *E. coli* XL1-blue.

2.5. *Subcloning and sequencing*

In general insert DNAs or subfragments thereof were purified and extracted from polyacrylamide gels after electrophoresis, subcloned into M13mp18 and sequenced by the dideoxy chain terminator method using [^{35}S]dATP (1400 Ci/mmol, New England Nuclear). Both strands of all sequences were determined. Sequence data were analyzed using Microgenie (Beckman).

3. RESULTS AND DISCUSSION

3.1. *Isolation of cDNAs which encode CCP family members*

A previously described cDNA library [8], constructed from MTL2.8.2 [14] mRNA in pUC13, was hybridized with purified insert B10 and C11 [9]. A number of positive clones were identified and preliminary analysis indicated that they were identical to the probe sequences. However, one clone which strongly cross-hybridized with B10 and C11 but was clearly distinguishable by restriction map analysis was found. Northern blot analysis (not shown) revealed that this gene hybridized with an mRNA of approx. 1000 nucleotides. This is the same size of mRNA that B10 detects [8], however, sequence analysis revealed that the cDNA was not B10 but a third member of the family. We named the cDNA D12. As D12 did not contain the complete coding sequence we used it as a probe to screen a size selected cDNA library constructed in λ Zap. A plaque with an insert of approx. 1000 base pairs corresponding to D12 was identified, and in addition another related cDNA was found (C134).

3.2. *The cDNAs D12 and C134 appear to encode serine proteases*

In fig.1 the nucleotide sequences of D12 and

C134 are presented on the upper line with the predicted amino acids below. Both protein sequences show a high degree of homology to the family of serine proteases and in addition contain the catalytic triad residues, His, Asp and Ser (marked by *), suggesting that they are indeed active serine esterases. The proteins encoded by D12 and C134 have predicted molecular masses of 27498 and 27646 Da and, in keeping with our original convention [9], have been named CCP3 and -4.

Both proteins have hydrophobic amino-termini which most likely act as signal peptides to direct the nascent proteins to the lumen of the rough endoplasmic reticulum [15]. Further evidence for this hypothesis comes from the occurrence of the signal peptidase cleavage sequence Ala-Gly-Ala [16] at the end of this stretch of residues. This would leave an amino-terminal sequence Glu-Glu-Ile-Ile after cleavage by the signal peptidase which we believe is activated during packaging by the removal of Glu-Glu. This conclusion is based in part on the sequencing studies performed on the granzymes [5], where this class of putative serine proteases all started with the sequence Ile-Ile. Indeed a comparison of these sequence data with our own predicted protein structures shows that, over the first 20 amino acid residues determined, CCP3 and CCP4 are identical with granzymes E and F, respectively. Our predicted molecular masses for the mature forms of CCP3 (25455 Da) and CCP4 (25490 Da) are in close agreement with those demonstrated for granzymes E and F (27000 Da). Furthermore the extensive glycosylation seen with these granzymes would agree with the presence of 4 potential *N*-linked glycosylation sites in the predicted CCP3 sequence and 3 in CCP4. This high degree of glycosylation together with the inherent basic nature of this family of proteases (approx. 15% basic and 7% acidic amino acids) may play a role in controlling their proteolytic activity. Within granules they may be sequestered by interaction with the acidic proteoglycans [4] and only become released, and therefore active, upon exocytosis.

3.3. *CCP1–4 are homologous*

At the nucleic acid level the genes C11, B10, D12 and C134 cross-hybridize even under relatively high stringency conditions. This indication of a high degree of similarity is borne out by a comparison of the predicted primary amino acid sequences given in table 1. Even the lowest level of homology is 55% (CCP1 vs CCP3) and this increases up to 75% (CCP3 vs CCP4). When these values are compared to values of 30–40% for the relatedness of CCP1 to serine proteases in general [9], it is clear that CCP1–4 belong to a closely related subfamily of serine proteases. This subfamily probably also includes cathepsin G [17] and rat mast cell protease (RMCP) II [18]. Indeed we have recently also shown that the genomic organizations of CCP1 and -2 are almost identical with each other and with the exon-intron structure described for RMCP II [19]. Thus these proteins are likely encoded by a set of structurally homologous and evolutionarily closely related genes.

The similarities between the CCPs is further highlighted in fig.2 which shows an alignment of the four predicted protein sequences. All four probably exist in a prepro-form with the signal sequence comprising of the amino-terminal 18 residues and removal of the next 2 amino acids being required for activation. In CCP2–4 this activation dipeptide is Glu-Glu, while for CCP1 it is Gly-Glu. Perhaps there is some basic difference between the activation of CCP1 versus the other CCPs. Cathepsin G also has the Gly-Glu sequence while in RMCPII it is again Glu-Glu. It is intriguing that in the genes encoding CCP1 and -2 [12] and RMCPII [19] there is an intron sequence which interrupts within this putative activation sequence.

Throughout the entire sequences these four proteins show a high degree of similarity. This is particularly noticeable over the first 150 residues where large stretches of amino acids are identical between all four proteins. After this point CCP1 and -2 remain similar (63%) but diverge somewhat from CCP3 and -4 (34–46%). This latter pair remain homologous (67%), however, it should be noted that even these two are less similar in their carboxy-termini than they are over the first 150 amino acids (81%). Perhaps the key to the specific roles played by the individual family members resides in their final 100 residues whereas the primary sequence of the first half of the molecules determines shared functions, targeting to granules for example.

D12

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GGT CAT CCT GGG CCT GGA GCA GCA GGT GCG AGG TAG GCT GCG CCG CCC CGT CTT GTT CAT

CCT GCT GGC CAG GGG CAG CCT CCG CAC AGC CCC GCC CCT CCT TCC TCC CCT TCC AAG GGC

TTT GTC TCC TTT GCT CTC CTT CAA CTG AGC AGC CTT CCT GGG AAG ATG CCA CCA GTC CTG
                                                        Met Pro Pro Val Leu

ATT CTC CTG ACC CTA CTT CTG CCT CTT GGA GCT GGA GCA GAG GAG ATC ATC GGC GGC CAT
Ile Leu Leu Thr Leu Leu Leu Pro Leu Gly Ala Gly Ala Glu Glu Ile Ile Gly Gly His

GTG GTG AAG CCA CAC TCC CGC CCC TAC ATG GCG TTT GTT AAG TCT GTG GAT ATT GAA GGT
Val Val Lys Pro His Ser Arg Pro Tyr Met Ala Phe Val Lys Ser Val Asp Ile Glu Gly

AAT AGG AGA TAC TGT GGA GGC TTC TTG GTT CAA GAT GAC TTT GTG CTG ACT GCT GCT CAC
Asn Arg Arg Tyr Cys Gly Gly Phe Leu Val Gln Asp Asp Phe Val Leu Thr Ala Ala His
                                                                              *

TGC AGG AAC AGG ACA ATG ACA GTC ACA CTG GGG GCC CAC AAC ATC AAG GCT AAG GAG GAG
Cys Arg Asn Arg Thr Met Thr Val Thr Leu Gly Ala His Asn Ile Lys Ala Lys Glu Glu

ACA CAG CAG ATC ATC CCT GTG GCA AAA GCC ATT CCC CAT CCA GAT TAT AAT GCC ACT GCC
Thr Gln Gln Ile Ile Pro Val Ala Lys Ala Ile Pro His Pro Asp Tyr Asn Ala Thr Ala

TTC TTC AGT GAC ATC ATG CTG TTA AAG CTG GAG AGT AAG GCC AAG AGA ACT AAA GCT GTG
Phe Phe Ser Asp Ile Met Leu Leu Lys Leu Glu Ser Lys Ala Lys Arg Thr Lys Ala Val
        *

AGA CCC CTC AAG TTG CCC AGA CCC AAT GCC CGG GTG AAG CCA GGG GAT GTG TGC AGT GTG
Arg Pro Leu Lys Leu Pro Arg Pro Asn Ala Arg Val Lys Pro Gly Asp Val Cys Ser Val

GCT GGC TGG GGG TCA AGG TCC ATC AAT GAC ACT AAA GCA TCT GCC CGC CTG CGA GAG GCT
Ala Gly Trp Gly Ser Arg Ser Ile Asn Asp Thr Lys Ala Ser Ala Arg Leu Arg Glu Ala

CAA CTG GTC ATC CAG GAG GAT GAG GAA TGC AAA AAA CGT TTC CGA CAC TAC ACT GAG ACC
Gln Leu Val Ile Gln Glu Asp Glu Glu Cys Lys Lys Arg Phe Arg His Tyr Thr Glu Thr

ACA GAG ATT TGT GCT GGA GAC TTG AAG AAA ATA AAG ACT CCT TTC AAG GGT GAC TCT GGG
Thr Glu Ile Cys Ala Gly Asp Leu Lys Lys Ile Lys Thr Pro Phe Lys Gly Asp Ser Gly
                                                                        *

GGA CCC CTC GTG TGT GAC AAC AAA GCT TAT GGA CTT TTA GCC TAT GCA AAA AAC AGG ACA
Gly Pro Leu Val Cys Asp Asn Lys Ala Tyr Gly Leu Leu Ala Tyr Ala Lys Asn Arg Thr

ATC TCT TCA GGA GTC TTC ACT AAG ATT GTG CAC TTC CTG CCG TGG ATA AGC AGG AAC ATG
Ile Ser Ser Gly Val Phe Thr Lys Ile Val His Phe Leu Pro Trp Ile Ser Arg Asn Met

AAG CTG CTC TAA CAG TGT TAA ACC ACC CGT GCC TGA CCA GCC TGT CCG ACC TCA GGC AAG
Lys Leu Leu

AAC CAC GTG GAG TGG GCA GCA AAG AAT GAA AAT TCA CAA TAA ATA ACC TCC AGA CTG CAA
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C134

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ATG CCA CCA ATC CTG ATT CTC CTG ACC CTT CTT CTG CCT CTC AGA GCT GGA GCA GAG GAG
Met Pro Pro Ile Leu Ile Leu Leu Thr Leu Leu Leu Pro Leu Arg Ala Gly Ala Glu Glu

ATC ATC GGG GGC CAT GAG GTC AAG CCC CAC TCC CGC CCT TAC ATG GCA CGT GTG AGG TTT
Ile Ile Gly Gly His Glu Val Lys Pro His Ser Arg Pro Tyr Met Ala Arg Val Arg Phe

GTG AAA GAT AAT GGA AAA AGA CAT TCC TGT GGA GGC TTC CTG GTT CAA GAC TAC TTT GTG
Val Lys Asp Asn Gly Lys Arg His Ser Cys Gly Gly Phe Leu Val Gln Asp Tyr Phe Val

CTG ACG GCT GCT CAC TGC ACT GGA AGC TCA ATG AGA GTC ATA CTG GGG GCC CAC AAC ATC
Leu Thr Ala Ala His Cys Thr Gly Ser Ser Met Arg Val Ile Leu Gly Ala His Asn Ile
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AGG GCT AAG GAA GAG ACA CAG CAG ATC ATC CCT GTG GCA AAA GCC ATT CCC CAC CCA GCT
Arg Ala Lys Glu Glu Thr Gln Gln Ile Ile Pro Val Ala Lys Ala Ile Pro His Pro Ala

TAT GAT GAT AAG GAC AAC ACC AGT GAC ATC ATG CTA TTA AAG CTG GAG AGT AAG GCC AAG
Tyr Asp Asp Lys Asp Asn Thr Ser Asp Ile Met Leu Leu Lys Leu Glu Ser Lys Ala Lys

AGA ACT AAA GCT GTG AGG CCC CTC AAG TTG CCC AGA CCC AAT GCC CGG GTG AAG CCA GGG
Arg Thr Lys Ala Val Arg Pro Leu Lys Leu Pro Arg Pro Asn Ala Arg Val Lys Pro Gly

CAT GTT TGC AGT GTG GCT GGC TGG GGG AGA ACA TCC ATC AAT GCA ACA CAA AGA TCT TCC
His Val Cys Ser Val Ala Gly Trp Gly Arg Thr Ser Ile Asn Ala Thr Gln Arg Ser Ser

TGC CTA CGA GAG GCT CAA CTG ATC ATC CAG AAG GAT AAG GAA TGC AAA AAA TAC TTC TAT
Cys Leu Arg Glu Ala Gln Leu Ile Ile Gln Lys Asp Lys Glu Cys Lys Lys Tyr Phe Tyr

AAG TAT TTC AAG ACC ATG CAG ATT TGT GCT GGA GAC CCA AAG AAA ATA CAG TCT ACT TAC
Lys Tyr Phe Lys Thr Met Gln Ile Cys Ala Gly Asp Pro Lys Lys Ile Gln Ser Thr Tyr

AGT GGT GAC TCC GGG GGA CCC CTC GTG TGT AAC AAC AAA GCT TAT GGA GTT TTA ACC TAT
Ser Gly Asp Ser Gly Gly Pro Leu Val Cys Asn Asn Lys Ala Tyr Gly Val Leu Thr Tyr

GGG CTA AAC AGG ACA ATC GGT CCA GGA GTC TTC ACT AAG GTT GTG CAC TAC CTG CCG TGG
Gly Leu Asn Arg Thr Ile Gly Pro Gly Val Phe Thr Lys Val Val His Tyr Leu Pro Trp

ATA AGC AGG AAC ATG AAG CTG CTC TAA CAG GAG TTA AAC CAC CCG TGC CTG ACC AGC CTG
Ile Ser Arg Asn Met Lys Leu Leu

TCC GAC CTC AGG CAA GAA CCA TGT GGA GTG AGC AGC AAA GAA TGA AAA TTC ATA ATA AAT

AAC CTC CAG AGT GCA AAA AAA AAA
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Fig.1. Nucleotide and inferred amino acid sequences derived from the cDNA inserts D12 and C134.

3.4. *The CCPs are structurally unusual*

In our first report of the predicted sequence for CCP1, we noted not only a high degree of homology with RMCPII but also that the two proteins shared some unusual structural features [9]. Both CCP1 and RMCPII lacked the cysteines which normally form a disulphide bond between residues 191 and 220 in chymotrypsin. CCP2–4 also lack this disulphide bridge. In CCP1–3 and RMCPII the cysteine in question before the active site serine is replaced with a Phe while in CCP4 another aromatic amino acid Tyr is used. The absence of this disulphide bond could lead to an increased flexibility for the substrate binding pocket and thus to a potentially extended substrate recognition sequence. Two other primary sequences, which could alter the substrate binding site, were also shared between CCP1 and RMCPII. The amino acid six residues prior to the active site serine, which is an Asp in trypsin-like and a Ser in chymotrypsin-like enzymes, is an Ala in CCP1 and RMCPII indicating a possible preference for a hydrophobic amino acid at the P_1 position of the substrate. In CCP2 this residue is also an Ala while in CCP3 it is a Thr and in CCP4 a Ser. Thus a chymotrypsin-like activity is suggested for the latter two proteases. The substitution for Ser-Trp-Gly 216 of chymotrypsin with Ser-Tyr-Gly in CCP1 and RMCPII is also observed with CCP2. However, in CCP3 and CCP4 it is changed further to Ala-Tyr-Ala and Thr-Tyr-Gly, respectively.

One unusual feature of the CCP1 sequence is the presence of an Arg residue at position 228 (equivalent to Gly-226 in chymotrypsin) suggestive of an acidic substrate specificity [11]. This

Table 1

Homologies between the predicted protein sequences for the four cytotoxic cell proteases

	CCP1	CCP2	CCP3	CCP4
CCP1	100%	66%	55%	57%
CCP2	66%	100%	59%	59%
CCP3	55%	59%	100%	75%
CCP4	57%	59%	75%	100%

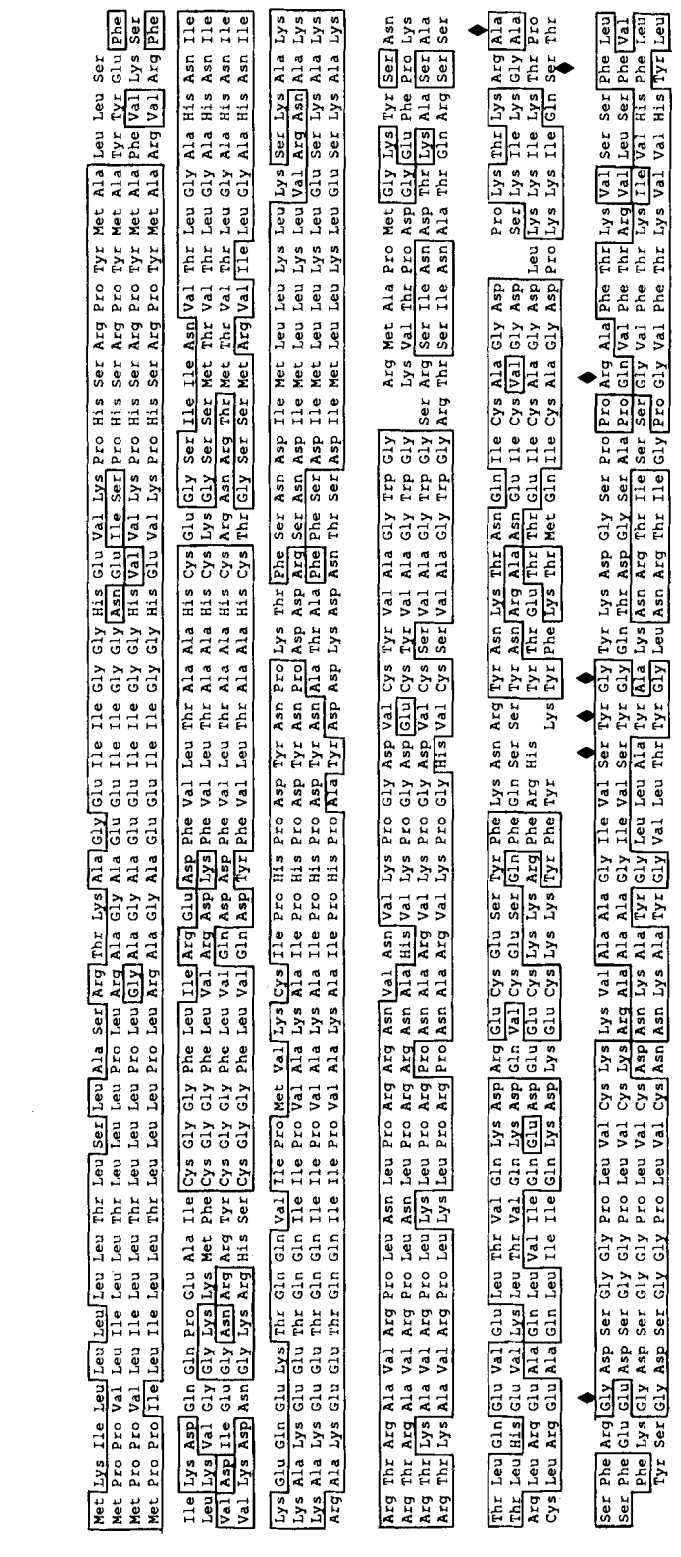

Fig.2. Comparison of the primary amino acid sequences of four cytotoxic cell proteases. Residues which are thought to be important in the structure of the binding site are marked (♦).

substitution is unique for CCP1, in CCP2 a Gln is found while in CCP3 and 4 a conventional Gly occurs. One intriguing specific substitution is found for CCP2 at position 202 (equivalent to Gly-193 in chymotrypsin). In the other three and indeed in all known serine proteases this residue is a Gly, thus suggesting that CCP2 may possess a most unusual activity.

The overall homology of all four proteases leaves little doubt that they are closely related, however discrete differences in residues known to play key roles in substrate interaction suggest that each has a different specificity. Possibly they play related but different roles in granule mediated cytotoxicity.

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