Identification of granzyme A isolated from cytotoxic T-lymphocyte-granules as one of the proteases encoded by CTL-specific genes

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A serine esterase called granzyme A, which is specifically expressed in cytolytic lymphocytes has been characterized. It is a disulfide-linked dimer and exhibits a trypsin-like specificity cleaving best after Arg. N-terminal sequence analysis revealed that granzyme A is identical to a protease recently predicted from a cloned CTL-specific gene.

CTL-specific protein *Serine esterase* *Cytotoxicity*

1. INTRODUCTION

In an attempt to understand the mechanism of target cell lysis mediated by cytolytic lymphocytes, three genes specific for CTLs have been recently isolated, using subtractive hybridization methods [1–3]. Sequence analysis predicts that all three proteins code for serine proteases and reveal the highest degree of homology with rat mast cell protease type II. In an alternative approach, proteins of the cytolytic granules of cytotoxic lymphocytes have been isolated [4,5]. At present the cytolytic, pore-forming protein perforin [6,7], which exhibits structural and functional homology with complement component C9 [8,9], and two serine esterases [10,11] have been characterized. However, the link between the isolated esterases and the genes is missing. Here, we report that one of these esterases, denoted granzyme A, contains an amino-terminal sequence identical to the one encoded by the CTL-specific gene (H-factor) isolated by Gershenfeld and Weissmann [1].

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2. MATERIALS AND METHODS

2.1. *Enzyme assays*

The amidase activity of granzyme A was tested on various peptide-7-amino-4-methyl-coumarin (AMC) substrates (Bachem, Bubendorf, Switzerland). Stocks (10 mM) of the substrates were prepared in DMSO and then diluted to 0.5 mM in a buffer containing 20 mM Tris-HCl, pH 7.8, 200 mM NaCl, 3 mM $CaCl_2$ and 1 mM $MgCl_2$. Granzyme A (500 ng) in 20 μl of a buffer containing 10 mM Bis-Tris, pH 6.0, 550 mM NaCl was added to 80 μl of the substrate solution and incubated at 37°C for 60 min. The reaction was stopped by adding 1 ml of 1 M ammonium acetate buffer, pH 4.5. Fluorescence intensity was measured in a spectrofluorometer SFM23 (Kontron, Zurich) at an excitation and emission wavelength of 380 and 460 nm, respectively. Esterase activity of granzyme A was measured using thiobenzyl-ester substrates [12] (Bachem). 360 μl of the substrate reaction mixture (0.2 M Tris-HCl, pH 8.1, 2×10^{-4} M thiobenzyl-ester substrate and 2.2×10^{-4} M dithiobis (nitrobenzoic acid)) was mixed with 40 μl granzyme A (25 ng).

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After 20 min at room temperature, absorbance was read at 405 using an automatic absorption reader (Gilford, Model PR-50). The protease activity on azocasein was tested according to Coombs [13]. In brief, granzyme A or trypsin as a reference (2 μg each) was added to 500 μg azocasein in a buffer containing 50 mM Tris, pH 8.2, and 150 mM NaCl (total volume, 500 μl). After 30 min incubation at 37°C undigested substrate and protease were precipitated by the addition of 500 μl of 10% trifluoroacetic acid. After centrifugation for 1 min at 10000 × g, the absorbance of the acid-soluble azocasein peptides was read at 366 nm. Relative activity for granzyme A as compared to trypsin was determined from a standard curve using different quantities of trypsin.

2.2. *Purification of granzyme A*

Lytic granules were isolated from the CTL-line B6.1. Approx. 10^9 cells were lysed by nitrogen cavitation and granules isolated on a Percoll density gradient (for exact procedure see [15]). After removal of Percoll by high speed centrifugation (3 h, 50000 rpm, 4°C, Beckman 60 Ti rotor) in the presence of 2 M NaCl, soluble granule proteins were passed over a TSK-4000 (2.5 cm × 60 cm) column (LKB). All fractions devoid of perforin were pooled, concentrated 10 times by ultrafiltration (Amicon) and dialyzed against buffer A (10 mM Bis-Tris, pH 6.0, 50 mM NaCl). The proteins were then applied to a FPLC Mono S anion-exchange column (HR 5/5, Pharmacia) equilibrated in buffer A [10]. Proteins were eluted at a rate of 1 ml/min with a linear salt gradient (buffer B: 10 mM Bis-Tris, pH 6.0, 1 M NaCl). The fractions containing granzyme A were pooled, concentrated by ultracentrifugation and were applied to a TSK-2000 gel chromatography column equilibrated in 150 mM CH_3COONH_4, pH 4.5. Proteins were eluted at a rate of 1 ml/min. 1 ml fractions were collected. Proteins were analysed in 10% polyacrylamide gels according to Laemmli [14]. The proteins were silver stained. 10 mM dithiothreitol was included in the sample buffer when the protein was analysed under reducing conditions.

2.3. *Protein sequencing*

Granzyme A isolated by gel chromatography using a TSK-2000 column (see fig.1) was lyophilized and 400 pmol of the non-reduced protein was subjected to sequencing. An Applied Biosystems 470 A protein sequencer was used, and the released phenylthiohydantoin-amino acids (PTH-AA) analysed by an online Applied Biosystems 120 A PTH-AA analyser.

3. RESULTS AND DISCUSSION

Granzyme A was isolated from cytoplasmic granules of the CTL-line B6.1 [11], which expresses cytolytic activity against H-2^d targets. Granules were isolated by means of Percoll density centrifugation. The soluble granule proteins were depleted of the pore-forming protein perforin/cytolysin using a TSK-4000 gel filtration column [6] and the remainder of the proteins passed through a FPLC Mono S cation-exchange column [10]. Two of the eluting proteins could be identified as serine esterases [10] on the basis of their reactivity with the radioactive affinity label [^{3}H]diisopropylfluorophosphate (DFP). One, designated granzyme A, has a molecular mass of 35 kDa under reducing conditions and cleaves very efficiently the substrate α-benzoyl-lysine-thiobenzylester (BLT), suggesting a trypsin-like substrate specificity.

Granzyme A is probably identical with the DFP-labeled esterase, the activity of which was detected in cytolytic lymphocytes by Pasternak and coworkers [11,16]. Their study indicates that this enzyme activity is specifically expressed in cytolytic lymphocytes, whereas our investigation showed that granzyme A is synthesized during the appearance of cytolytic granules in a CTL-hybrid line [10], suggesting a possible role for the protease in cytolysis.

In order to characterize granzyme A better, we purified it to homogeneity. Granzyme A-containing fractions of the FPLC Mono S column were pooled and passed through a TSK-2000 gel filtration column. Fig.1 shows the SDS-PAGE analysis of the column fractions. Granzyme A separated well from the two impurities of lower molecular mass. Granzyme A is disulfide linked with an apparent molecular mass of 60 kDa. Under reducing conditions, only one band of 35 kDa is detected, suggesting that granzyme A is a homo-dimer.

Earlier studies have indicated that granzyme A

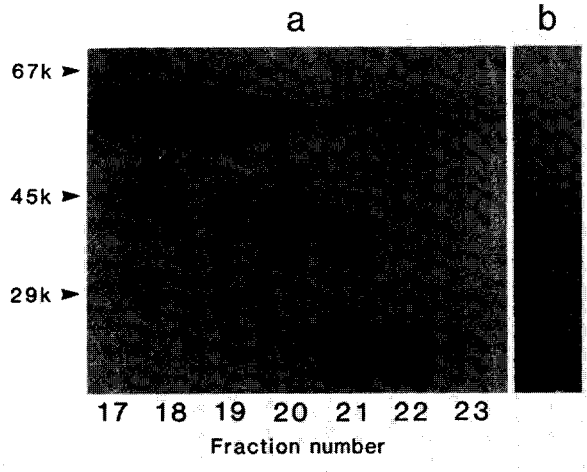

Fig.1. SDS-polyacrylamide electrophoresis of granzyme A under non-reducing (a) and reducing conditions (b). (a) Analysis of fractions eluting from the TSK-2000 column, which represents the last purification step of granzyme A. As shown in a previous report, the 60 kDa protein exhibits trypsin-like esterase activity and reacts with the serine esterase affinity label [^{3}H]diisopropyl-fluorophosphate. Granzyme A elutes in fractions 17–19 well separated from two other CTL-granule proteins. (b) Fraction 18 was used for sequence analysis and for further SDS-polyacrylamide analysis under reducing conditions. The following M_r standards were used to calibrate the gel: bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

has a trypsin-like specificity [10]. It cleaves BLT (substrate for trypsin) much more efficiently than the analogous substrate for chymotrypsin (SPT) (table 1). Therefore, several known synthetic substrates for trypsin containing the fluorogenic group 7-amino-4-methyl-coumarin (AMC) were tested. As can be seen in table 1, granzyme A is highly active on the substrates Pro-Phe-Arg-AMC, Val-Pro-Arg-AMC and to a lesser extent on substrates in which Lys instead of Arg is coupled to the fluorogenic group (P_1 position), such as Ala-Phe-Lys-AMC. Little or no cleavage was observed with other substrates not containing Lys or Arg at the P_1 position. Casein, a protein often used as a substrate for demonstrating proteolytic activity was also cleaved by granzyme A, although at least 20 times less efficient than by trypsin.

Granzyme A seems to be present only in cytolytic lymphocytes [16], suggesting that this protein could be identical with one of the three

Table 1

Relative amidase (esterase) activity of granzyme A

Substrate		Relative fluorescence units	Relative activity (%)
H-Pro-Phe-Arg-AMC		177.0	100
BOC-Val-Pro-Arg-AMC		59.4	34
BOC-Leu-Gly-Arg-AMC		8.8	5
Suc-Ala-Phe-Lys-AMC		18.1	10
BOC-Val-Leu-Lys-AMC		8.3	5
Suc-Gly-Pro-Leu-Gly-Pro-AMC		0.8	0
Suc-Ala-Ala-Ala-AMC		0.4	0
Suc-Ala-Ala-Pro-Val-AMC		4.1	2
SBz-Cys-Bzl-AMC		0.6	0
		Absorbance$_{405}$	
N-α-benzyloxoycarbonyl-Lys-thiobenzyl ester (BLT)		0.86	100
Suc-Ala-Ala-Pro-Phe-thiobenzyl ester (SPT)		0.07	8
		Absorbance$_{366}$	
Azocasein	Granzyme A	0.26	4
	Trypsin	0.907	100

predicted proteases encoded by the reported CTL-specific genes [1,3]. Microsequencing of the intact granzyme A showed that the first 25 amino acids are identical with the N-terminal of one of the predicted CTL-specific proteases [1], designated H-factor (fig.2). The sequence is clearly distinct from the second predicted protease [2], denoted CCPI by Lobe and colleagues [2], in spite of the fact that H-factor and CCPI have 15 out of 25 amino acid residues of the presumptive N-terminal in common. It is also very unlikely that the N-terminal sequence of granzyme A corresponds to the one of CCPII, the third predicted CTL-specific protease [2], whose N-terminal sequence has not yet been determined: CCPII is highly homologous to CCPI on the segment containing the reactive center and quite distinct from H-factor [1–3]. Moreover, in contrast to H-factor, CCPI and CCPII have no predicted trypsin-like specificity.

Granzyme A possesses several interesting features. Although we have no definite proof, it is almost certainly a disulfide-linked homo-dimer,

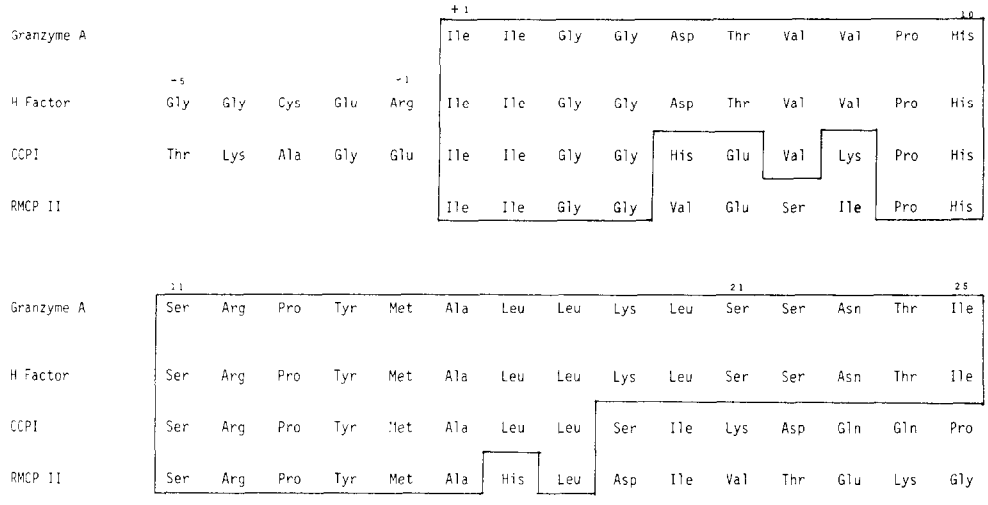

Fig.2. N-terminal sequence analysis of granzyme A. The amino acid residues of granzyme A are compared to the primary structure of H-factor, cytotoxic-T-cell specific protein I (CCPI). For the third predicted CTL-specific protease (CCPII), no N-terminal sequence has been determined. Due to the highest homology of CCPI and H-factor to rat mast cell protease II (RMCP II), this sequence is included for comparison. The regions containing identical residues are boxed.

since N-terminal amino acid sequence and electrophoretic analysis reveal only one distinct polypeptide chain. Most likely, cysteine-76 of granzyme A is involved in the interchain disulfide bridge. The molecule predicted by the gene contains nine cysteines, and by analogy to other members of the serine protease superfamily [17], such as elastase, Cys-26 and Cys-44, Cys-120 and Cys-189, Cys-150 and Cys-168, and Cys-179 and Cys-204 of granzyme A form intrachain disulfide bonds.

By analogy to the substrate binding site of trypsin, Asp-177 confers upon granzyme A a trypsin-like specificity, cleaving best after Arg and Lys. Of the AMC substrates tested, Pro-Phe-Arg-AMC was the most sensitive one. It contains a prolyl residue at the P_3 position. Peptides of this type are not cleaved by most other serine proteases in a way which releases the chromogenic group [18]. There are however, some exceptions: e.g. rat mast cell proteases (RMCP) catalyze the release of *p*-nitroanilide from *N*-Suc-Ala-Pro-Leu-Phe-*p*-nitroanilide, and it is interesting to note that granzyme A shows the highest sequence homology with RMCP II [1–3].

Gershenfeld and Weissmann [1] deduced from the CTL-specific gene a protein sequence containing 246 amino acid residues. By analogy to RMCP II, they concluded that the active enzyme starts at position 15 (Ile), which we confirmed with the N-terminal sequence analysis of granzyme A. Since the preceding amino acid residues do not have the characteristics of a leader peptide, they most likely represent the pre-sequence of granzyme A. Interestingly, the amino acid before the N-terminus of the active enzyme is an arginine, suggesting the possibility that granzyme A activates itself. This may occur during exocytosis of the granules, where the acidic pH of the granules is changed to neutral pH. Granzyme A is active at pH 7.0, but not at pH 5.0 and is therefore probably inactive in granules. In addition, due to its basic nature, granzyme A binds very well to proteoglycans, such as heparin and chondroitin-sulfate A (not shown). The latter molecule is a constituent of granules of cytolytic lymphocytes [19] and may further contribute to maintain granzyme A in an inactive form within the granules.

As to the actual physiological role of this protease, nothing is known at present. In our previous report we demonstrated that granule-mediated cytolysis is not affected by inhibitors of granzyme A [10]. This observation is in contrast to several reports indicating that high and low molecular

mass protease inhibitors block NK- and CTL-mediated target cell lysis [20–22]. Two other CTL-specific genes coding for proteases [2,3], and a second esterase (granzyme B) in CTL-granules [10] have been characterized. This may mean that a protease cascade similar to the complement and clotting systems may be involved in lymphocyte cytotoxicity. Future studies using the purified enzymes may allow one to examine this notion.

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